SU11248 Inhibits KIT and Platelet-derived Growth Factor Receptor β in Preclinical Models of Human Small Cell Lung Cancer

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Abstract

The purpose of this study was to evaluate the activity of the indolinone kinase inhibitor SU11248 against the receptor tyrosine kinase KIT *in vitro* and *in vivo*, examine the role of KIT in small cell lung cancer (SCLC), and anticipate clinical utility of SU11248 in SCLC. SU11248 is an oral, multitargeted tyrosine kinase inhibitor with direct antitumor and antiangiogenic activity through targeting platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor, KIT, and FLT3 receptors. Treatment of the KIT-expressing SCLC-derived NCI-H526 cell line *in vitro* with SU11248 resulted in dose-dependent inhibition of stem cell factor-stimulated KIT phosphotyrosine levels and proliferation. The biological significance of KIT inhibition was evaluated *in vivo* by treating mice bearing s.c. NCI-H526 tumors with SU11248 or another structurally unrelated KIT inhibitor, STI571 (Gleevec), which is also known to inhibit Bcr-Abl and PDGFRβ. SU11248 treatment resulted in significant tumor growth inhibition, whereas inhibition from STI571 treatment was less dramatic. Both compounds reduced phospho-KIT levels in NCI-H526 tumors, with a greater reduction by SU11248, correlating with efficacy. Likewise, phospho-PDGFRβ levels contributed by tumor stroma and with known involvement in angiogenesis were strongly inhibited by SU11248 and less so by STI571. Because platinum-based chemotherapy is part of the standard of care for SCLC, SU11248 was combined with cisplatin, and significant tumor growth delay was measured compared with either agent alone. These results expand the profile of SU11248 as a KIT signaling inhibitor and suggest that SU11248 may have clinical potential in the treatment of SCLC via direct antitumor activity mediated via KIT as well as tumor angiogenesis via vascular endothelial growth factor receptor FLK1/KDR and PDGFRβ.

Introduction

SCLC[2] is an aggressive disease representing approximately 20% of lung cancers (1). Two-thirds of patients present with extensive stage disease, which is highly responsive to first-line therapy, such as etoposide and cisplatin. However, the vast majority of cases of SCLC recur and then rapidly progress, with a median survival time of 7–10 months (2). New therapeutic agents for SCLC are urgently needed.

The molecular abnormalities underlying SCLC are not well understood. One feature of SCLC is coexpression of the KIT RTK with its ligand, SCF, in up to 70% of SCLC cell lines and clinical SCLC samples. SCF-stimulated KIT signaling has been most extensively studied in its role of regulating proliferation and survival of hematopoietic progenitor cells (3). However, KIT activation has also been shown to stimulate proliferation of SCLC-derived cell lines in culture (4), and dysregulated KIT activation has been implicated in driving some tumors; for example, activating mutations of KIT have been identified in gastrointestinal stromal tumors (5, 6), acute myeloid leukemia, and some cases of mastocytosis (7). Whereas activating KIT mutations have not been observed in SCLC, the coexpression of KIT and SCF in SCLC suggests that an autocrine activation loop may result in elevated or prolonged KIT signaling in this malignancy and that interrupting this signaling pathway may provide a novel therapeutic approach to treat this devastating disease. As evidence for KIT signaling effects on growth in SCLC, stable transfection of KIT in a SCLC line that naturally produced SCF led to autocrine growth stimulation (4). In addition, expression of a kinase-defective form of KIT in a cell line that naturally coexpresses ligand and receptor led to a marked decrease in the ability of the cells to grow under growth factor-free conditions (4). Several KIT inhibitors have also been shown to block SCF-stimulated SCLC proliferation *in vitro* (8–11). However, the role of KIT activity in SCLC *in vivo* is not known.

The intention of these studies was to evaluate the activity of the indolinone kinase inhibitor SU11248 against KIT *in vitro* and *in vivo* and to model the utility of SU11248 for the treatment of SCLC. SU11248 is a selective, oral, multitargeted RTK kinase inhibitor of PDGFR, VEGFR, and FLT3 with

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[2] The abbreviations used are: SCLC, small cell lung cancer; SCF, stem cell factor; RTK, receptor tyrosine kinase; PDGFR, platelet-derived growth factor receptor, VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; IGF-I, insulin-like growth factor I, IGF-IR, IGF-I receptor; FBS, fetal bovine serum; IP/W, immunoprecipitation and Western blot; ERK, extracellular signal-regulated kinase; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; IHC, immunohistochemical.

5–50 nM IC_{50} values in cellular autophosphorylation assays (12, 13). SU11248 was also designed to target KIT and was expected to inhibit KIT kinase activity in cells.

SU11248 has shown antitumor activity by inhibiting both RTKs expressed by tumor cells and involved in tumor proliferation and survival and RTKs expressed on endothelial or stromal cells. As an example of the former, SU11248 blocks the activity of wild-type and activated FLT3 expressed by acute myelogenous leukemia-derived cell lines (13). The latter mechanism of inhibition is supported by the fact that SU11248 also inhibits the VEGFR FLK/KDR and PDGFRβ, both of which play a prominent role in angiogenesis (14).

As a comparator KIT inhibitor in the *in vivo* studies, the aminopyrimidine STI571 (imatinib mesylate, Gleevec; Novartis Pharmaceuticals, East Hanover, NJ), a structurally distinct tyrosine kinase inhibitor, was used. STI571, like SU11248, inhibits both KIT and PDGFR, but with an IC_{50} of ~100 nM in cellular assays (15, 16). STI571 is an effective inhibitor of KIT autophosphorylation and SCF-stimulated proliferation in SCLC cellular assays (10).

Materials and Methods

Cell Lines. The NCI-H526 and NCI-H82 human SCLC cell lines were obtained from American Type Culture Collection (Manassas, VA). KIT expression in these cell lines was initially determined by fluorescence-activated cell-sorting analysis using an antihuman antibody KIT (CD117; Becton Dickinson, Franklin Lakes, NJ) and FACScan.

Cells were cultured using standard technique in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and 1 mM sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD) and maintained routinely in a humidified chamber at 37°C and 5% carbon dioxide. When grown under conditions for serum deprivation, 0.1% FBS (Life Technologies, Inc.) was added to the medium.

