Plasma vascular endothelial growth factor and interleukin-8 as biomarkers of antitumor efficacy of a prototypical erbB family tyrosine kinase inhibitor

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Abstract

CI-1033 (*N*-[4-[*N*-(3-chloro-4-fluorophenyl)amino-7-[3-(4-morpholynyl)propoxy]quinazolin-6-yl]acrylamide, PD 0183805-mesylate salt) was identified as a potent, selective inhibitor of erbB family tyrosine kinases, which are overexpressed in a number of solid tumors and have been shown to be involved in tumor progression. Because objective response of clinical patients to erbB-targeted therapies like CI-1033 has been observed only in a subset of cancer patients that exhibit the intended molecular targets, much emphasis has been placed on the identification of biomarkers of antitumor efficacy. Vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) were considered as potential biomarkers for CI-1033 due to ease of detection in patient plasma and showed roles in angiogenesis and cancer progression and positive regulation by the erbB receptor family. In the present studies, mice bearing established xenografts (A431 epidermoid carcinoma, H125 non–small cell lung carcinoma, SF767 glioblastoma, and MDA-MB-468 mammary carcinoma) were treated with efficacious and subefficacious doses of CI-1033, and plasma levels and xenograft gene expression of VEGF and IL-8 were evaluated. Oral administration of CI-1033 to tumor-bearing mice at efficacious doses resulted in markedly decreased levels of VEGF and/or IL-8 plasma levels and tumor mRNA levels relative to vehicle-treated control mice in xenograft models that exhibited evaluable levels of these markers. In contrast, subefficacious doses of CI-1033 did not significantly affect VEGF or IL-8 levels in any of the xenograft models. These studies indicate that plasma VEGF and IL-8 may have use as biomarkers of antitumor efficacy for epidermal growth factor receptor/erbB–targeted therapies such as CI-1033 and suggest that further clinical study of these markers in cancer patients are warranted. [Mol Cancer Ther 2005;4(6):938–47]

Introduction

The erbB or epidermal growth factor (EGF) family of tyrosine kinase receptors plays an important role in a wide variety of cellular functions including regulation of mitogenesis, cell death, angiogenesis, and cell differentiation (reviewed in ref. 1). The diverse effects of these receptors on cell function and signaling pathways are mediated by activation of their receptor tyrosine kinase activity by receptor-selective ligands (EGF, transforming growth factor-α, heregulins, and betacellulins) and subsequent formation of a variety of homodimers and heterodimers by erbB family members (2, 3). Several members of the erbB family of receptors were shown to be overexpressed or altered in a number of human cancers, including mammary, ovarian, non–small cell lung, glioblastoma, prostate, pancreas, head and neck, and other cancers (4, 5). In addition, a number of studies have implicated erbB family members in the development, progression, and treatment outcome in certain human cancers (6–9). The extensive information on the involvement of erbB receptors in human cancers and the role of erbB receptors in the wide variety of cellular processes that are dysregulated during human cancer make this receptor family attractive candidates for targeted therapy.

CI-1033 (*N*-[4-[*N*-(3-chloro-4-fluorophenyl)amino-7-[3-(4-morpholynyl)propoxy]quinazolin-6-yl]acrylamide, PD 0183805-mesylate salt) is an irreversible, highly selective small-molecule inhibitor of the erbB family of tyrosine kinase receptors (10, 11). The evaluation of the clinical response to targeted therapies such as CI-1033 is a key issue in determining the utility of this class of kinase-targeted anticancer drugs. CI-1033 treatment resulted in tumor stasis in the majority of preclinical cancer models suggesting that chronic, long-term treatment may be necessary for the optimal benefit in a clinical setting (10, 12). In addition, similar therapeutic entities including Herceptin, Iressa, Tarceva, and Cetuximab have shown clinical response and/or therapeutic benefit in only a subset of patients displaying the intended therapeutic targets, HER-2 and

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EGF receptor (EGFR), respectively (13). The molecular determinants that affect the response to therapy, including missense mutations in the drug targets, are in still in the process of becoming identified (14, 15). Therefore, the identification and validation of surrogate biomarkers of successful treatment with CI-1033, and like agents, will be useful in the monitoring of clinical response, design of treatment schedules, and selection of the appropriate clinical dose.

Some important features of surrogate biomarkers of anticancer efficacy include the following: (a) correlation of biomarker modulation with the antitumor response, (b) the modulation of the biomarker supports the proposed antitumor mechanism of the drug, (c) the biomarker is linked with a causal pathway in human cancer, and (d) the biomarker is clinically accessible and accurately measured. The vascular endothelial growth factor (VEGF) is an appropriate biomarker candidate for efficacy of erbB family inhibitors for several reasons. First, erbB family receptor signaling positively regulates VEGF expression, whereas erbB family inhibitors block VEGF expression (16–18). Second, increased VEGF expression and secretion has been shown to correlate with disease progression, treatment outcome, and prognosis in a wide variety of human cancers (19–22). In addition, VEGF has been shown to be an important positive regulator of tumor angiogenesis and growth, whereas inhibition of VEGF receptors has been shown to inhibit tumor angiogenesis and growth (23–25). Furthermore, because VEGF is a secreted factor and its plasma levels were elevated in cancer patients, it can be easily measured in a clinical setting. Similar to VEGF, interleukin-8 (IL-8) has been shown to be positively regulated by EGFR signaling, whereas EGFR inhibitors block IL-8 expression (17, 26, 27). IL-8 is secreted by a variety of tumor cells, has been shown to be a positive regulator of tumor cell proliferation and angiogenesis, and correlates with the metastatic potential of tumor cells (28–31). Like VEGF, IL-8 is a secreted factor and IL-8 plasma levels can be easily measured in a clinical setting. Interestingly, VEGF and IL-8 gene expression have been shown to be regulated via common elements including activator protein binding sites and hypoxia-inducible elements (31–33).

The goals of the present study are to investigate the effects CI-1033 on tumor expression and secretion of VEGF and IL-8 into plasma and to correlate the modulation of these factors with antitumor efficacy. These studies indicated that tumor-secreted VEGF and IL-8 in plasma and gene expression were down-regulated by CI-1033 in multiple human tumor xenograft models. Furthermore, the down-regulation of VEGF and IL-8 correlated with the antitumor efficacy of CI-1033. These studies indicate that plasma VEGF and IL-8 may have use as biomarkers of antitumor efficacy for EGFR/erbB-targeted therapies such as CI-1033. Furthermore, the involvement of VEGF and IL-8 in tumor angiogenesis suggests that a partial mechanism of the antitumor effects of CI-1033 are mediated by inhibition of angiogenesis.

