Contribution of individual targets to the antitumor efficacy of the multitargeted receptor tyrosine kinase inhibitor SU11248

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Abstract

Recent achievements in the development of multitargeted molecular inhibitors necessitate a better understanding of the contribution of activity against individual targets to their efficacy. SU11248, a small-molecule inhibitor targeting class III/V receptor tyrosine kinases, including the platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptors, KIT and FLT3, exhibits direct effects on cancer cells as well as antiangiogenic activity. Here, we investigated the contributions of inhibiting individual SU11248 target receptors to its overall antitumor efficacy in tumor models representing diverse signaling paradigms. Consistent with previous results, SU11248 was highly efficacious (frequently cytoreductive) in all models tested. To elucidate the specific contributions of inhibition of PDGF and VEGF receptors to the *in vivo* efficacy of SU11248, we employed two selective inhibitors, SU10944 (VEGF receptor inhibitor) and Gleevec (PDGF receptor inhibitor). SU10944 alone induced a tumor growth delay in all models evaluated, consistent with a primarily antiangiogenic mode of action. In contrast, Gleevec resulted in modest growth inhibition in tumor models in which the cancer cells expressed its targets (PDGFRβ and KIT), but was not efficacious against tumors not driven by these target receptor tyrosine kinases. Strikingly, in all but one tumor model evaluated, the antitumor efficacy of SU10944 combined with Gleevec was similar to that of single-agent SU11248, and was greatly superior to that of each compound alone, indicating that the antitumor potency of SU11248 in these models stems from combined inhibition of both PDGF and VEGF receptors. The one exception was a model driven by an activated mutant of FLT3, in which the activity of SU11248, which targets FLT3, was greater than that of SU10944 plus Gleevec. Moreover, SU10944 combined with Gleevec inhibited tumor neoangiogenesis to an extent comparable to that of SU11248. Thus, the potent efficacy of SU11248 in models representing diverse signaling paradigms results from simultaneous inhibition of individual target receptors expressed both in cancer cells and in the tumor neovasculature, supporting the hypothesis that multitargeted inhibitors have the cumulative antitumor efficacy of combined single-target inhibitors. [Mol Cancer Ther 2006;5(5):1280–9]

Introduction

Therapeutic interest in class III and class V receptor tyrosine kinases (RTK), notably the platelet-derived growth factor receptors (PDGF receptors) and the vascular endothelial growth factor receptors (VEGF receptors), KIT and FLT3, is supported by their central roles in human cancer. Autocrine loops mediated by class III/V receptor-ligand interactions have been implicated in cancer cell growth and survival, e.g., PDGF receptors in glioblastoma (1, 2), VEGF receptors in melanoma (3), and KIT in small cell lung cancer (4, 5). Some solid tumors and hematologic malignancies are driven by deregulated KIT, PDGF receptor, and FLT3 signaling [e.g., KIT in gastrointestinal stromal tumors (6, 7), PDGF receptor in dermatofibrosarcoma protuberans (8), and FLT3-ITD in acute myelogenous leukemia (9)]. In addition to their roles in cancer cell growth and survival, the PDGF and VEGF receptors facilitate the transmission of proliferation, migration, differentiation, and survival signals from cancer cells and neighboring host-derived stromal cells to the endothelial cells of the tumor neovasculature (10, 11).

Initial efforts to identify kinase inhibitors with therapeutic potential have focused on the inhibition of signaling by individual kinases or kinase families using diverse strategies, such as blocking of VEGF binding to its receptors (12) or selective inhibition of target kinase activity using small-molecule inhibitors (13). Some of these approaches have yielded promising results in preclinical models and encouraging signs of clinical activity, as most successfully exemplified by Gleevec in Bcr/Abl-driven chronic myelogenous leukemia (14) and KIT-driven gastrointestinal stromal tumor (15). However, in recent studies using the small-molecule kinase inhibitor SU6668, which functions as a potent antiangiogenic agent by targeting the VEGF, PDGF, and fibroblast growth factor receptors, it became evident that simultaneous inhibition

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of more than one kinase family could result in greatly augmented single-agent antitumor activity in preclinical models relative to specifically targeting one kinase family alone (16, 17).

We previously described a novel tyrosine kinase inhibitor, SU11248, which selectively inhibits class III and class V RTKs, including PDGF receptors, VEGF receptors, KIT, and FLT3 with low nanomolar potency (18–20). The activity and selectivity of SU11248, initially determined *in vitro*, has been confirmed *in vivo* (18–20). SU11248 is highly efficacious (frequently cytoreductive) in all tumor xenograft models investigated (18–20), and its full antitumor efficacy against solid tumor xenografts was associated with prolonged (at least 12 of 24 hours), but not continuous, inhibition of VEGFR2 and PDGFRβ (18).

To better understand the antitumor activity of multitargeted agents such as SU11248, and in order to aid in the optimal selection of clinical indications, it is necessary to understand the contribution of inhibiting individual targets to the overall antitumor activity of the compound. We have undertaken this type of study with SU11248 as an example of a multitargeted agent. Specifically, we have evaluated the effects of SU11248 in various tumor cell lines expressing single/multiple target RTK(s), distinguished the contributions of inhibition of individual target RTKs in cancer cells to SU11248 efficacy *in vivo*, and determined the relative contributions of PDGF and VEGF receptor inhibition to the antiangiogenic activity of SU11248. To achieve these goals, we employed two selective small-molecule inhibitors, SU10944 (VEGF receptor inhibitor; ref. 21) and Gleevec (PDGF receptor and KIT inhibitor; refs. 22–24), which were administered as single agents, or in combination, to mice bearing established tumors derived from human and rat cancer cells. The activity of SU11248 was compared with that of the selective inhibitors, and the hypothesis that the potent *in vivo* antitumor activity of SU11248 could be recapitulated by combining VEGF and PDGF receptor inhibitors was tested.