IP/W Analysis *in Vitro*. NCI-H526 cells were starved in low serum (0.1% FBS) overnight before treatment with SU11248 for 2 h. After compound treatment, cells were stimulated with 250 ng/ml recombinant human SCF or 20 ng/ml IGF-I (R&D Systems, Minneapolis, MN) for 15 min. Cells were then lysed with HNTG lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 1 mM EGTA) containing protease and phosphatase inhibitors [10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM Pefabloc (Roche Molecular Biochemicals); 2 mM bestatin, 280 μM E-64, 200 μM leupeptin, 200 μM pepstatin A, and 60 μM aprotinin], and protein concentration in lysates was determined using the BCA assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). One mg of protein from each sample was immunoprecipitated overnight at 4°C with an anti-KIT antibody (SC-1493; Santa Cruz Biotechnology, Santa Cruz, CA) and protein G-agarose beads (16-266; Upstate Biotechnology, Lake Placid, NY). Immune complexes were washed with HNTG lysis buffer containing inhibitors. Proteins were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were probed with an anti-phosphotyrosine antibody (SC-7020; Santa Cruz Biotechnology) and then stripped with Restore Western Blot Stripping Buffer (Pierce). To detect total KIT levels, membranes were reprobed with the same anti-KIT antibody that was used for the immunoprecipitation. Lysates were also immunoprecipitated with an anti-IGF-IR antibody (SC-713; Santa Cruz Biotechnology), and Western blot analysis was performed using antibodies against phosphotyrosine (SC-7020; Santa Cruz Biotechnology) or total IGF-IR, as described above. Western blot analysis of ERK1/2 (Thr 202/Tyr 204; 9106; Cell Signaling, Beverly, MA) was performed on whole cell lysates (40 μg) as described above.

Cell Proliferation Assay. NCI-H526 cells were starved in low serum overnight in a medium containing 0.1% FBS. Cells were plated in 96-well plates at a density of 1×10^4 cells/well and treated for 30 min with SU11248 before ligand stimulation. Subsequently, cells were stimulated for 15 min with 100 ng/ml recombinant human SCF or 20 ng/ml IGF-I (R&D Systems) and cultured continuously in the presence of SU11248. After 72 h, cellular metabolism was assessed using the XTT colorimetric dye reduction method (1465015; Roche Molecular Biochemicals). XTT assays were also performed on NCI-H526 cells growing in 10% FBS.

Apoptosis Assay. NCI-H526 cells were cultured in low serum (0.1% FBS) overnight. A total of 5×10^4 cells/well were treated with SU11248 for 30 min before the addition of ligand and then stimulated with 100 ng/ml recombinant human SCF. Cells were incubated for 24 h at 37°C. Then cells were pelleted by centrifugation (1500 rpm for 5 min), washed in 1 ml of culture medium, and pelleted again. Cell pellets were processed according to the manufacturer's protocol using the Cell Death Detection ELISA KIT (Roche Molecular Biochemicals) for determination of histone-associated DNA fragments.

***In Vivo* Implantation of SCLC and Efficacy Studies.** Cells to be implanted in mice were harvested from cell culture flasks during exponential growth, washed once with sterile PBS, counted, and resuspended in PBS to a suitable concentration before implantation.

All animal studies were carried out with the approval of the SUGEN Institutional Animal Care and Use Committee in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited vivarium and in accordance with the Institute of Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals, NIH, Bethesda, MD). Female 9–12-week-old athymic *nu/nu* mice purchased from Charles River Laboratories (Wilmington, MA) were used.

Mice received s.c. injections into the hind flank on day 0 with 5×10^6 NCI-H526 or NCI-H82 cells. Animals with established tumors (approximately 300–500 mm^3) were randomized into treatment groups of 10 mice each for efficacy studies. SU11248, STI571, or their vehicle was administered as indicated in the figure legends. SU11248 and STI571 were delivered p.o. in a carboxymethylcellulose suspension by gavage. Tumor growth was measured twice weekly using Vernier calipers for the duration of the treatment. Tumor volumes were calculated as the product of length $\times$ width $\times$ height. For all studies, *P* values were calculated using the two-tailed Student's *t* test.

Combination Treatment of SU11248 and Cisplatin against NCI-H526 SCLC Tumors. Mice bearing s.c. tumors established to 250–300 mm^3 in volume were treated either p.o. once daily with SU11248 to the end of the study, i.p. once daily with cisplatin for the first 5 days, or a combination of the two. Cisplatin was prepared in 0.9% saline. Compounds or their vehicles were administered as indicated in the figure legends. Tumor growth was measured twice weekly using Vernier calipers for the duration of the treatment. Tumor volumes were calculated as the product of length × width × height. For all studies, *P*s were calculated using the two-tailed Student's *t* test.

***In Vivo* Target Modulation Studies in NCI-H526 SCLC Tumors.** At the end of the efficacy studies evaluating the effects of SU11248 and STI571 on NCI-H526 tumor volume, mice bearing tumors were treated with a final oral dose of SU11248 or STI571 at the indicated concentrations. Control animals received an oral dose of vehicle. Four h after SU11248 treatment and 2 h after STI571 treatment (C_{max}), individual mice were euthanized, and tumors were resected. The resected tumors were immediately pulverized using a liquid nitrogen-cooled cryomortar and pestle and stored at −70°C until use. Pulverized tumors were later homogenized in cold HNTG lysis buffer with protease and phosphatase inhibitors (see "IP/W Analysis *in Vitro*"). The lysates were centrifuged at 14,000 rpm for 25 min at 4°C. The resulting supernatant was collected, and the amount of protein in each lysate was immediately determined using the BCA Protein Assay (Pierce). One mg of lysate from each sample was then immunoprecipitated overnight at 4°C using an agarose-conjugated antibody to KIT (SC-1493AC; Santa Cruz Biotechnology) or PDGFRβ (06-498; Upstate Biotechnology). Immunoprecipitated protein was resolved by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was then blocked for 1 h at room temperature in 5% nonfat milk in TBST [1 mM Tris (pH 7.4), 150 mM NaCl, and 1% Triton X-100]. The amount of phosphorylated KIT in each sample was determined by probing the membranes with an antibody to phosphotyrosine (UBI4G10; Upstate Biotechnology) overnight at 4°C; for the analysis of total KIT or PDGFR, the blots were stripped, reblocked, and reprobed with an antibody to KIT (A-4542; DAKO Corp., Carpinteria, CA) or PDGFRβ (SC-432; Santa Cruz Biotechnology) overnight at 4°C. After washing, the membranes were incubated with either antimouse or antirabbit horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h at room temperature, washed again, and developed by the enhanced chemiluminescence method according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech).