Materials and Methods

Tumors and Animals

Solid human tumor models of A431 (human epidermoid carcinoma, American Type Culture Collection), H125 (non–small cell lung carcinoma, American Type Culture Collection, Rockville, MD), SF767 (glioblastoma, donated by Michelle Garrett, Onyx Pharmaceuticals), and MDA-MB-468 (mammary carcinoma, American Type Culture Collection) were developed from cell lines and were maintained and tested in either athymic NCr *nu/nu* mice or ICR-SCID mice. All tumors were serially passed as s.c. implants of tumor fragments (~30 mg) obtained from tumors weighing ~1 g. This tumor panel was selected because of the overexpression of at least two EGFR/erbB family members as determined by Western blot analysis (12). Mice used for all experiments were obtained from Charles River Breeding Laboratories (Raleigh, NC). Animals were examined before the initiation of experiments to ensure that they were healthy and acclimated to the laboratory environment. Animals were housed in barrier facilities with food and water provided ad libitum on a 12-hour light/dark cycle. All treatment protocols were reviewed and approved by the Institutional Animal Care and Use Committee and comply with NIH publication No. 85-23, revised 1985.

Drug

CI-1033 was synthesized as described previously (10) and dissolved in 50 mmol/L sodium lactate buffer (pH 4.0) for administration by oral gavage administration in a volume of 0.5 mL/mouse. Vehicle control groups were included in each study to show that the vehicles used to deliver the drugs had no significant effects on tumor growth.

In vivo

The general methods used in these studies have been described previously (34). For all tests, the mice (19-24 g) were randomized and implanted with tumor fragments (~30 mg) s.c. into the right axilla. Treatment was initiated when tumors were 150 to 250 mg in size (advanced stage). CI-1033 was given daily by oral gavage based on group average weight for the duration of each experiment. The animals were weighed every 3 to 4 days during the treatment period. Tumors were measured 2 days per week and tumor mass [(length $\times$ width^2) / 2] was calculated from caliper measurements as described previously (34). A gross visual necropsy of each animal was done at death or terminal sacrifice. CI-1033 was given between 9 and 11 a.m. daily, over a range of doses representing the maximum tolerated dose, moderately efficacious dose, minimal efficacious dose, and subefficacious dose. For each time point, groups of six mice were given CI-1033 or vehicle (lactate buffer) alone. Data from each dose level were reported as median tumor mass for each time point. Antitumor efficacy calculations were based on percent treated versus control (T/C) when T and C are the median treated tumor mass and median control tumor mass, respectively, on the final treatment day when the median tumor burden in the control animals reached ~1 g (smaller percent T/C = greater effect). At each time point, mice were humanely euthanized and plasma was collected from the cardiac left ventricle using

a syringe primed with heparin sulfate. Plasma samples were centrifuged at 3,000 × *g* for 10 minutes in an Eppendorf 5417R refrigerated centrifuge at 4°C and stored at −80°C. Tumors were excised using a scalpel and forceps, weighed, pulverized using a liquid nitrogen–cooled cryomortar and pestle, and stored at −80°C.

VEGF and IL-8 ELISA

Plasma levels of VEGF and IL-8 were evaluated using ELISA kits from R&D Systems (Minneapolis, MN; QuantiGlo human VEGF Chemiluminescent ELISA kit and QuantiGlo human IL-8 Chemiluminescent ELISA kit). The VEGF ELISA kit recognizes the $VEGF_{121}$ and $VEGF_{165}$ isoforms. Briefly, standards and plasma samples were added to antibody-coated ELISA plate wells. Plates were then incubated for 2 hours on an orbital shaker set at 500 rpm at room temperature. Plates were washed four times and incubated with a horseradish peroxidase conjugated secondary antibody for 3 hours on the shaker at room temperature. After a second wash, plates were incubated in substrate solution for 10 to 30 minutes at room temperature and relative luminescence in each well was quantitated using VICTOR 1420 time-resolved luminometer (Perkin Elmer, Inc., Boston, MA). Luminescence units were converted into plasma VEGF or IL-8 concentrations by extrapolation from the standard curve generated for each ELISA plate. VEGF or IL-8 plasma concentrations were calculated for a minimum of six mice/treatment group/time point. Results were reported as means ± SDs plasma concentration for each treatment group and time point. Significant differences in VEGF or IL-8 plasma concentrations were assessed by ANOVA and Dunnett's test.

VEGF and IL-8 Northern Blots

Total RNA was isolated from pulverized xenograft tissue by the RNAzol B method (TEL-TEST, Inc., Friendswood, TX). Equal amounts of total RNA were electrophoresed on a 1% denaturing formaldehyde agarose gel, transferred to a Nytran nylon membrane by downward capillary blotting (Schleicher and Schuell, Keene, NH), and cross-linked to the membrane using a UV Stratalinker 180 (Stratagene, La Jolla, CA). The membranes were then hybridized with probes specific for VEGF, IL-8, or β-actin (loading control). The cDNA probes used were (a) a full-length, sequence-verified, cDNA probe for $VEGF_{165}$ (600 bp) generated by PCR screening of a PC-3 prostate carcinoma cell cDNA library; (b) a *Hin*dIII-*Eco*RI cDNA fragment corresponding to IL-8 from cloning vector BBG 44 (R&D Systems, Abingdon, United Kingdom); (c) β-actin mouse DECAprobe template (Ambion, Inc., Austin, TX). Probes were radiolabeled using random primers with [α-^{32}P]dCTP (Stratagene). Membranes were washed with 0.1 to 2× sodium citrate buffer and 0.1% to 1% SDS to remove background and visualized by autoradiography.

Results

In vivo Efficacy

Experiments were first done to qualitatively assess antitumor efficacy of selective erbB inhibition by CI-1033 in human tumor xenograft models. Tumor models evaluated included the A431 human epidermoid carcinoma xenograft model that expresses high levels of EGFR; the H125 human non–small cell lung carcinoma xenograft model that expresses moderate levels of EGFR, erbB2 and erbB3, and SF767; human glioma xenograft model that expresses high levels of EGFR and erbB4; and the MDA-MB-468 human mammary carcinoma xenograft model that expresses EGFR, erbB2, and erbB4.