Materials and Methods

Compounds

SU11248 has been described in detail elsewhere (18). SU10944 is a selective inhibitor of VEGF receptors (21). STI571 (imatinib, Gleevec) is a selective small-molecule inhibitor of Abl, PDGF receptors, and c-KIT kinase activity, whose structure and activity have been previously reported (22, 24, 25).

Cells

Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). C6 (rat glioma); U87, U118MG, and LN-18 (human glioblastoma); HT-29 and Colo205 (human colon carcinoma); 786-O (human renal carcinoma); WM266-4 (human melanoma); H226 (human lung carcinoma); and MV4;11 (FLT3-ITD-mutated human acute myelogenous leukemia) cells were obtained from and cultured as recommended by the American Type Culture Collection (Manassas, VA). All cells were routinely maintained in a humidified chamber at 37°C with 5% carbon dioxide.

Immunoprecipitation and Western Blot Analysis

To determine the activity of SU11248 against intrinsic target receptor phosphorylation *in vitro*, cells maintained either under normal growth conditions (10% fetal bovine serum) or starved overnight in low serum (0.5% fetal bovine serum) were treated with a designated amount of SU11248 overnight, lysed with HNTG whole-cell lysis buffer (18), and protein concentrations were determined using the bicinchoninic acid (BCA) assay kit (Pierce Chemical Company, Rockford, IL). For the detection of phosphorylated PDGFRβ, 500 μg of protein was immunoprecipitated overnight at 4°C with anti-PDGFRβ antibody (SC-432, Santa Cruz Biotechnology, Santa Cruz, CA) and protein A agarose beads, Western blotted and probed using an anti-phospho-PDGFRβ (Y716) antibody (07-021, Upstate Biotechnology, Lake Placid, NY). Membranes were subsequently stripped and reprobed to detect total PDGFRβ using the same anti-PDGFRβ antibody used for immunoprecipitation.

Soft Agar Assay

Culture dish wells (24-well plates) were coated with 0.5 mL of the bottom agar mixture (10% fetal bovine serum, DMEM, and 0.6% agar). After the bottom layer had solidified, 0.5 mL of the top agar mixture (10% fetal bovine serum, DMEM, and 0.3% agar) containing 1,500 cells/well was added into each well with twice the concentration of SU11248 indicated in the figure legends. Plates were incubated for 3 weeks, and stained overnight at 37°C, 5% CO_2 in a solution of 1 μg/mL iodonitrotetrazolium salt (Sigma Chemical, St. Louis, MO) in 50% ethanol. Colonies were visualized at 400× magnification, manually counted, and the average number of colonies per well was calculated.

Mouse Xenograft Models

Female *nu/nu* mice (8–12 weeks old, 25 g), obtained from Charles River (Wilmington, MA), were used for *in vivo* studies as previously described (17). Animals were maintained under clean-room conditions in sterile filter-top cages housed on high-efficiency particulate air–filtered ventilated racks, and received sterile rodent chow and water ad libitum. Animal experiments were conducted in accordance to Institutional Animal Care and Use Committee guidelines in the SUGEN Animal Facility, which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Briefly, for efficacy studies, 3 to 10×10^6 tumor cells were implanted s.c. into the hindflank region of mice [in some cases, 50% Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA) was added to tumor cells to aid initial tumor formation]. Oral treatment of tumor-bearing mice with compounds, all formulated as carboxymethylcellulose suspensions, was initiated once tumors reached the indicated average volume. SU11248 and SU10944

were administered once a day. Based on published studies demonstrating its relatively short half-life in mice (22, 26), Gleevec was administered twice daily (b.i.d.). Tumor growth was evaluated as described previously (17). Typically, studies were terminated when tumors in vehicle-treated animals reached an average size of 1,000 mm^3, or when the tumors could adversely affect the well-being of the animals. Where established tumors (250–400 mm^3) were used to determine the inhibitory effect of various treatments, the percentage of tumor inhibition was calculated as $100 \times (1 - [(\text{tumor volume}_{\text{final}} - \text{tumor volume}_{\text{initial}}$ for the compound-treated group) / (tumor $\text{volume}_{\text{final}} - \text{tumor volume}_{\text{initial}}$ for the vehicle-treated group)]). The percentage of tumor regression for each group was calculated as $100 \times [(\text{tumor volume}_{\text{initial}} - \text{tumor volume}_{\text{final}}) / (\text{tumor volume}_{\text{initial}})]$.

Immunohistochemistry, Microvessel Density Assessment, and Automated Slides Analysis

Xenograft tumor specimens were harvested at the end of an efficacy study or at the indicated time points and fixed in 10% buffered formalin for 24 hours or, alternatively, in Streck's tissue fixative (Streck Laboratories, La Vista, NE). After embedding in paraffin and sectioning, general tissue morphology was visualized by H&E staining. The induction of apoptosis was assessed by visualizing the cleaved 17 kDa fragment of caspase 3 (antibody 9661, Cell Signaling Technologies, Beverly, MA). To identify proliferating tumor cells, cell nuclei were stained with antibodies recognizing Ki-67 [anti-rat antibody NCL-Ki67p (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) for C6 xenografts, and monoclonal anti-human clone MIB-1 (DakoCytomation, Glostrup, Denmark) for all other models].

The intensity and distribution of cleaved/activated caspase-3 and the percentage of Ki-67-positive nuclei were measured using the Chromavision ACIS system (Chromavision Advanced Cellular Imaging, San Luis Obispo, CA) with generic tissue scoring and Ki-67 protocols, respectively. At least three animals per treatment group were evaluated, and a minimum of five fields per tumor section were scored. To calculate levels of apoptosis, the average percentage of the area positive for caspase-3 staining was multiplied by the average intensity of the signal in each section; these values were averaged and normalized to values in the untreated group. To calculate changes in the proliferation index, the average percentage of Ki-67-positive nuclei in treatment groups were normalized to values obtained for vehicle-treated control tumors.

Tumor microvessels were visualized by immunohistochemical staining of mouse CD31 using the rat anti-mouse monoclonal antibody MEC 13.3 (BD PharMingen, San Diego, CA) at 1:100 dilution. All immunostained sections were counterstained using hematoxylin. Five fields per tumor were scored in blinded fashion for tumor microvessels at 100× magnification, and the average number of microvessels/field was calculated.