KIT and PDGFRβ Immunohistochemistry. Sections were prepared from formalin-fixed, paraffin-embedded s.c. tumors. For detection of KIT, a rabbit polyclonal antihuman antibody (A-4502; DAKO Corp.) and horseradish peroxidase enzyme-labeled polymer conjugated to polyclonal rabbit secondary antibodies (EnVision+ Systems; DAKO Corp.) were used. For PDGFRβ detection, a rabbit polyclonal antibody (SC-432; Santa Cruz Biotechnology) and a biotinylated goat antirabbit secondary antibody (Vector Laboratories, Burlingame, CA), followed by a peroxidase-based immunostaining protocol (Vectastain ABC Elite KIT; Vector Laboratories), were used. In both cases, peroxidase enzyme was developed with 3,3′-diaminobenzidine as a substrate.

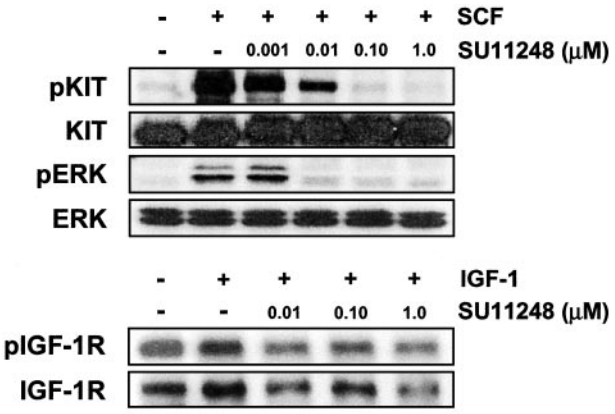

Fig. 1. Dose-dependent inhibition of SCF-stimulated phosphorylation of KIT and downstream ERK1/2 by SU11248 in NCI-H526 cells. Cells were serum starved in 0.1% FBS overnight and then incubated with the indicated concentrations of SU11248 for 2 h. Control cells received the drug vehicle DMSO. Cells were then stimulated (+) or not stimulated (−) with a final saturating concentration of 100 ng/ml SCF or 20 ng/ml IGF-I for 15 min, lysed, immunoprecipitated with anti-KIT or anti-IGF-IR antibodies, and then used for Western blot analysis. Blots were immunostained with an anti-phosphotyrosine antibody. The membranes were stripped and reprobed with an anti-KIT or anti-IGF-IR antibody. Additionally, whole cell lysates from SCF-stimulated cells were Western blotted and immunostained with anti-ERK1/2 and anti-phospho-ERK1/2 (Thr 202/Tyr 204).

Results

SU11248 Reduces KIT Phosphotyrosine Levels *in Vitro*. The NCI-H526 SCLC cell line used in these studies has been reported to express KIT (8). We confirmed KIT expression by fluorescence-activated cell-sorting analysis (data not shown) and immunoblotting against human KIT (Fig. 1). The KIT ligand, SCF, has been shown to induce tyrosine phosphorylation of KIT and proliferation of the SCLC cell line NCI-H526 (8). To investigate the ability of SU11248 to inhibit KIT kinase activity, SCF-induced tyrosine phosphorylation of KIT in the presence of varying concentrations of SU11248 was monitored by IP/W analysis. As shown in Fig. 1, SU11248 reduced SCF-induced phosphotyrosine levels on KIT in a dose-dependent manner, with an IC_{50} value between 0.001 and 0.01 μM. To examine the effect of SU11248 treatment on a SCF-mediated signaling event downstream of KIT activation, ERK1/2 phosphorylation was investigated. As shown in Fig. 1, SU11248 also inhibited SCF-stimulated ERK1/2 phosphorylation in a dose-dependent manner, with an IC_{50} value consistent with that for inhibition of KIT phosphotyrosine levels. NCI-H526 cells also express IGF-IR, which is not a target of SU11248. To demonstrate compound selectivity in NCI-H526 cells, SU11248 did not significantly inhibit IGF-IR phosphotyrosine levels up to 1 μM, as monitored by IP/W analysis (Fig. 1).

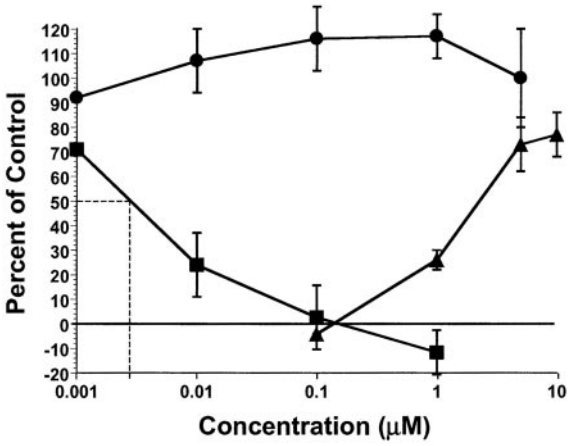

Fig. 2. SU11248 selectively inhibits SCF-induced proliferation of KIT-expressing SCLC cells. NCI-H526 SCLC cells were incubated in medium containing 0.1% serum in the presence of increasing concentrations of SU11248. Thirty min after compound administration, cells were stimulated to proliferate with addition of either 100 ng/ml SCF (■) or 20 ng/ml IGF-I (●). The number of metabolically active cells present after 72 h was determined by XTT assay. Data are presented as the percentage change in cell number during drug treatment relative to DMSO control and represent mean values obtained from three to four independent experiments. For apoptosis induction, NCI-H526 cells were incubated for 24 h with the indicated concentrations of SU11248 in serum-free medium plus SCF and then analyzed by ELISA for apoptotic cells. Data are expressed as the percentage of apoptotic cells relative to DMSO vehicle control (▲).