In the A431 model, daily oral administration of CI-1033 at 3.6, 12, and 40 mg/kg/d resulted in T/C values of 41.8%, 24.7%, and 19.0 %, respectively, on treatment day 19 indicating a dose-response relationship (Fig. 1A). Pharmacodynamic studies investigating the effect of CI-1033 showed inhibition of EGFR phosphorylation at doses as low as 2.5 mg/kg (data not shown). In a second study to further resolve the dose-response relationship, CI-1033 did not significantly affect A431 tumor mass at 0.15 and 1.5 mg/kg/d, whereas treatment with 15 mg/kg/d resulted in a 33.9% T/C value at treatment day 19 (Fig. 1B). In the H125 model, daily oral administration of CI-1033 at 10, 20, and 40 mg/kg resulted in T/C values of 55.2%, 46.0%, and 39.9%, respectively, on treatment day 16 also indicating a dose-response relationship (Fig. 1C). In a second study, CI-1033 at 1 and 5 mg/kg did not significantly affect H125 tumor mass, whereas treatment with 25 mg/kg/d resulted in a 51.3% T/C on treatment day 13 (Fig. 1D). In the SF767 model, daily oral administration of CI-1033 at 15 and 30 mg/kg resulted in T/C values of 20.5% and 11.2%, respectively, on treatment day 22 (Fig. 1E). In the relatively slower-growing MDA-MB-468 xenograft model, daily oral administration of CI-1033 at 25 mg/kg/d resulted in a 67.2% T/C value on treatment day 15, whereas 5 mg/kg did not significantly affect tumor mass (Fig. 1F). CI-1033 was generally well tolerated in each efficacy study with no overt signs of toxicity at all dose levels and only minor weight loss (<10% body weight) observed at 40 mg/kg/d.

Collectively, these results indicate that CI-1033 produced measurable antitumor effects ranging from apparent tumor regressions (A431 and SF767) to delayed tumor progression (H125 and MDA-MB-468) in a variety of human tumor xenograft models expressing various erbB family members. Furthermore, the high level of selectivity of this molecule compared with other kinases in addition to target modulation data *in vivo* indicate that the antitumor efficacy observed at the doses evaluated is likely due to inhibition of erbB family (35).

Selection of VEGF and IL-8 as Potential Biomarkers of CI-1033 Antitumor Efficacy

A panel of plasma biomarkers was initially considered for further evaluation in tumor models that were responsive to CI-1033. This panel was based on evaluation of literature describing regulation of downstream gene and protein expression targets as well as Affymetrix experiments profiling the effects of CI-1033 on gene expression in A431 and H125 xenografts (36). Potential plasma markers initially considered based on this approach included VEGF,

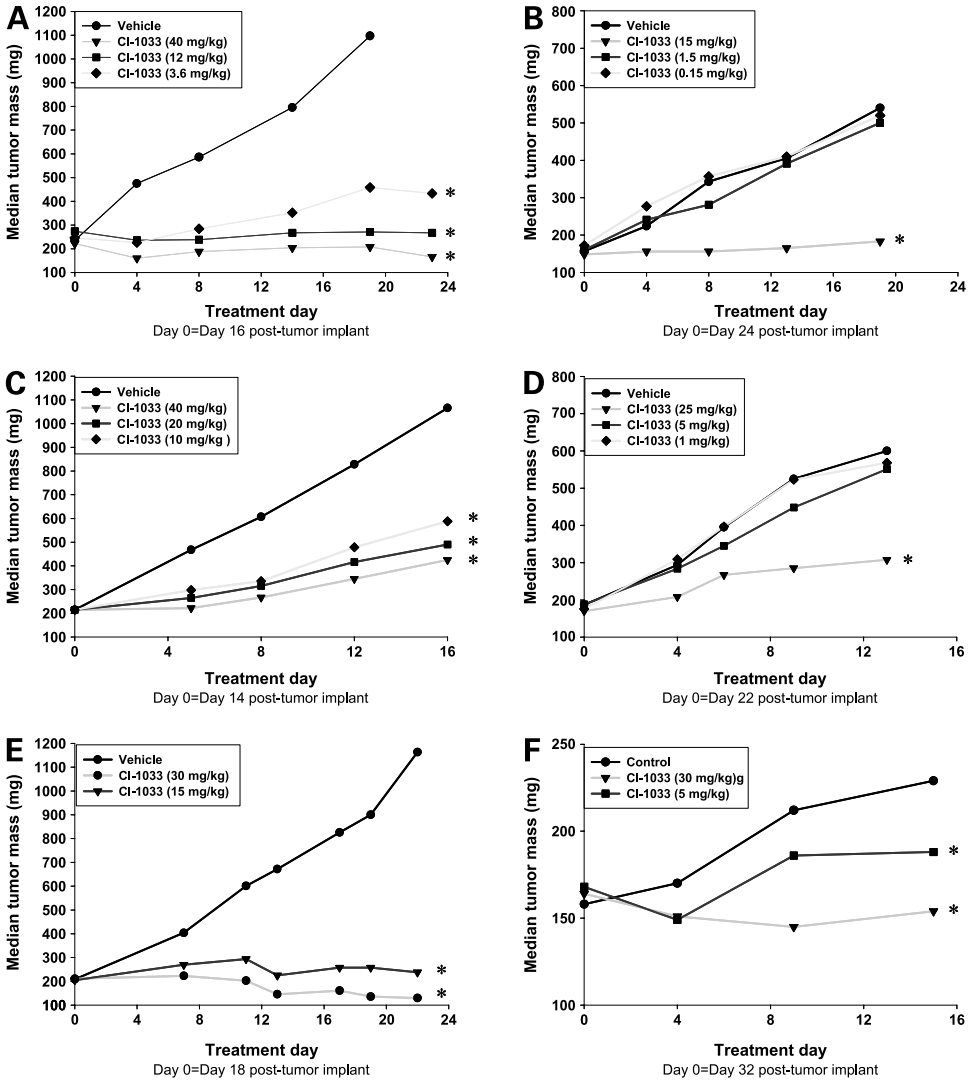

Figure 1. Effect of oral administration of CI-1033 on tumor mass in mice bearing established A431, H125, MDA-MB-468, or SF767 xenografts. Efficacy studies: (**A**) A431 xenograft-bearing mice treated with vehicle or efficacious doses of CI-1033, (**B**) A431 xenograft-bearing mice treated with vehicle or subefficacious doses of CI-1033, (**C**) H125 xenograft-bearing mice treated with vehicle or efficacious doses of CI-1033, (**D**) H125 xenograft-bearing mice treated with vehicle or subefficacious doses of CI-1033, (**E**) SF767 xenograft-bearing mice treated with vehicle or CI-1033, and (**F**) MDA-MB-468 xenograft-bearing mice treated with vehicle or CI-1033. Tumor fragments were implanted s.c. as described in Materials and Methods on day 0. Xenografts were allowed to grow to ~200 mg at which point therapy was initiated. CI-1033 was administered orally, daily for the duration of the experiment. Xenografts were measured with calipers and tumor mass was estimated twice weekly. A minimum of six mice were included in the evaluation of median tumor mass at each time point. *, statistical significance as determined by two-tailed *t* test comparison of CI-1033 and vehicle-treated control groups.