Results

Profiling of Tumor Cell Lines for Expression of SU11248 Targets

In order to explore the contribution of inhibiting individual receptors expressed on tumor cells to the potent antitumor efficacy of SU11248, we profiled more than 20 human tumor cell lines for expression and activation status (assessed by phosphotyrosine content) of the SU11248 targets PDGFRα, PDGFRβ, VEGFR2, KIT, and FLT3. The profiles for the cell lines selected for use during the course of studies described in this report are presented in Table 1.

Direct Inhibition of Cancer Cell Growth by SU11248 in Soft Agar and *In vivo*

The target profile of SU11248 suggests potential for exerting direct effects on tumor cells expressing activated class III/V RTKs. However, we were initially unable to show any selective effects of SU11248 on diverse tumor cells cultured on plastic (18). To more decisively explore the ability of SU11248 to directly inhibit growth of cancer cells, we evaluated anchorage-independent cell growth, a hallmark of the transformed state, in the presence of SU11248. Inhibition of anchorage-independent growth of glioblastoma cells ($IC_{50} = 0.05–0.1$ μmol/L) was observed (Fig. 1B) at concentrations sufficient to inhibit PDGFRβ phosphorylation (Fig. 1A), indicating a direct target-dependent activity of the compound. However, the same doses of SU11248 had no effect on the growth of EGF-dependent HT-29 cells (Fig. 1B; ref. 27), indicating compound selectivity and the absence of generic cytotoxicity. Of note, SU11248 caused only ~50% inhibition of anchorage-independent growth of C6 cells at concentrations between 0.1 μmol/L, its IC_{50} value in soft agar, and 1.5 μmol/L, although it exerted a pronounced growth-inhibitory effect at higher concentrations. In this case, residual C6 colony growth at concentrations in which PDGF receptors are inhibited may be the result of signaling via non–class III/V kinases, which are ultimately inhibited at high, less selective concentrations of SU11248.

In order to determine whether the anti–cancer cell activity of SU11248 could be reproduced *in vivo*, C6 rat glioma tumors were resected from SU11248-treated animals and immunostained for Ki-67 (proliferation marker), and an activation-specific cleavage-derived neoepitope of caspase-3 (apoptosis marker). The proliferation of cancer cells was reduced in a dose-dependent manner by SU11248 treatment with a 60% reduction ($P < 0.006$, Student's t test) at the fully efficacious dose of 40 mg/kg/d following 3 days of treatment (Fig. 2). In contrast, no induction of apoptosis was observed.

Recapitulating the Antitumor Activity of SU11248 through Simultaneous Inhibition of VEGF and PDGF Receptors

To determine the specific contributions of selectively inhibiting PDGF receptors versus VEGF receptors directly on cancer cells, as well as in the tumor vasculature, we employed two tool compounds: SU10944, a selective VEGF receptor inhibitor (21), and Gleevec, which has been used

Table 1. Expression levels and *in vitro* activation status of SU11248 target RTKs in cancer cell lines

Cell line	Tumor type	SU11248 Target RTK			
		PDGFRβ	VEGFR2	KIT	FLT3
C6	Glioma (rat)	High, constitutively activated*	Low	No	No
HT-29	Colon	No	No	No	No
U118MG	Glioblastoma	High, constitutively activated	Moderate	High, constitutively activated	No
LN-18	Glioblastoma	High, constitutively activated	Low	No	No
786-O	Renal	High, constitutively activated	Moderate	No	No
WM-266-4	Melanoma	Low	No	No	No
H226	Lung	High, constitutively activated	Moderate	Low	No
MV4;11	Leukemia	No	No	Very low	High, constitutively activated through ITD mutation

NOTE: Cells were grown in 10% fetal bovine serum to ~90% confluency and Western blot analyses were done as described in Materials and Methods. Data for MV4;11 cells are adapted from ref. 19.

*Constitutively activated; RTK is considered constitutively activated if its phosphorylation was detected after incubation of cells under low-serum conditions in the absence of ligand.

here to selectively inhibit PDGF receptors (and KIT, in some models; ref. 22). We then compared their antitumor effect, alone or in combination, with the antitumor efficacy of SU11248. Doses of SU10944 (150 mg/kg once daily) and Gleevec (200 mg/kg twice daily) used in these studies were selected to provide inhibition of their respective targets similar in extent and duration to that achieved with SU11248 at its fully efficacious dose of 40 mg/kg/d (inhibition of PDGF and VEGF receptors for 12 of 24 hours; ref. 18). It should be noted, however, that the inhibition of PDGFRβ by Gleevec at 200 mg/kg twice daily (the maximum tolerated dose in mice) was somewhat less in extent and duration than that achieved with SU11248 at 40 mg/kg once daily (19). As an important control, limited last-day pharmacokinetic analyses undertaken at the end of the efficacy studies confirmed that single-agent SU10944