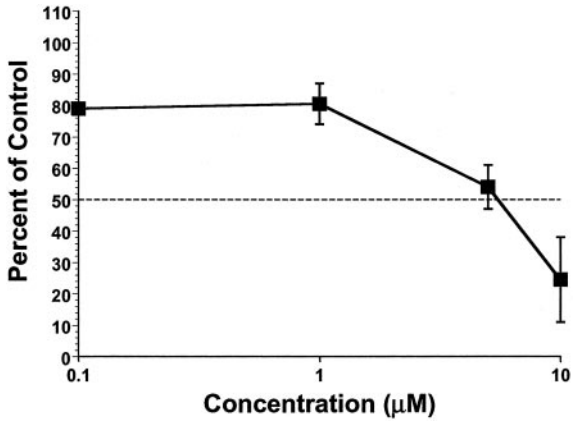

Fig. 3. Higher concentrations of SU11248 are required to inhibit proliferation of KIT-expressing SCLC cells in the presence of serum. NCI-H526 cells were incubated in medium containing 10% FBS in the presence of increasing concentrations of SU11248 (■). The number of metabolically active cells present after 72 h was determined by XTT assay. Data are presented as the percentage change in cell number during drug treatment relative to DMSO control and represent mean values obtained from three independent experiments.

SU11248 Inhibits SCF-induced Proliferation, but Higher Concentrations Are Required to Inhibit Growth in Complete Medium. Consistent with the demonstration that SU11248 inhibited SCF-induced tyrosine phosphorylation of KIT, it also inhibited SCF-stimulated proliferation in NCI-H526 cells, as measured by XTT assay. As shown in Fig. 2, inhibition of NCI-H526 cell proliferation was dose dependent, with an IC_{50} value of 0.002 μM. Concentrations of SU11248 below 0.1 μM did not result in absorbance readings below the level of the untreated control cells, suggesting that at these drug concentrations, inhibition of proliferation occurred without a cytotoxic response. Additionally, an ELISA assay detecting histone-associated DNA fragments revealed that apoptosis occurred at doses higher than those required to inhibit SCF-induced proliferation. In contrast, SU11248 did not inhibit IGF-I-stimulated proliferation of NCI-H526 cells.

In the studies above, the effects of SU11248 were examined in a cell system designed to define the consequences of specifically inhibiting SCF-stimulated KIT activity. *In vivo*, tumors are exposed to a complex mixture of growth factors. To determine the effect of SU11248 on growth in the presence of multiple growth factors, NCI-H526 SCLC cells were treated with increasing concentrations of SU11248 in medium containing 10% serum (Fig. 3). Proliferation was normalized to a control culture treated only with the DMSO vehicle. Little growth suppression was observed in cultures treated with $\leq$1 μM SU11248. At higher concentrations, dose-dependent growth inhibition was observed.

***In Vivo* Growth Inhibition by SU11248 and STI571.** The inhibition of KIT phosphotyrosine levels and SCF-driven proliferation prompted us to test SU11248 against tumor growth *in vivo*. STI571, a known inhibitor of KIT, was also included as a comparator in the *in vivo* studies. Both compounds were evaluated in s.c. NCI-H526 (KIT-positive) and NCI-H82 (KIT-negative) tumor xenograft models. Mice received SU11248 p.o. at 40 and 80 mg/kg/day, initiated when SCLC tumors of approximately 300–500 mm^3 were established. STI571 was administered p.o. at 160 mg/kg twice daily, a dose within the range reported to be efficacious (17–19).

Tumor volumes were measured and compared with tumors from animals that received drug vehicle alone. Fig. 4*A* shows that SU11248 p.o. administered at 40 and 80 mg/kg/day had significant *in vivo* activity against the NCI-H526 human SCLC tumor xenograft, resulting in inhibition of growth [day 36, 57% inhibition ($P = 0.001$) and 74% inhibition ($P = 0.0002$), respectively]. STI571 p.o. administered twice daily at 160 mg/kg also demonstrated efficacy, exhibiting up to 35% inhibition ($P = 0.04$) on day 31. In Fig. 4*B*, SU11248 administered at 40 and 80 mg/kg/day also significantly inhibited the KIT-negative NCI-H82 SCLC tumor model [day 39, 46% inhibition ($P = 0.05$) and 75% inhibition ($P = 0.0013$), respectively]. STI571 had no effect on tumor growth in this model.

SU11248 and STI571 Inhibit KIT and PDGFRβ Phosphotyrosine Levels *in Vivo*. To investigate KIT inhibition *in vivo*, SU11248-, STI571-, and vehicle-treated NCI-H526 tumors were collected at the end of the efficacy study. Treatment with SU11248 resulted in a significant dose-dependent reduction in KIT phosphotyrosine levels as compared with tumors from vehicle-treated mice (Fig. 5). Treatment with STI571 also resulted in evident reduction of KIT phosphotyrosine relative to the control, although to a lesser extent than SU11248.

Because activities of SU11248 and STI571 (12, 20) are known to overlap in targeting not only KIT, but PDGFRβ as well, the expression of KIT and PDGFRβ was evaluated by IHC methods to determine localization of their expression in