basic fibroblast growth factor, IL-8, matrix metalloproteinases 2 and 9, plasminogen activator inhibitor-1, sHER-2, transforming growth factor-α, tumor necrosis factor–related apoptosis-inducing ligand, and cystatin-C (36). VEGF and IL-8 were prioritized for further study due to observed modulation in multiple cell lines as well as their low intrapatient variability of plasma levels in human healthy volunteers and cancer patients (data not shown).

Effects of CI-1033 on VEGF Secretion and Expression

The effect of CI-1033 on levels of human VEGF in the plasma of tumor-bearing mice, gene expression of VEGF in tumor tissue, and the correlation of plasma VEGF with antitumor efficacy was assessed at several time points following daily oral administration. Human VEGF was detectable in plasma from mice bearing A431, H125, and SF767 xenografts. VEGF was not detectable in plasma of mice bearing MDA-MB-468 xenografts or nontumor-bearing mice suggesting that the source of measured plasma VEGF is from the tumor tissue.

In the A431 xenograft model, daily administration of CI-1033 at the efficacious doses of 3.6, 12, and 40 mg/kg/d resulted in significantly reduced plasma VEGF levels compared with vehicle-treated controls as early as day 2 of treatment and lasting throughout the duration of administration (Fig. 2A). All three efficacious doses tested produced essentially, equivalent reductions in VEGF levels at each time point examined (Fig. 2A). In a second study, subefficacious doses of 0.15 and 1.5 mg/kg/d did not have a significant effect on plasma VEGF levels at any of the time points evaluated, whereas the efficacious dose of 15 mg/kg/d caused a significant reduction of plasma VEGF at each time point (Fig. 2B). In each A431 study, a correlation of plasma VEGF levels with untreated control tumor mass was observed. In general, animals bearing relatively smaller A431 xenografts exhibited lower levels of plasma VEGF whereas animals bearing relatively larger A431 xenografts had higher levels of plasma VEGF indicating a relationship of tumor size to plasma VEGF (Fig. 3A). To further elucidate

the acute molecular mechanism of reduction of plasma VEGF by CI-1033, the effects of CI-1033 on VEGF gene expression was investigated in excised xenograft tissue by Northern blot analysis. VEGF RNA levels were decreased in A431 xenografts at early time points and undetectable by day 12 following oral administration of CI-1033 at 12 and 40 mg/kg/d compared with xenografts from vehicle-treated mice at these time points (Fig. 4A). No change of expression was observed in the housekeeping gene, *β-actin*, during treatment with CI-1033.

Similarly, in the H125 xenograft model, daily oral administration of CI-1033 at the efficacious doses of 10, 20, and 40 mg/kg/d caused a significant reduction in plasma VEGF levels at days 5 and 12 (Fig. 2C). All three efficacious doses similarly affected plasma VEGF levels at these early time points (Fig. 2C). In contrast to A431, at dosing day 18, CI-1033 at 40 mg/kg/d but not 20 or 10 mg/kg/d was associated with significantly reduced plasma VEGF levels. Furthermore, levels of plasma VEGF in each treatment group at treatment day 18 were elevated relative to earlier time points in a dose-dependent pattern (Fig. 2C). The elevated levels of VEGF in each treatment group correlated with increases in the apparent tumor mass of H125 xenografts observed during CI-1033 treatment at later time points (Fig. 1C and Fig. 2C). In a second study in the H125 xenograft model, the subefficacious doses of 1 and 5 mg/kg/d did not have a significant effect on plasma VEGF levels at any time point evaluated, whereas the efficacious dose of 25 mg/kg/d caused a significant reduction of plasma VEGF (Fig. 2D). Similar to the studies in A431 xenograft-bearing mice, a correlation of plasma VEGF levels with tumor mass was observed in untreated H125 xenograft-bearing mice (Fig. 3B). Similar to the A431 model, oral administration of CI-1033 caused an acute reduction in VEGF tumor gene expression in H125 xenografts at 20 and 40 mg/kg/d in xenografts at as