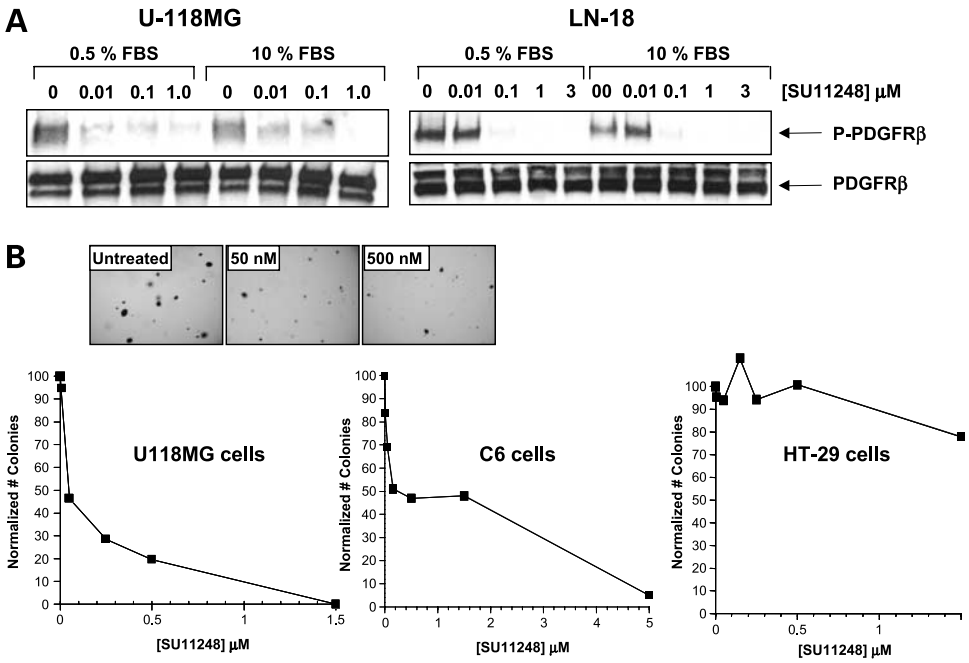

Figure 1. SU11248 inhibits *in vitro* PDGF receptor signaling and anchorage-independent growth of human glioblastoma cells. **A,** SU11248 treatment induces dose-dependent inhibition of phosphorylation of PDGFRβ in human tumor cells. Human glioblastoma U118MG and LN-18 cells were treated with the indicated concentration of SU11248 for 24 h, and levels of activated and total PDGFRβ were visualized using Western blot analysis. **B,** SU11248 inhibits *in vitro* anchorage-independent growth of tumor cells expressing target RTKs. Cells were seeded in soft agar containing the indicated dose of SU11248 and viable colonies were counted 3 wks later, as described in Materials and Methods. Representative images are shown for U118MG cells.

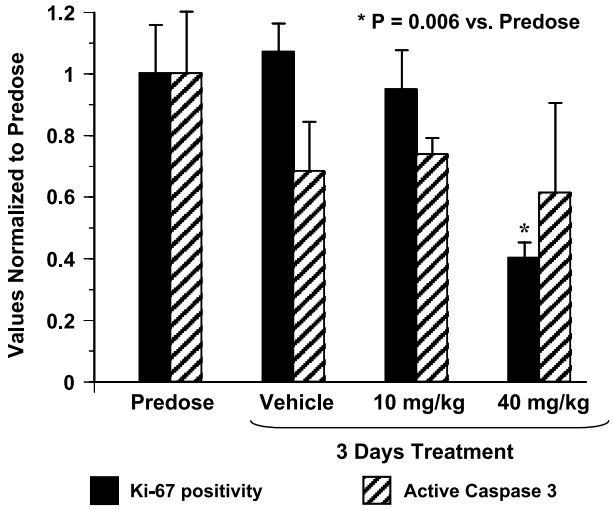

Figure 2. SU11248 treatment of C6 rat glioma xenografts results in the inhibition of cancer cell proliferation. Portions of tumors harvested at the indicated times from xenograft-bearing mice treated with SU11248 at 40 mg/kg/d were fixed in 10% buffered formalin and paraffin-embedded. Tumor sections were immunostained to visualize markers of proliferation (Ki-67) and apoptosis (activated caspase-3). Slides were analyzed using the Chromavision ACIS system, and results are plotted as mean ± SE of measurements in three to six tumors/group/treatment.

and Gleevec plasma exposures were comparable to those achieved when the agents were combined (data not shown), indicating that there was no evidence of drug-drug interactions between these two compounds when they were dosed together.

Using these tool compounds, the hypothesis that the antitumor efficacy of a multitargeted RTK inhibitor (i.e., SU11248, inhibits PDGFR + VEGFR + KIT + FLT3) would be the same or better than combined treatment with selective inhibitors of subsets of class III/V RTKs (SU10944, inhibiting VEGFR, and Gleevec, inhibiting PDGFR + KIT) was tested in a panel of tumor xenograft models representing different signaling paradigms (Table 1). The models used were selected based on target RTK expression to specifically investigate a situation in which SU11248 was functioning purely as an antiangiogenic agent (HT-29 model in which no target RTKs were detected on the tumor cells) or several models in which SU11248 targeted both tumor vasculature and the tumor cells themselves because the tumor cells express one or more target RTKs (e.g., C6, 786-O, and H226).

Consistent with previous findings (18, 19), treatment with SU11248 resulted in pronounced antitumor efficacy, including either growth inhibition, stasis, or regression of established xenografts, in all models tested, regardless of their *in vitro* target RTK expression profile (Table 2). Single-agent Gleevec had moderate antitumor activity (growth delay) against xenografts in which cancer cells expressed PDGFRβ, a target of Gleevec (Fig. 3; Table 2). Somewhat enhanced activity (tumor stasis) was observed against H226 tumors, in which two Gleevec-targeted receptors are expressed on the tumor cells (PDGFRβ and KIT), and no antitumor effect was observed in Gleevec-treated animals bearing HT-29 and MV4;11 xenografts, in which Gleevec-targeted receptors are not detectably expressed on the tumor cells (Figs. 3 and 4). In contrast, treatment with single-agent SU10944 resulted in tumor growth delay in all models evaluated (Figs. 3 and 4; Table 2). This result was associated with a modest reduction in tumor microvessel density (MVD; Table 3), indicative of antiangiogenic activity, presumably mediated by inhibition of VEGF receptors in the tumor vasculature.