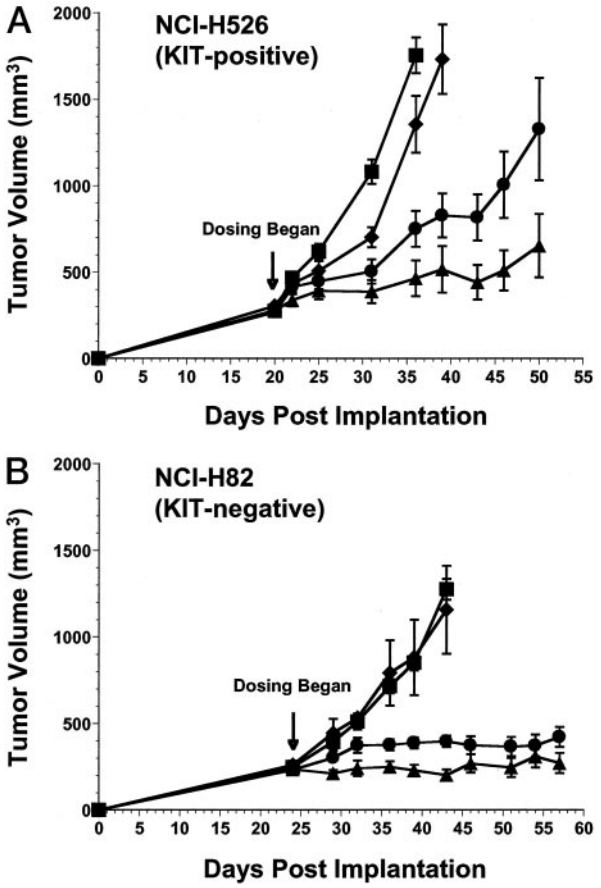

Fig. 4. Antitumor activity of SU11248 and STI571 on established human SCLC xenografts in athymic mice. NCI-H526 (*A*) or NCI-H82 (*B*) cells were inoculated s.c. into athymic mice (5×10^6 cells/mouse). Oral administration of SU11248 at 80 (▲) or 40 (●) mg/kg/day, STI571 (♦) at 160 mg/kg/twice daily, or vehicle (■) was initiated when tumors reached an average of approximately 300–400 mm^3 in volume (indicated by *arrow*) and continued through the end of the experiment. Tumor volume was measured on the indicated days, with the mean tumor volume ± SE indicated for each group (10 mice/group).

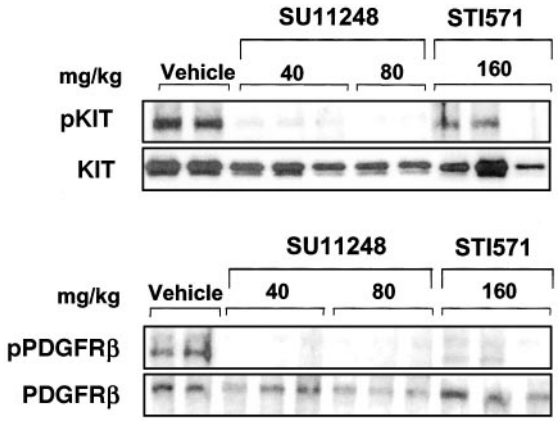

Fig. 5. SU11248 inhibits KIT and PDGFRβ phosphorylation *in vivo*. From the end of the efficacy studies, athymic mice bearing NCI-H526 SCLC s.c. tumors were given a single oral dose of SU11248 at 40 or 80 mg/kg, STI571 at 160 mg/kg, or vehicle control. Tumors were resected and lysed at the expected C_{max} for each compound (4 h post-dose for SU11248 and 2 h post-dose for STI571). Lysates were immunoprecipitated with an anti-KIT or anti-PDGFRβ antibody. Phosphotyrosine and total KIT and PDGFRβ levels were determined by Western blotting.

the tumors. As expected, KIT expression was localized to the SCLC tumor epithelial cells in NCI-H526 (Fig. 6*A*) and was not present in NCI-H82 tumors (Fig. 6*B*). Whereas PDGFRβ expression was not detectable by IP/W in either cell line grown in culture, PDGFRβ was detected in tumor stroma as shown by IHC methods (Fig. 6, *C* and *D*). PDGFRβ phosphorylation in SU11248- and STI571-treated NCI-H526 tumors from the end of the efficacy study was evaluated. PDGFRβ phosphotyrosine levels in the tumors were reduced by SU11248 and reduced to a lesser extent by STI571 (Fig. 5). Thus, the compounds may be acting not only against the SCLC tumor epithelium, but also against its supporting stroma, including the vasculature.

Combination of SU11248 with Cisplatin Improved Inhibition of NCI-H526 SCLC Tumors. Treatment of NCI-H526 tumors with SU11248 alone resulted in a tumor growth delay. Because platinum-based drugs are the standard of care for SCLC (21), SU11248 was combined with cisplatin in the NCI-H526 s.c. tumor model. SU11248 was administered p.o. daily at a dose of 40 mg/kg to the end of the study. Cisplatin was administered i.p. at 1.5 mg/kg for the first 5 days of SU11248 treatment. Tumor volumes were compared with those in animals receiving each agent alone. Fig. 7 shows that the antitumor activity by monotherapy of SU11248 or cisplatin was enhanced by coadministration of the two compounds, resulting in a 29-day delay of tumor growth to reach a volume of 900 mm^3 compared with SU11248 monotherapy and a 22-day delay compared with cisplatin monotherapy ($P < 0.0001$ and $P = 0.004$, respectively). The combination was well tolerated.

Discussion

SU11248 is an oral, multitargeted, small molecule with antitumor and antiangiogenic activity through selectively targeting PDGFR, VEGFR, KIT, and FLT3. By inhibiting the activity of these receptors, SU11248 directly targets tumor cell proliferation and survival in cancers where these receptors are involved. SU11248 has been demonstrated to inhibit FLK/KDR and PDGFRβ phosphorylation *in vivo* (12) and exhibits antitumor activity in a large number of preclinical tumor xenograft models, including leukemia expressing FLT3-activating mutations (12, 13).