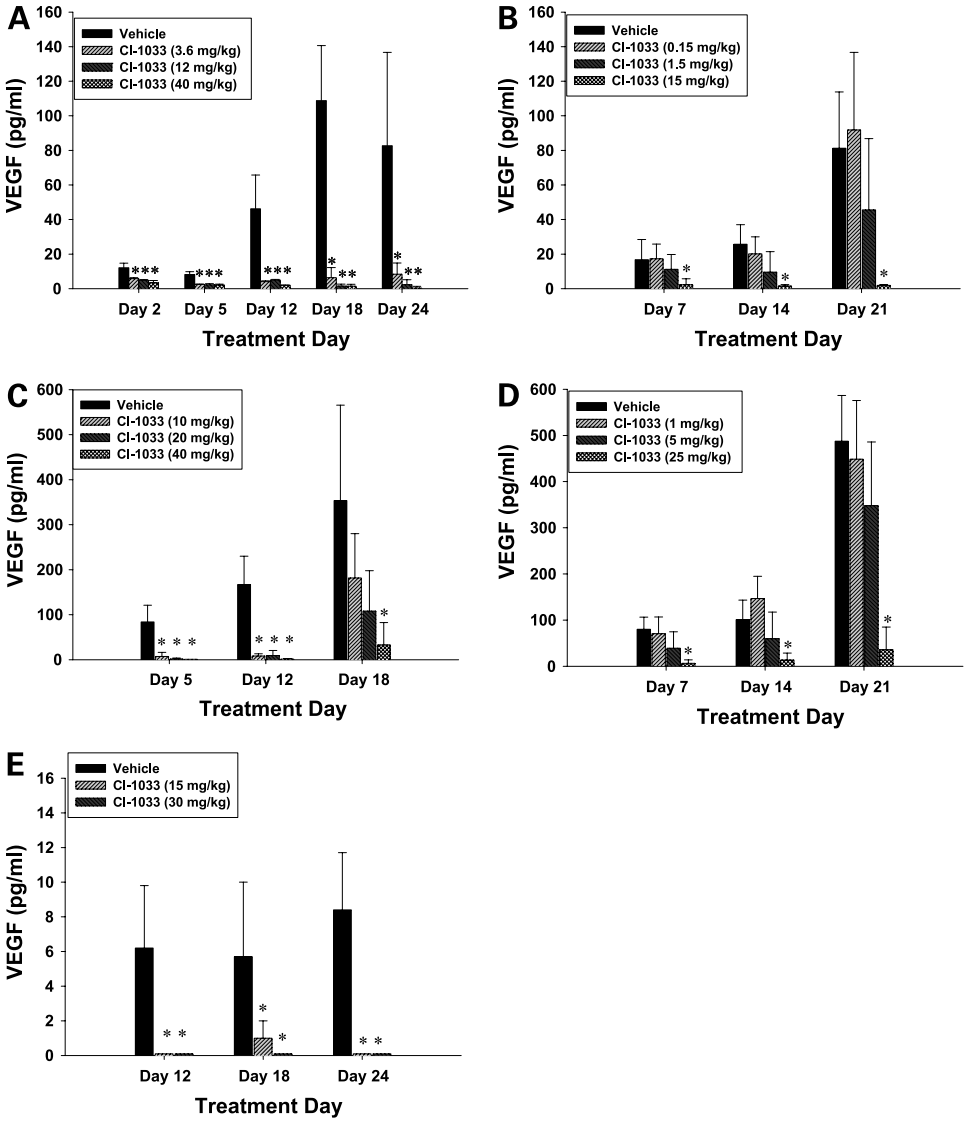

Figure 2. Effect of oral administration of CI-1033 on plasma VEGF levels in mice bearing established A431, H125, or SF767 xenografts. Plasma VEGF levels in (**A**) A431 xenograft-bearing mice treated with vehicle or efficacious doses of CI-1033, (**B**) A431 xenograft-bearing mice treated with vehicle or subefficacious doses of CI-1033, (**C**) H125 xenograft-bearing mice treated with vehicle or efficacious doses of CI-1033, (**D**) H125 xenograft-bearing mice treated with vehicle or subefficacious doses of CI-1033, and (**E**) SF767 xenograft-bearing mice treated with vehicle or CI-1033. At each time point, mice were sacrificed and plasma was isolated using an EDTA-primed syringe. Mouse plasma was analyzed for VEGF concentration by ELISA. *, significant difference from control.

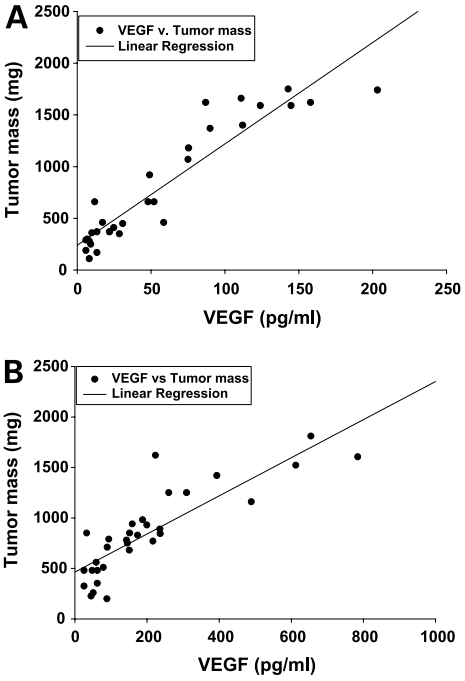

Figure 3. Correlation of plasma VEGF levels with A431 and H125 tumor mass. Plasma VEGF levels in relation to tumor mass in (**A**) vehicle-treated A431 xenograft-bearing mice or (**B**) vehicle-treated H125 xenograft-bearing mice. At each time point, vehicle-treated mice were sacrificed and plasma was isolated using an EDTA-primed syringe. Mouse plasma was analyzed for VEGF concentration by ELISA.

early as day 2 and continued through day 12 of treatment compared with xenografts from vehicle-treated mice at these time points compared with no change in expression observed for β-actin (Fig. 4B).

In addition to reduction of plasma VEGF by CI-1033 in A431 and H125 xenograft-bearing mice, a reduction of plasma VEGF following CI-1033 was also observed in SF767 xenograft-bearing mice. VEGF was detected in plasma of SF767 xenograft-bearing mice following daily oral administration of vehicle only at days 12, 18, and 24 of treatment (Fig. 2E). In contrast, VEGF levels were below the limit of detection in the plasma of SF767 xenograft-bearing mice following daily oral administration of CI-1033 at the efficacious doses of 15 or 30 mg/kg (Fig. 2E). VEGF mRNA was below the limit of detection in SF767 xenografts.

Collectively, these results indicate that CI-1033 treatment causes a reduction of tumor VEGF gene expression and secretion into plasma in several erbB family–expressing human tumor xenograft models. In addition, the reduction of plasma VEGF by CI-1033 in A431 and H125 xenograft models correlated with the efficacious doses of CI-1033. The acute changes in VEGF also suggest that CI-1033 exerts a direct effect on VEGF expression and secretion into plasma.

Effects of CI-1033 on IL-8 Secretion and Expression

The effects of CI-1033 on levels of human IL-8 in the plasma of tumor-bearing mice, gene expression of IL-8 in tumor tissue, and the correlation of plasma IL-8 with antitumor efficacy were also assessed at several time points following daily administration of the compound. Human IL-8 was detectable in plasma from mice bearing SF767, MDA-MB-468, and H125 xenografts. No IL-8 was detectable in plasma of A431 tumor-bearing mice or nontumor-bearing mice suggesting that the source of plasma IL-8 is from the tumor tissue.

In the SF767 xenograft model, daily administration of CI-1033 at the efficacious doses of 15 and 30 mg/kg/d caused a significant reduction in plasma IL-8 levels at treatment days 6 through 24 (Fig. 5A). A slight dose response was observed at days 12, 18, and 24 of treatment. Levels of plasma IL-8 in SF767 xenograft-bearing mice following daily oral administration of 30 mg/kg CI-1033 were 4.6%, 2.3%, and 2.1% of control at 12, 18, and 24 days compared with 10.5%, 10.7%, and 6.8% of control at the 15 mg/kg/d dose level at the same time points (Fig. 5A). In untreated SF767 xenograft-bearing mice, a correlation of plasma IL-8 levels with tumor mass was also observed indicating a relationship of the amount of human IL-8 detected in mouse plasma to mass of the tumor (Fig. 6A). To further examine the molecular mechanism of plasma IL-8 reduction, investigation of tumor gene expression by Northern blot analysis indicated that IL-8 mRNA levels were markedly decreased in SF767 xenografts at days 6 through 18 following oral administration of CI-1033 at 15 and 30 mg/kg/d compared with xenografts from vehicle-treated mice, whereas no effect of β-actin gene expression by CI-1033 was observed (Fig. 7A).