Notably, in most models, a close concordance (Pearson correlation coefficient, R^2 = 0.87–0.997) was observed between the antitumor effect of SU11248 and that of SU10944 + Gleevec, as assessed by comparison between

Table 2. In solid tumor models, combined treatment with a selective VEGF receptor inhibitor (SU10944) and a selective PDGF receptor inhibitor (Gleevec) recapitulates the antitumor efficacy of SU11248, which inhibits both PDGF receptors and VEGF receptors

Xenograft tumors (days of treatment)	Antitumor activity (%)								Treatment similarity†
	SU10944		Gleevec		SU11248		SU10944 + Gleevec		
	Effect*	*P*	Effect*	*P*	Effect*	*P*	Effect*	*P*	
C6 (d12)	**76**	0.001	**72**	0.001	**92**	0.001	**97**	0.001	0.969
MV4;11 (d15)	**95**	0.00001	No effect	—	100	0.00001	52	0.003	Not applicable
HT-29 (d18)	**55**	0.23	No effect	—	35	0.13	22	0.14	0.886
786-O (d14)	**90**	0.013	**40**	0.18	46	0.05	40	0.08	0.994
WM-266-4 (d29)	**82**	0.0005	**35**	0.06	37	0.04	27	0.13	0.871
H226 (d14)	28	0.11	**104**	0.01	69	0.001	64	0.0001	0.997

NOTE: Tumors were established as s.c. xenografts. Number of cells implanted per animal: C6, 3×10^6; Colo205, WM-266-4, H226, HT-29, and MV4;11, 5×10^6 (last two plus 50% basement Matrigel matrix); 786-O, 1×10^7 plus 50% basement Matrigel matrix. Once tumors reached the indicated average size, daily treatment with 40 mg/kg of oral SU11248 was initiated. The results of representative experiments are shown here.

*Percentage of tumor regression (not bolded) and percentage of growth inhibition (bolded) are shown for representative experiments. *P*, Student's *t* test (two-tailed distribution)—in cases when $P < 10^{-5}$, $P = 0.00001$ is shown.

†Overall treatment similarity between SU11248-treated and combination (SU10944 + Gleevec)-treated groups. Calculated as R^2, Pearson coefficient between average tumor sizes in the two groups at each measurement throughout the length of the experiment.

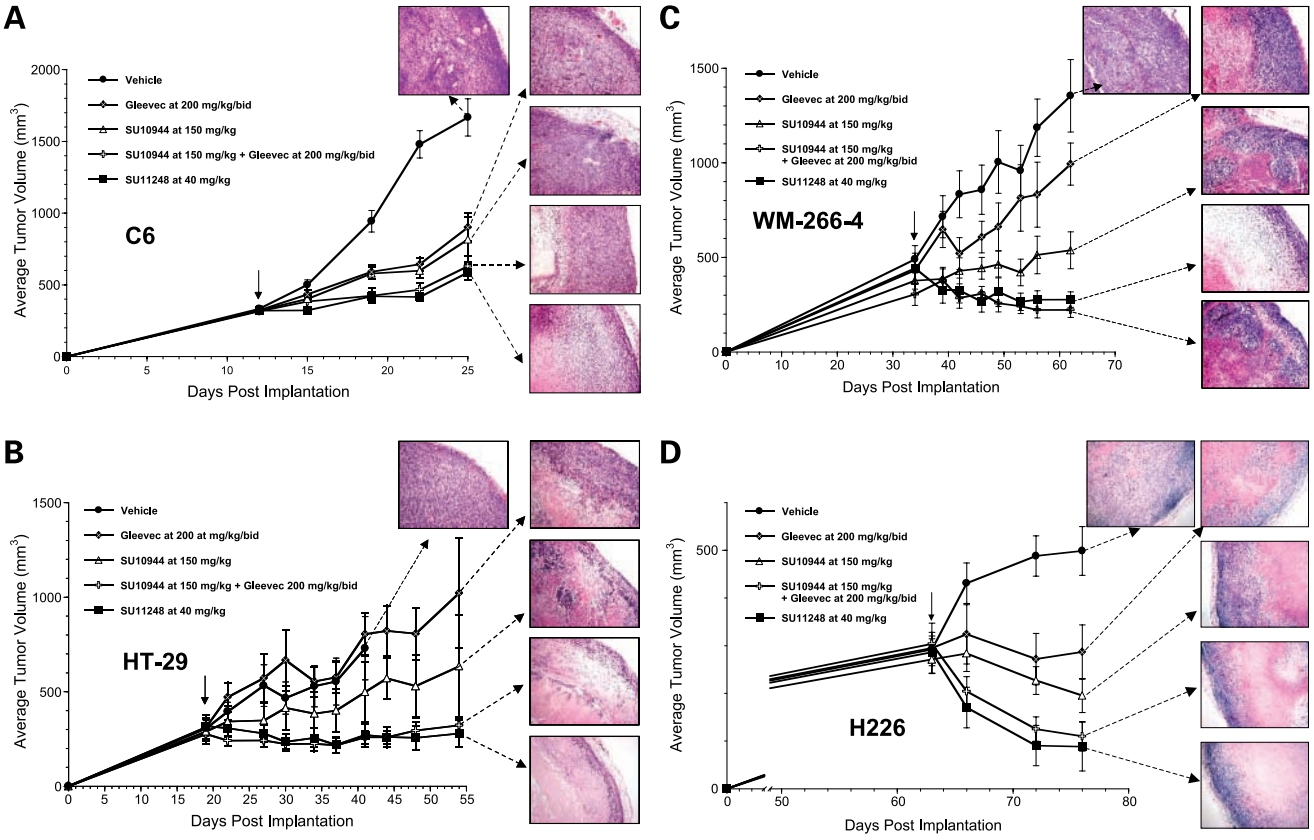

Figure 3. Combined administration of SU10944 and Gleevec recapitulates the antitumor effect of SU11248. C6 rat glioma (**A**), HT-29 human colon carcinoma (**B**), WM-266-4 human melanoma (**C**), and H226 lung carcinoma (**D**) tumor xenografts were established in athymic mice. Oral administration of indicated compounds was initiated (*arrows*) when tumors reached an average size of 320 mm^3 (*C6*), 300 mm^3 (*HT-29*), 410 mm^3 (*WM-266-4*), and 290 mm^3 (*H226*), and was continued through the end of the experiment. *Points,* mean tumor volume for groups of 8 to 10 (treated) or 16 to 20 (vehicle control) mice; *bars,* ±SE. A representative H&E-stained tumor tissue section taken at the end of the experiment for each treatment group (original magnification, ×200; connected with the efficacy plot by a dotted line to indicate the treatment group from which it originated).

average tumor volumes in the two treatment groups throughout the length of the experiment. In addition to tumor volume measurements, the morphologic appearance of tumors and histologic markers were evaluated. In all solid tumor models, xenografts from mice treated with SU11248 or with the combination of SU10944 and Gleevec, exhibited a similar morphologic appearance (Fig. 3, *insets*). For instance, HT-29 xenograft sections from both of these treatment groups were comprised of an inner acellular, necrotic tumor mass surrounded by a thin outer layer of viable cancer cells (Fig. 3B).