Activating mutations in KIT have been implicated in a number of neoplasias, in particular gastrointestinal stromal tumors, adult-onset mastocytosis, and acute myelogenous leukemia (5, 6). The extent to which growth and survival of these diseases rely on KIT signaling remains to be determined. However, exploitation of the anti-KIT activity of STI571 (Gleevec) resulted in objective responses in patients with gastrointestinal stromal tumors, and Gleevec is now approved for this indication. KIT activity has also been implicated in regulating the proliferation *in vitro* of cell lines derived from human SCLC through autocrine and/or paracrine stimulation of the receptor by its ligand (4). This, to-

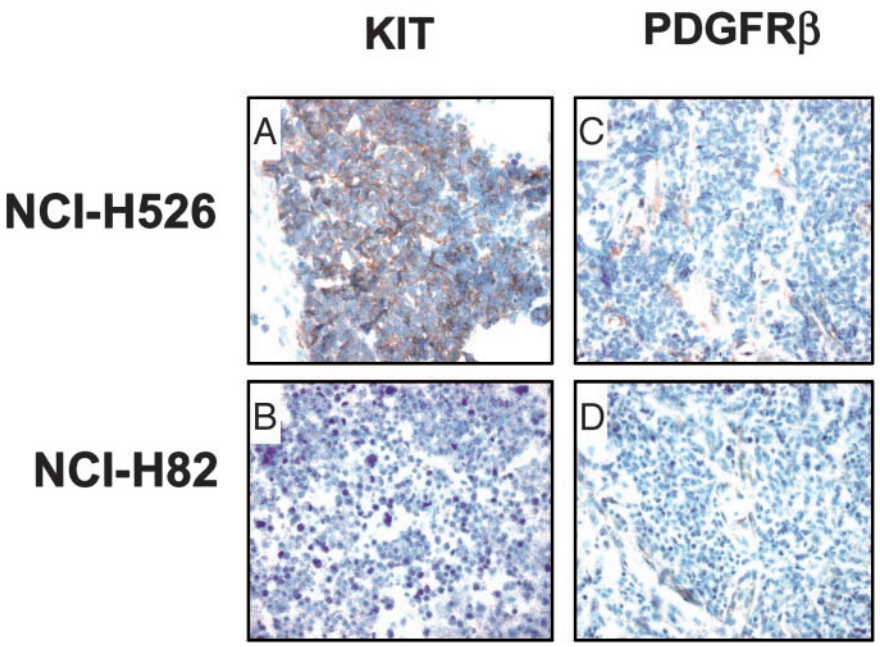

Fig. 6. IHC characterization of KIT and PDGFRβ in s.c. SCLC tumors. Representative slides from NCI-H526 tumors immunostained with antibodies to KIT and PDGFRβ (*A* and *C*, respectively), demonstrating expression of KIT in the SCLC and PDGFRβ in the stroma. Representative slides from NCI-H82 tumors immunostained for KIT and PDGFRβ (*B* and *D*, respectively), demonstrating lack of KIT expression in SCLC and PDGFRβ expression in the stroma.

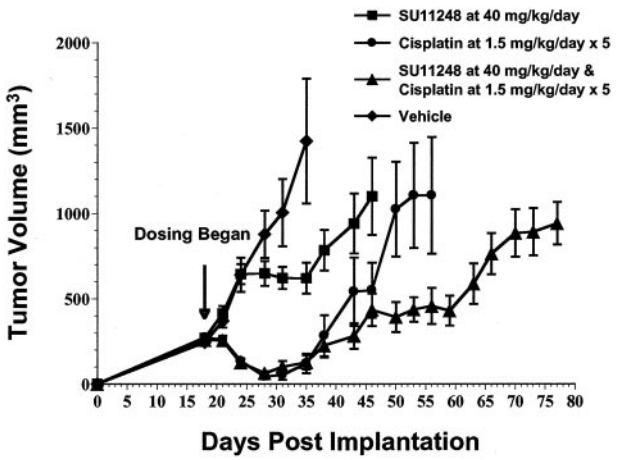

Fig. 7. Combination of SU11248 with cisplatin improves inhibition of NCI-H526 SCLC tumors. NCI-H526 cells were inoculated s.c. into athymic mice (5×10^6 cells/mouse). Oral administration of SU11248 at 40 mg/kg/day to the end of the study, i.p. administration of cisplatin at 1.5 mg/kg for the first 5 consecutive days of dosing, or a combination of the two was initiated when tumors reached an average of approximately 200–300 mm^3 in volume (indicated by *arrow*). Tumor volume was measured on the indicated days, with the mean tumor volume ± SE indicated for each group (10 mice/group).

gether with data on frequent KIT overexpression in human SCLC (22–25), at times associated with SCF coexpression (22–24), suggests that blocking the function of KIT may be a useful therapeutic approach for SCLC.

Using the KIT-positive NCI-H526 SCLC cell line, we demonstrated that SU11248 inhibited SCF-dependent KIT phosphorylation, signal transduction, and cell proliferation *in vitro*. In the presence of complete serum, the IC_{50} value for inhibition of proliferation was much higher than that for SCF-stimulated proliferation, as reported previously for STI571 (22–24). This is likely due to the contribution of multiple growth factors in serum stimulating multiple signaling pathways including those not inhibited by SU11248 at submicromolar concentrations.

KIT signaling has been shown to be important for survival in hematopoietic cells (26). However, we found no effect of SU11248 on apoptosis in SCF-stimulated cultured cells at levels that block KIT activity. SCLC tumor-derived cells may be less dependent on KIT signaling for survival than are hematopoietic cells. Many tumor-derived lines secrete autocrine growth factors, some of which are likely to maintain survival in the absence of serum (27).

Consistent with the cytostatic nature of the drug *in vitro* against this cell line, SU11248 treatment significantly inhibited the growth of established s.c. KIT-expressing tumors in athymic mice, but it did not induce tumor regression. As a comparator in the *in vivo* studies, we used the structurally distinct aminopyrimidine tyrosine kinase inhibitor STI571 (Gleevec), known to inhibit KIT as well as Bcr-Abl and PDGFR (15, 16). STI571 is an effective inhibitor of KIT autophosphorylation and SCF-stimulated proliferation in SCLC cellular assays (10). In these efficacy studies, STI571 moderately inhibited tumor growth of NCI-H526.

These findings led to an evaluation of KIT phosphotyrosine levels in NCI-H526 tumor samples collected at the end of the efficacy studies. As predicted from *in vitro* assays, KIT phosphotyrosine levels were reduced by SU11248 in established SCLC tumors in a dose-dependent manner. STI571 partially reduced KIT phosphotyrosine levels in the these tumors, although it was less effective than SU11248 at the doses used in this study, consistent with the relative activities *in vitro* and lower degree of efficacy against NCI-H526 tumor growth.