In the MDA-MB-468 xenograft model, daily oral administration of CI-1033 at the efficacious dose of 30 mg/kg/d caused a significant reduction in plasma IL-8 levels at

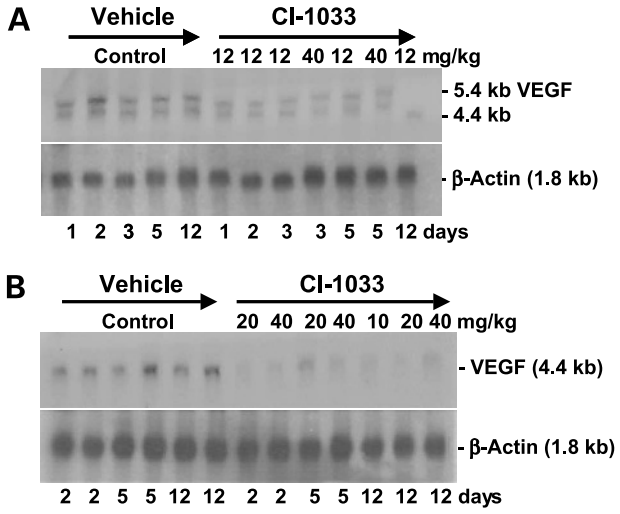

Figure 4. Effect of oral administration of CI-1033 on VEGF mRNA levels in xenografts. Xenograft VEGF (4.4/5.4 kb transcript) or β-actin (loading control) expression in (**A**) A431 xenograft-bearing mice treated with vehicle or CI-1033 (12 or 40 mg/kg) at treatment days 1, 2, 3, 5, and 12 or (**B**) H125 xenograft-bearing mice treated with vehicle or CI-1033 1033 (20 or 40 mg/kg) at treatment days 2, 5, and 12. Expression of VEGF and β-actin were determined by Northern blotting.

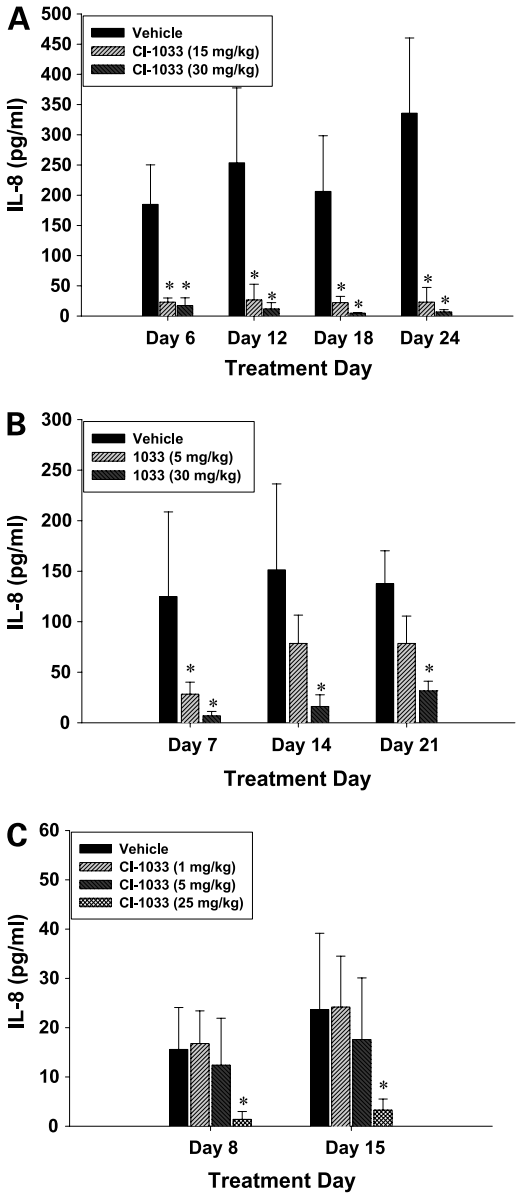

Figure 5. Effect of oral administration of CI-1033 on plasma IL-8 levels in mice bearing established SF767, MDA-MB-468, or H125 xenografts. Plasma VEGF levels in (**A**) SF767 xenograft-bearing mice treated with vehicle or CI-1033, (**B**) MDA-MB-468 xenograft-bearing mice treated with vehicle or CI-1033, or (**C**) H125 xenograft-bearing mice treated with vehicle or CI-1033. At each time point, mice were sacrificed and plasma was isolated using an EDTA-primed syringe. Mouse plasma was analyzed for IL-8 concentration by ELISA. *, significant difference from control.

treatment days 7, 14, and 21 (Fig. 5B). Oral administration of CI-1033 at 5 mg/kg/d caused a significant reduction in plasma IL-8 levels at day 7 but not days 14 or 21 correlating with the pattern of tumor growth observed at this dose in this model (Fig. 1F and Fig. 5B). Levels of plasma IL-8 in MDA-MB-468 xenograft-bearing mice following daily oral administration of 30 mg/kg CI-1033 were 5.5%, 10.6%, and 18.9% of control at 7, 14, and 21 days, respectively (Fig. 5B). In contrast, levels of IL-8 following treatment with 5 mg/kg CI-1033 were 22.5%, 51.9%, and 57.0% of control at 7, 14 and 21 days, respectively (Fig. 5B). Similar to the studies in SF767 xenograft-bearing mice, a correlation of plasma IL-8 levels with untreated tumor mass was observed in MDA-MB-468 xenograft-bearing mice (Fig. 6B). Also similar to the SF767 studies, administration of CI-1033 at 30 mg/kg/d but not 5 mg/kg/d caused a reduction of IL-8 expression in MDA-MB-468 xenografts at days 7, 14, and 21 of treatment compared with xenografts from vehicle-treated mice, whereas no effect of β-actin gene expression by CI-1033 was observed (Fig. 7B).