To further test the hypothesis that the overall efficacy of the multitargeted inhibitor SU11248 stems from its combined activity against individual targets, the efficacies of treatments with SU11248 alone and SU10944 + Gleevec were compared in a model heavily dependent on an RTK target, FLT3, which is inhibited by SU11248, but not by the other two compounds (ref. 20, data not shown). The human acute myelogenous leukemia model MV4;11, which is driven by constitutively activated FLT3 and is correspondingly highly sensitive to SU11248 (20), was used in these experiments. As anticipated, Gleevec treatment had no effect on the growth rates of these tumors, whereas treatment with SU10944 resulted in tumor stasis (Fig. 4; Table 2). As predicted, combined treatment with Gleevec and SU10944 (52% reduction in tumor volume) was more effective than treatment with either agent alone, but did not recapitulate the dramatic effect of SU11248 on MV4;11 xenografts (complete tumor debulking).

Antiangiogenic Activity of Combined (SU10944 + Gleevec) Treatment Is Similar to That of SU11248

Tumor MVD was assessed in several models (Fig. 5; Table 3) where there was sufficient preservation of viable tumor mass posttreatment. Treatment with single-agent Gleevec did not significantly inhibit MVD in any of the models evaluated, whereas administration of SU10944 alone resulted in a modest MVD decrease (22–54%), supporting the importance of the antiangiogenic effects of this compound for its antitumor activity. In contrast, treatment with SU11248 resulted in profound reductions in tumor MVD (68–89% inhibition). Consistent with its similar antitumor efficacy, the combination of SU10944 with Gleevec reduced tumor MVD to an extent comparable to that seen with SU11248 (Fig. 5; Table 3).

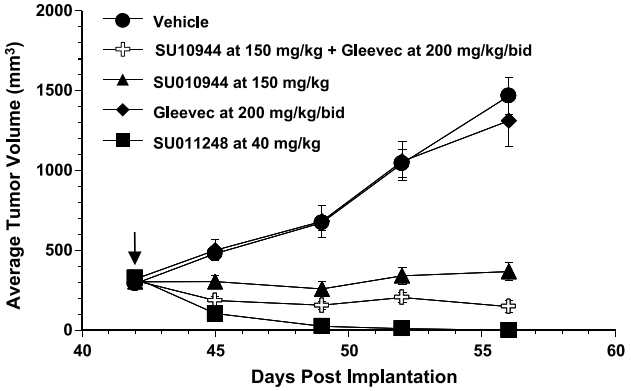

Figure 4. SU11248 treatment is more effective than all other treatments evaluated against FLT3-ITD-driven MV4;11 xenografts. Oral administration of the indicated compounds were initiated (*arrow*) when xenografts reached an average size of 310 mm^3, and was continued through the end of the experiment. Data were analyzed as described in Fig. 3.

Discussion

Over the past decade, much attention has been devoted to the development of novel molecular therapeutics targeting kinase pathways important for tumor growth and survival (13). A paradigm of inhibiting a specific pathway (single-target therapies) has been extensively explored, and is best exemplified by Herceptin (anti-HER2 antibody), targeting cancer cells overexpressing HER2 in breast cancer (28), and Gleevec (small-molecule kinase inhibitor) in Bcr/Abl-driven chronic myelogenous leukemia (14), and KIT-driven gastrointestinal stromal tumors (15). More recently, there has been growing interest in multitargeted agents which inhibit several related pathways in multiple cell types to achieve better single-agent efficacy in a broader range of tumors. This paradigm is illustrated by such agents as SU11248 (class III/V RTK inhibitor; refs. 18–20) and ZD6474 (EGF and VEGF receptor inhibitor; ref. 29), as well as many other agents currently in clinical development. For the most effective application of such multi-targeted agents in the clinic, it is imperative that the mechanisms underlying their efficacy be well-understood, in particular, whether the *in vivo* efficacy of such a compound could be understood in terms of its inhibition of one or a combination of its targets in various tumor models. As an example of this approach, the primary goal of the studies described here was to elucidate the mechanism of action of SU11248 *in vivo*, and to determine the contributions of inhibiting individual targets to its antitumor activity.

By virtue of the biological role of the class III and class V RTKs targeted by SU11248, this compound can exert both a direct antitumor activity, if the tumor cells express and are dependent on the target RTKs, as well as an indirect antitumor activity through inhibition of tumor-dependent angiogenesis by inhibiting VEGF and PDGF receptors on tumor-associated endothelial and stromal cells. In order to evaluate the contributions of inhibiting the individual targets to the *in vivo* activity of SU111248 in the broadest sense, we used xenograft models derived from tumor cell lines that did or did not express the target RTKs VEGFR-2, PDGFRβ, c-Kit, and FLT3 to be able to evaluate both the antitumor and the antiangiogenic activity of SU11248.