The marked tumor growth inhibition of the KIT-negative SCLC line NCI-H82 by SU11248 prompted an IHC evaluation

of tumor xenograft sections for the expression of the angiogenic receptor PDGFRβ, another SU11248 target, expressed on pericytes surrounding endothelial cells and fibroblasts. PDGFRβ expression was identified in the supporting stroma of tumors derived from these SCLC lines. Furthermore, PDGFRβ phosphorylation was completely suppressed in SU11248-treated NCI-H526 tumor samples even at the lower dose used in this study. As observed with inhibition of KIT phosphotyrosine levels, STI571 partially reduced PDGFRβ phosphotyrosine levels in the NCI-H526 tumors. This may be due to the lower potency of STI571 against KIT and PDGFR and/or to pharmacokinetic properties in mice that prohibit tumor exposure sufficient to suppress the molecular targets of STI571. Additionally, the inhibitory target profile of SU11248 is different from that of STI571, and inhibition of SU11248 targets not affected by STI571, such as the VEGFR FLK1/KDR, may contribute to growth suppression.

Whereas SU11248 has been shown to inhibit targets expressed by tumor cells, as well as targets expressed in the supporting stromal cells (12), several factors suggest that the efficacy of SU11248 in these SCLC preclinical models may be conferred in part by targeting PDGFR and the VEGFR FLK1/KDR. First, SU11248 inhibits tumor growth at drug exposures that fully inhibit PDGFR while only partially suppressing KIT activity. PDGFR signaling on the pericytes supporting tumor vasculature is known to contribute to the process of neovascularization in growing tumors (14), as does FLK1/KDR signaling in endothelial cells (28, 29). Second, SU11248 has previously been demonstrated to inhibit FLK1/KDR phosphorylation *in vivo* and was similarly found to inhibit VEGF-induced vascular permeability in mice (12). VEGF expression and serum levels of VEGF have been found to be associated with poor prognosis (30–32) and shorter survival in SCLC patients (30). Although we have not examined the contribution of FLK1/KDR in these SCLC-derived tumors, it is a target of SU11248 that is likely contributing to the inhibition observed by SU11248 in this study.

The advent of molecular targeted oncology therapies offers the opportunity to selectively inhibit key molecular defects in human cancers and tailor therapies to distinct tumor types. The challenge in developing such therapies is to understand the relative contributions of different molecular defects to tumor growth, survival, and metastasis. In SCLC, KIT signaling has been implicated in pathogenesis based on KIT overexpression and coexpression of its ligand, SCF, in a majority of tumors.

We found that SU11248 inhibited KIT activity in SCLC-derived tumor xenografts and also inhibited tumor growth but was equally efficacious in blocking the growth of KIT-negative tumors. Thus, although KIT overexpression is common in SCLC tumors, it is unclear whether KIT is a major factor in driving the growth of SCLC, and it has yet to be established in the clinic that inhibition of wild-type KIT activity in a tumor will have an impact on that cancer.

The advantage of using multitargeted inhibitors is the opportunity to block tumor growth by multiple mechanisms. Despite the unclear role for KIT activity in these SCLC tumor xenografts, KIT signaling may contribute to clinical cases of the disease. SU11248 offers a potential novel therapy for this disease because beyond providing direct antitumor activity, SU11248 could provide additional significant benefit in the clinic via inhibition of PDGFRβ and FLK1/KDR in tissues supporting the cancer (12). In addition, we have shown that SU11248 in combination with cisplatin, the standard of care for first-line therapy in SCLC, was well tolerated and caused a significant delay in tumor regrowth compared with either agent alone. Small molecule inhibitors that target several distinct molecular targets regulating both tumor cell growth and survival directly and the supporting stroma of the tumor may provide the greatest impact in the clinic.

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References

1. Chia, M. M., Gazdar, A. F., Carone, D. P., and Minna J. P. Biology of lung cancer. *In*: J. F. Murray and J. A. Nadel (eds.). Textbook of Respiratory Medicine, 2nd ed., pp. 1485–1503. Philadelphia: W. B. Saunders, 1994.

2. Murren, J., Glatstein, E., and Pass, H. I. Small cell lung cancer. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles & Practice of Oncology, 6th ed., pp. 983–1018. Philadelphia: Lippincott Williams & Wilkins, 1993.

3. Lev, S., Blechman, J. M., Givol, D., and Yarden, Y. Steel factor and c-kit protooncogene: genetic lessons in signal transduction. Crit. Rev. Oncog., *5:* 141–168, 1994.

4. Krystal, G. W., Hines, S. J., and Organ, C. P. Autocrine growth of small cell lung cancer mediated by coexpression of c-kit and stem cell factor. Cancer Res., *56:* 370–376, 1996.

5. Lux, M. L., Rubin, B. P., Biase, T. L., Chen, C. J., Maclure, T., Demetri, G., Xiao, S., Singer, S., Fletcher, C. D., and Fletcher, J. A. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. Am. J. Pathol., *156:* 791–795, 2000.

6. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Muhammad Tunio, G., Matsuzawa, Y., Kanakura, Y., Shinomura, Y., and Kitamura, Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science (Wash. DC), *279:* 577–580, 1998.

7. Boissan, M., Feger, F., Guillosson, J. J., and Arock, M. c-Kit and c-kit mutations in mastocytosis and other hematological diseases. J. Leukocyte Biol., *67:* 135–148, 2000.

8. Krystal, G. W., Carlson, P., and Litz, J. Induction of apoptosis and inhibition of small cell lung cancer growth by the quinoxaline tyrphostins. Cancer Res., *57:* 2203–2208, 1997.

9. Krystal, G. W., Honsawek, S., Kiewlich, D., Liang, C., Vasile, S., Sun, L., McMahon, G., and Lipson, K. E. Indolinone tyrosine kinase inhibitors block Kit activation and growth of small cell lung cancer cells. Cancer Res., *61:* 3660–3668, 2001.

10. Krystal, G. W., Honsawek, S., Litz, J., and Buchdunger, E. The selective tyrosine kinase inhibitor STI571 inhibits small cell lung cancer growth. Clin. Cancer Res., *6:* 3319–3326, 2000.