A reduction of plasma IL-8 following CI-1033 treatment was also observed in H125 xenograft-bearing mice. Daily oral administration of CI-1033 at the efficacious dose of 25 mg/kg/d but not the subefficacious doses of 1 or 5 mg/kg/d caused a significant reduction in plasma IL-8 levels at treatment days 8 and 15 (Fig. 5C). Levels of plasma IL-8 in H125 xenograft-bearing mice following daily oral administration of 25 mg/kg CI-1033 were 11.1% and 13.5% of control at 8 and 15 days, respectively (Fig. 5C). IL-8 mRNA was below the limit of detection in H125 xenografts.

Collectively, these results indicate that CI-1033 treatment causes a reduction of tumor IL-8 gene expression and secretion into plasma in several erbB family–expressing human tumor xenograft models. In addition, the reduction of plasma IL-8 by CI-1033 in MDA-MB-468 and H125 xenograft models correlated with the antitumor

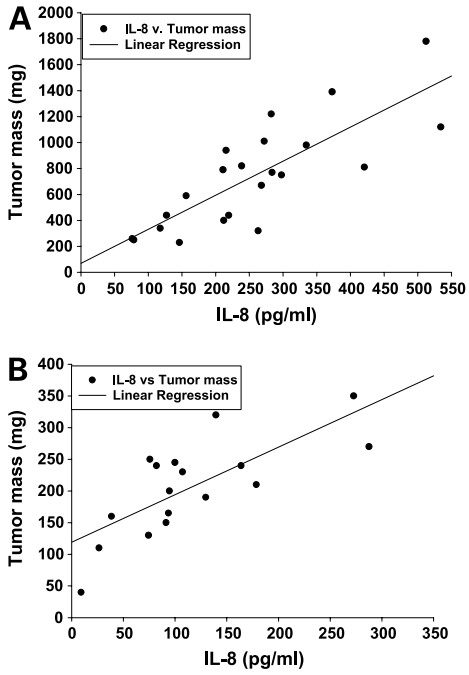

Figure 6. Correlation of plasma IL-8 levels with SF767 and MDA-MB-468 tumor mass. Plasma IL-8 levels in relation to tumor mass in (**A**) vehicle-treated SF767 xenograft-bearing mice or (**B**) vehicle-treated MDA-MB-468 xenograft-bearing mice. At each time point, vehicle-treated mice were sacrificed and plasma was isolated using an EDTA-primed syringe. Mouse plasma was analyzed for IL-8 concentration by ELISA.

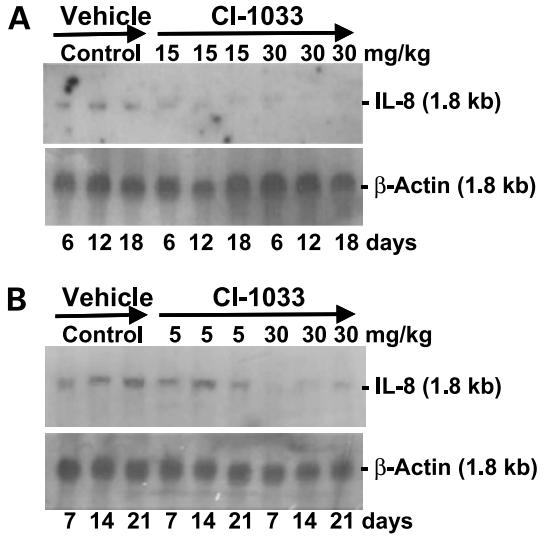

Figure 7. Effect of oral administration of CI-1033 of IL-8 mRNA levels in xenografts. Xenograft IL-8 (1.8-kb transcript) or β-actin (loading control) expression in (**A**) SF767 xenograft-bearing mice treated with vehicle or CI-1033 (15 or 30 mg/kg) at treatment days 6, 12, and 18 or (**B**) MDA-MB-468 xenograft-bearing mice treated with vehicle or CI-1033 (5 or 30 mg/kg) at treatment days 7, 14, and 21. Expression of IL-8 and β-actin were determined by Northern blotting.

efficacy of CI-1033. The down-regulation of IL-8 mRNA levels by CI-1033 at early time points also suggests that CI-1033 exerts a direct effect on IL-8 expression and secretion into plasma.

Discussion

The increasing amount of research dedicated to characterization of gene products and function has resulted in development of molecular-targeted therapies for a number of diseases including human cancers. The development of effective targeted therapies for human cancers may result in a paradigm shift for treatment of cancers from short-term treatment with cytotoxic drugs to long-term treatment with targeted therapies that have a static effect on tumor growth. The evaluation of clinical response to targeted therapies may, thus, be challenging depending on the nature of the predicted response to treatment. Therefore, the identification and validation of surrogate biomarkers of efficacious treatment with targeted therapies such as CI-1033 will be useful in the monitoring of clinical response, design of treatment schedules, and selection of the appropriate clinical dose.

The present studies investigating the effects of the erbB family inhibitor CI-1033 on potential biomarkers of antitumor efficacy indicate that VEGF and IL-8 are modulated by CI-1033 *in vivo*. Neither VEGF nor IL-8 was detectable in plasma of nontumor-bearing mice indicating that the presence of these factors in the plasma of xenograft-bearing mice was due to their secretion by cells from tumor tissue *in vivo*. Therefore, the decrease in plasma concentrations of VEGF and IL-8 following CI-1033 treatment was due to the inhibition of secretion of VEGF and IL-8 by tumors. In addition, the decrease in VEGF mRNA levels in A431 and H125 xenografts and the decrease in IL-8 mRNA levels in SF767 and MDA-MB-468 following CI-1033 treatment indicate that the direct down-regulation of these factors contributes to decreases in plasma levels of these factors. The direct modulation of VEGF and IL-8 at time points before the observation of an effect on tumor mass suggest that these proteins could potentially be used as predictors of an antitumor effect. Another factor potentially contributing to the decrease in plasma levels of VEGF and IL-8 is the inhibition of tumor growth by CI-1033. In untreated animals, plasma levels of VEGF correlated with A431 and H125 tumor mass, whereas plasma levels of IL-8 correlated with SF767 and MDA-MB-468 tumor mass. Thus, the inhibition of xenograft growth by CI-1033 would also be predicted to affect plasma levels of VEGF and IL-8. Collectively, these results indicate that reduction of plasma VEGF and IL-8 may be due to direct down-regulation of gene expression as well as VEGF- and IL-8-independent inhibition of tumor growth.