Consistent with our previous demonstration that SU11248 is active against a broad range of tumor models *in vivo* (18–20), SU11248 was efficacious against the C6, 786-O, WM-266-4, and H226 tumor models that express the SU11248 targets PDFGR and/or VEGFR and/or c-Kit on the tumor cells. Although SU11248 was invariably more efficacious, Gleevec or SU10944 showed single-agent activity in these models, consistent with the expression of the particular RTK target on the tumor cells, whereas SU10944 showed some level of efficacy in all tumor models, consistent with the role of VEGF in tumor-dependent angiogenesis (30), and the broad-based efficacy of other VEGF-selective inhibitors shown in mouse xenograft tumor models (12). Although it could be argued that off-target

Table 3. Extent of inhibition of tumor neoangiogenesis by combined treatment with a VEGF receptor inhibitor (SU10944) and a PDGF receptor inhibitor (Gleevec) is similar to that seen with SU11248

Xenograft tumors (days of treatment)	Carboxymethylcellulose	SU10944			Gleevec			SU11248			SU10944 + Gleevec		
	MVD	MVD	% Inh	*P*	MVD	% Inh	*P*	MVD	% Inh	*P*	MVD	% Inh	*P*
C6 (day 12)	24.6	19.8	20	0.19	35.9	—	—	31.8	—	—	36.0	—	—
786-O (day 14)	106.2	49.4	54	0.036	85.6	19	0.22	25.3	76	0.027	13.3	87	NA
WM-266-4 (day 29)	43.2	33.3	23	0.19	49.4	—	—	13.7	68	0.001	30.0	31	0.034
H226 (day 14)	74.2	43.2	42	0.09	76.2	—	—	8.4	89	0.012	21.2	71	0.013
MV4;11 (day 15)	46.9	28.1	40	0.12	86.1	—	—	NA	NA	NA	9.3	80	0.015

NOTE: *P*, Student's *t* test (one-tailed distribution).
Abbreviations: MVD, average number of microvessels per 100× field. % Inh, percentage of inhibition of MVD in xenografts from treated animals compared with that of xenografts from vehicle-treated controls. NA, not applicable.

effects might contribute to the greater antitumor efficacy of SU11248, our previous demonstration (18) that the selectivity of SU11248 is maintained *in vivo* at doses in excess of the fully efficacious 40 mg/kg dose used in the current studies suggests that this is not likely the case. Moreover, the observation that both the extent and time course of antitumor activity with SU11248 is recapitulated by combined treatment with SU10944 and Gleevec in these models supports the conclusion that the potent activity of SU11248 in these models was due to its ability to simultaneously inhibit VEGF and PDF receptors (and c-KIT in the case of the H226 tumor model).

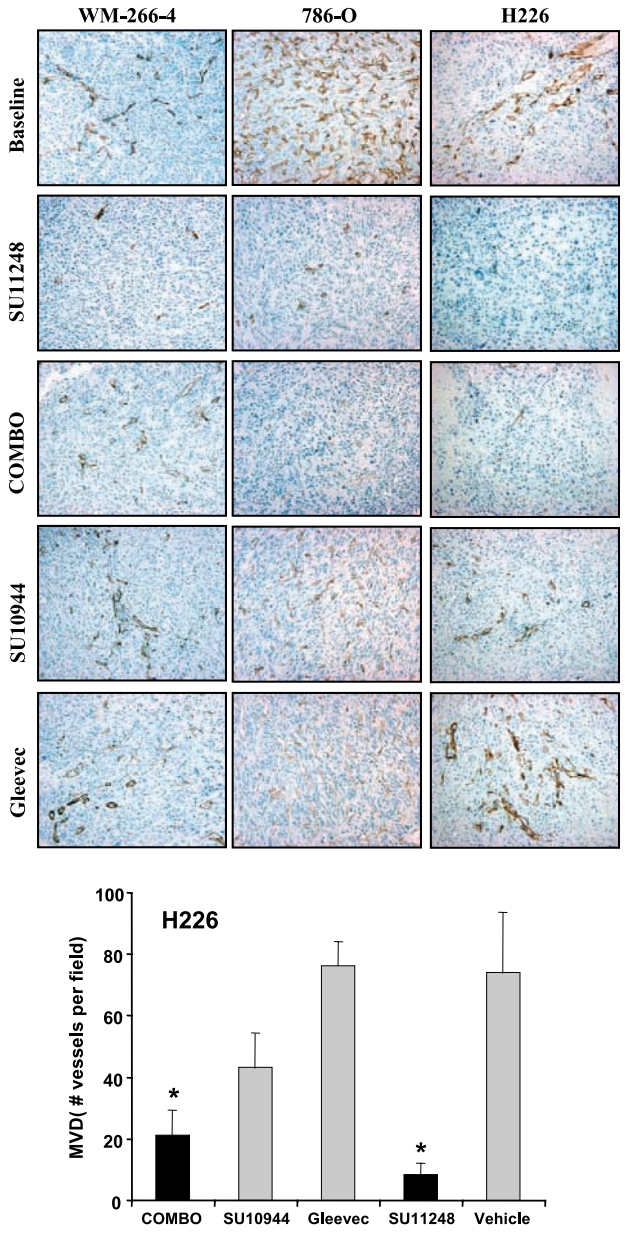

Figure 5. Treatment with SU11248 or with SU10944 + Gleevec results in potent inhibition of tumor neoangiogenesis. *Top,* images of representative end-of-study tumor sections immunostained with CD31 (original magnification, ×100). *Bottom,* an example of MVD reduction. End-of-study tumor MVD was determined as described in Materials and Methods.

In the models described above, SU11248 is presumably exerting both direct antitumor activity by inhibiting target RTKs expressed on the tumor cells that play a role in regulating tumor cell proliferation, and indirect antitumor activity by inhibiting tumor-dependent neoangiogenesis. Similar results were obtained when SU11248 was tested for *in vivo* activity against the HT-29 human colon carcinoma cell line, which does not express any functional SU11248 target receptors and is not affected by SU11248 *in vitro* in anchorage-independent growth assays. In this model, SU11248 and the combination of SU10944 and Gleevec induced regression of established tumors, an effect presumably due solely to inhibition of tumor neoangiogenesis (MVD was not evaluable in these groups in this model, owing to the extent of tumor cytoreduction). SU10944 also showed single-agent efficacy in the HT-29 model, as expected for a VEGF-selective, antiangiogenic inhibitor, although this efficacy was neither qualitatively nor quantitatively similar to that seen with SU11248, and Gleevec was not efficacious as a single agent. In other models in which tumor MVD was evaluable posttreatment, combined treatment with SU10944 and Gleevec could recapitulate the potent antiangiogenic activity of SU11248 (Table 3). This observation that the simultaneous inhibition of VEGF and PDGF receptors greatly augments antiangiogenic activity, and hence, antitumor activity, is consistent with previous reports (17, 31). Gleevec exhibited no single agent efficacy in the HT-29 model even though Gleevec-mediated inhibition of PDGF receptor on pericytes, which mechanically supports tumor vasculature (32), and on tumor stromal fibroblasts, which supply growth factors to other cells in tumors (33), could conceivably have resulted in antiangiogenic/antitumor activity in models in which expression/activation of target RTKs was not detected on cancer cells. The lack of a discernible antiangiogenic effect of Gleevec treatment in any of the models we evaluated was consistent with previous reports that Gleevec might affect the shape and size of microvessels (vessel area, boundary length, and diameter) but not MVD (34). Although these other variables were not directly evaluated in our studies, no obvious changes in vessel shape were evident.