11. Wang, W. L., Healy, M. E., Sattler, M., Verma, S., Lin, J., Maulik, G., Stiles, C. D., Griffin, J. D., Johnson, B. E., and Salgia, R. Growth inhibition and modulation of kinase pathways of small cell lung cancer cell lines by the novel tyrosine kinase inhibitor STI 571. Oncogene, *19:* 3521–3528, 2000.

12. Mendel, D. B., Laird, A. D., Xin, X., Louie, S. G., Christensen, J. G., Li, G., Schreck, R. E., Abrams, T. J., Ngai, T. J., Lee, L. B., Murray, L. J., Carver, J., Chan, E., Moss, K. G., Haznedar, J. O., Sukbuntherng, J., Blake, R. A., Sun, C., Tang, C., Miller, T., Shirazian, S., McMahon, G. and Cherrington, J. M. *In vivo* anti-tumor activity of SU11248, a novel tyrosine

kinase inhibitor targeting VEGF and PDGF receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin. Cancer Res., *9:* 327–337, 2002.

13. O'Farrell, A. M., Abrams, T. J., Yuen, H. A., Ngai, T. J., Louie, S. G., Yee, K. W. H., Wong, L. M., Hong, W. M., Lee, L. B., Town, A., Smolich, B. D., Manning, W. C., Murray, L. J., Heinrich, M. C., and Cherrington, J. M. SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity *in vitro* and *in vivo*. Blood, in press, 2003.

14. Cherrington, J. M., Strawn, L. M., and Shawver, L. K. New paradigms for the treatment of cancer; the role of anti-angiogenesis agents. *In:* G. Klein and G. F. Vande Woude (eds.), Advances in Cancer Research, 1st ed., pp. 1–38. San Diego, CA: Academic Press, 2000.

15. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat. Med., *2:* 561–566, 1996.

16. Carroll, M., Ohno-Jones, S., Tamura, S., Buchdunger, E., Zimmermann, J., Lydon, N. B., Gilliland, D. G., and Druker, B. J. CGP 57148, a tyrosine kinase inhibitor, inhibits the growth of cells expressing BCR-ABL, TEL-ABL, and TEL-PDGFR fusion proteins. Blood, *90:* 4947–4952, 1997.

17. Wolff, N. C., and Ilaria, R. L., Jr. Establishment of a murine model for therapy-treated chronic myelogenous leukemia using the tyrosine kinase inhibitor STI571. Blood, *98:* 2808–2816, 2001.

18. Sjoblom, T., Shimizu, A., O'Brien, K. P., Pietras, K., Dal Cin, P., Buchdunger, E., Dumanski, J. P., Ostman, A., and Heldin, C-H. Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. Cancer Res., *61:* 5778–5783, 2001.

19. le Coutre, P., Mologni, L., Cleris, L., Marchesi, E., Buchdunger, E., Giardini, R., Formelli, F., and Gambacorti-Passerini, C. *In vivo* eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. J. Natl. Cancer Inst. (Bethesda), *91:* 163–168, 1999.

20. Buchdunger, E., Cioffi, C. L., Law, N., Stover, D., Ohno-Jones, S., Druker, B. J., and Lydon, N. B. Abl protein-tyrosine kinase inhibitor STI571 inhibits *in vitro* signal transduction mediated by c-kit and platelet-derived growth factor receptors. J. Pharmacol. Exp. Ther., *295:* 139–145, 2000.

21. Schiller, J. H. Current standards of care in small-cell and non-small-cell lung cancer. Oncology (Basel), *61* (Suppl.)*:* 3–13, 2001.

22. Hibi, K., Takahashi, T., Sekido, Y., Ueda, R., Hida, T., Ariyoshi, Y., and Takagi, H. Coexpression of the stem cell factor and the c-kit genes in small-cell lung cancer. Oncogene, *6:* 2291–2296, 1991.

23. Plummer, H., III, Catlett, J., Leftwich, J., Armstrong, B., Carlson, P., Huff, T., and Krystal, G. c-myc expression correlates with suppression of c-kit protooncogene expression in small cell lung cancer cell lines. Cancer Res., *53:* 4337–4342, 1993.

24. Rygaard, K., Nakamura, T., and Spang-Thomsen, M. Expression of the proto-oncogenes c-met and c-kit and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts. Br. J. Cancer, *67:* 37–46, 1993.

25. Siziopikou, K. P., Cosar, E., Morgan, M., and Husain, A. N. Differential expression of c-kit in lung carcinomas; implications for possible use of the tyrosine kinase inhibitor STI571 in lung cancer. Proc. Am. Assoc. Cancer Res., *43:* 755–756, 2002.

26. Leflunomide approved for rheumatoid arthritis; other drugs nearing approval. Am. J. Health Syst. Pharm., 55: 2225–2226, 1998.

27. Pederson, L., Winding, B., Foged, N. T., Spelsberg, T. C., and Oursler, M. J. Identification of breast cancer cell line-derived paracrine factors that stimulate osteoclast activity. Cancer Res., *59:* 5849–5855, 1999.

28. Dvorak, H. F., Detmar, M., Claffey, K. P., Nagy, J. A., van de Water, L., and Senger, D. R. Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. Int. Arch. Allergy Immunol., *107:* 233–235, 1995.

29. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.

30. Salven, P., Ruotsalainen, T., Mattson, K., and Joensuu, H. High pretreatment serum level of vascular endothelial growth factor (VEGF) is associated with poor outcome in small-cell lung cancer. Int. J. Cancer, *79:* 144–146, 1998.

31. Mall, J. W., Schwenk, W., Philipp, A. W., Meyer-Kipker, C., Mall, W., Muller, J., and Pollmann, C. Serum vascular endothelial growth factor levels correlate better with tumour stage in small cell lung cancer than albumin, neuron-specific enolase or lactate dehydrogenase. Respirology, *7:* 99–102, 2002.

32. Lucchi, M., Mussi, A., Fontanini, G., Faviana, P., Ribechini, A., and Angeletti, C. A. Small cell lung carcinoma (SCLC): the angiogenic phenomenon. Eur. J. Cardio-thoracic Surg., *21:* 1105–1110, 2002.

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