One important feature of surrogate biomarkers is correlation of biomarker modulation with an antitumor response. Treatment with efficacious doses of CI-1033 modulated plasma VEGF and IL-8 levels, whereas treatment with subefficacious doses did not affect their plasma levels. For plasma VEGF, treatment of A431 and H125 xenograft-bearing mice with doses of CI-1033 that inhibited tumor growth caused a decrease in plasma levels of VEGF, whereas treatment of mice with doses of CI-1033 that did not have an effect on xenograft growth also had no effect on VEGF plasma levels. Interestingly, although a dose-response was observed with tumor growth inhibition by CI-1033, a dose-response pattern was not observed with plasma VEGF levels in these tumor models. The lack of a dose-response pattern associated with VEGF levels may indicate that inhibition of VEGF secretion by tumor cells is a sensitive molecular response to CI-1033 treatment. The sensitivity of VEGF to down-regulation by CI-1033 may indicate that modulation of other molecular responses or pathways may be necessary to attain a maximum response to this compound. In terms of biomarker identification, this lack of dose-response may suggest that the monitoring of a battery of biomarkers may be necessary to fully predict the response to drugs belonging to this class. For plasma IL-8, treatment of MDA-MB-468 and H125 xenograft-bearing mice with doses of CI-1033 that inhibited tumor growth caused a decrease in plasma levels of IL-8, whereas treatment of mice with subefficacious doses of CI-1033 had no effect on IL-8 plasma levels. These results indicate that reduction of plasma both VEGF and IL-8 levels by CI-1033 correlated with antitumor efficacy.

In addition to correlation of biomarkers with antitumor efficacy, correlations of the biomarker with the proposed antitumor mechanism of the drug and with causal pathways in cancer are hallmarks of predictive surrogate markers. Several studies suggest that VEGF and IL-8 correlate with cancer progression and severity and that they

may be downstream regulators of erbB family–mediated angiogenesis. The importance of neovascularization and vascular permeability in tumor progression has been well established. Both VEGF and IL-8 have been shown to be positive regulators of tumor angiogenesis in several types of human cancer (19–22, 28–30). Several lines of information suggest that the EGFR/erbB receptor family and its ligands also regulate the process of angiogenesis in tumors. Ligands for the erbB receptor family, including EGF, transforming growth factor-α, and heregulins, were shown to act as chemoattractants for endothelial cell lines (37). In addition, expression of erbB family members and their ligands correlated with microvessel density in certain human cancers (38–40). Furthermore, erbB family signaling was shown to be a positive regulator of both VEGF and IL-8 expression, whereas inhibition of EGFR or HER-2 (by Gemfitinib, Cetuximab, or Trastuzumab) down-regulated VEGF and IL-8 expression (16, 17, 27, 41–44). The presence of activator protein sites in the promoters of the *VEGF* and *IL-8* genes may at least partially contribute to their regulation by erbB receptor signaling suggesting that other genes that are similarly regulated may be modulated during the response to CI-1033 (32, 33).

Because both human VEGF and IL-8 function on murine receptors, it is feasible that at least part of the antitumor mechanism of CI-1033 observed in these studies is due to the inhibition of VEGF- and IL-8-dependent angiogenesis. The antiangiogenic mechanism of CI-1033 was supported by imaging studies using Lisca Doppler laser ultrasonography indicating the A431 tumor xenograft blood flow and tissue perfusion was markedly reduced (as early as day 4) following repeat-dose administration of the compound (data not shown). Previous studies have reported good correlation of microvessel density and tumor VEGF with Doppler ultrasonography imaging of tumor blood flow in preclinical tumor models (45–47). Collectively, these studies support the involvement of VEGF and IL-8 in angiogenesis as well as in the antitumor mechanism of CI-1033 and implicate these markers in the pathogenesis of human cancers. Although other erbB family inhibitors were not evaluated in the present studies, the strong link established between the erbB receptor family and VEGF and IL-8 regulation and the observed effect of other erbB inhibitors on these markers suggest that there is greater applicability for this biomarker approach to other erbB family inhibitors outside of CI-1033 (17, 27, 44).

In addition to the present experimental data characterizing plasma markers for erbB inhibitor, the use of VEGF and IL-8 as biomarkers is also supported by preliminary studies indicating VEGF and IL-8 are detectable in both tumor biopsy material and plasma samples taken from patients with cancers from a variety of indications (data not shown). Furthermore, although variability was observed across cancer patients from different indications, levels in a given patient over time were fairly consistent suggesting that pre–drug administration plasma biomarker levels in a selected patient could serve as controls over a course of therapy. However, one caveat associated with the approach to monitoring plasma markers such as VEGF and IL-8 is that several factors independent of drug treatment could potentially influence plasma levels of these markers in a in clinical setting. Multiple factors, including (a) multiple cell and tissue types as potential sources of these plasma proteins; (b) alteration of their levels by other physiologic influences such as presence of other disease conditions, wound healing, or immune responses; and (c) complexity of genetic regulation of these factors, could each lead to intrapatient variability resulting in increased complexity of analysis of the effects of the drug on plasma levels of certain biomarkers. Potential solutions to these complexities in a clinical setting could include the analysis of a battery of biomarkers, analysis of multiple tissues, and use of multiple approaches in evaluation of response (e.g., analysis of target activity using tumor biopsy material, tumor imaging, classic tumor staging, etc). The cross-validation of several approaches in a clinical setting and later validation using patient response and survival benefit data will determine the utility of biomarker-based approaches in a clinical oncology setting over time. Although potential early positive trends with soluble biomarkers in individual patients have been observed in ongoing clinical studies with CI-1033, the true clinical use of VEGF and IL-8 (or similar approaches) as biomarkers will only be determined by their correlation to durable response to therapy in a large patient cohort in a clinical setting.

In conclusion, CI-1033 was efficacious against in a variety of tumor models. Oral administration of CI-1033 caused a decrease in plasma levels of VEGF or IL-8 in a number of models. The decreases of plasma levels of VEGF or IL-8 correlated with antitumor efficacy in these models. In addition, a direct effect on gene expression as well as effects on tumor mass caused the decrease in VEGF or IL-8 plasma levels. Both VEGF and IL-8 fit several criteria for biomarkers, including involvement in causal pathways of cancer, association with drug mechanism, and ease of detection in accessible tissues. These studies indicate that plasma VEGF and IL-8 may have use as biomarkers of antitumor efficacy for EGFR/erbB targeted therapies such as CI-1033. Furthermore, the involvement of VEGF and IL-8 in tumor angiogenesis suggests that a partial mechanism of the antitumor effects of CI-1033 are mediated by inhibition of angiogenesis.

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