Unique among the tumor models tested, the MV4:11 leukemia tumor model expressed a mutationally activated allele of the SU11248 target FLT3. This FLT3-ITD mutant is constitutively activated and provides both proliferative and survival signals for these cells. In this model, SU11248 showed potent activity leading to rapid regression of large established tumors. Similar to the HT-29 model, simultaneous treatment of MV4:11 tumors with SU10944 and Gleevec was more efficacious than treatment with either agent alone, presumably through a more potent combined antiangiogenic effect, but did not give the regressions seen following treatment with SU11248, most likely because neither of these agents effectively inhibit FLT3-ITD.

Therefore, the inability of Gleevec and SU10944 to recapitulate the activity of SU11248 in this model supports the conclusion that the activity of SU11248 must be viewed in terms of its activity against all the relevant targets involved in the particular tumor being evaluated.

The qualitative nature of the tumor response in these tumor models (growth delay versus regression) did not seem to be strictly dependent on the expression level of SU11248 RTK targets on the xenograft tumor cells. Two of the tumor models least sensitive to SU11248 are the A375 and C6 tumor models (18), which exhibit, respectively, VEGF- and PDGF-dependent autocrine loops. Instead, tumor response is more likely to depend on the biological role of the target RTK in the tumor cell. If the target RTK provides a survival signal, inhibition will likely result in tumor cell apoptosis and tumor regression, as seen in the FLT3-ITD-driven MV4;11 tumor model. If the target RTK is the major factor responsible for tumor cell proliferation *in vivo*, inhibition of that target may confer stasis, which may be the case for the C6 tumor model, in which reduction of xenograft tumor growth by SU11248 was associated with a rapid reduction in cancer cell proliferation. Alternatively, if the target RTK provides one of several growth signals to the tumor cell, inhibition may cause only a reduction in the rate of growth of the tumor. Other important determinants such as the status of p53, and/or PTEN-mediated tumor suppression pathways, may also be involved in governing the response to SU11248 treatment. For example, p53 status has been shown to determine the extent of response of otherwise genetically matched HCT-116 human colorectal carcinoma tumor xenografts to treatment with the anti-angiogenic therapy of DC101 (anti-VEGFR2 monoclonal antibody) combined with low-dose vinblastine (35).

The results described in this report show that the use of multitargeted inhibitors may allow for improved overall efficacy, and that the antiangiogenic consequences of inhibiting VEGF receptor signaling (12, 17) could be markedly augmented by simultaneously inhibiting PDGF receptor signaling. The administration of a multitargeted agent, such as SU11248, which simultaneously targets several facets of the transformed state is conceptually attractive, and offers practical advantages over combining several more selective targeted therapeutics. Conversely, combination therapy with single-targeted agents could offer the possibility of titration of each agent to optimize the balance between efficacy and tolerability, if that is an issue.

In conclusion, we have shown that the *in vivo* preclinical mechanism of action of the class III/V RTK inhibitor SU11248 is a combination of direct antitumor cell effects and antiangiogenic inhibition of tumor neovasculature, resulting from inhibition of SU11248 RTK targets expressed both by tumor cells and normal cells in the surrounding tumor microenvironment. Although this study was undertaken to determine the contributions of individual targets to efficacy, a similar approach could be taken to determine the contribution of individual targets to the preclinical toxicity of multitargeted agents. Preclinical pharmacologic studies such as those we describe could be valuable for all selective multitargeted agents in order to better understand the role of individual targets to their activity, thus contributing to rational selection of clinical indications and of patients within those indications most likely to benefit from treatment.

References

1. Maher EA, Furnari FB, Bachoo RM, et al. Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001;15:1311 – 33.

2. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, Giese NA. Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer Res 2002;62:3729 – 35.

3. Lazar-Molnar E, Hegyesi H, Toth S, Falus A. Autocrine and paracrine regulation by cytokines and growth factors in melanoma. Cytokine 2000; 12:547 – 54.

4. Pietsch T, Nicotra MR, Fraioli R, Wolf HK, Mottolese M, Natali PG. Expression of the c-Kit receptor and its ligand SCF in non-small-cell lung carcinomas. Int J Cancer 1998;75:171 – 5.

5. Micke P, Basrai M, Faldum A, et al. Characterization of c-kit expression in small cell lung cancer: prognostic and therapeutic implications. Clin Cancer Res 2003;9:188 – 94.

6. Miettinen M, Majidi M, Lasota J. Pathology and diagnostic criteria of gastrointestinal stromal tumors (GISTs): a review. Eur J Cancer 2002;38: S39 – 51.

7. Heinrich MC, Blanke CD, Druker BJ, Corless CL. Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies. J Clin Oncol 2002;20:1692 – 703.

8. Rubin BP, Schuetze SM, Eary JF, et al. Molecular targeting of platelet-derived growth factor B by imatinib mesylate in a patient with metastatic dermatofibrosarcoma protuberans. J Clin Oncol 2002;20:3586 – 91.

9. Gilliland DG, Griffin JD. Role of FLT3 in leukemia. Curr Opin Hematol 2002;9:274 – 81.

10. Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes Dev 1999;13:1055 – 66.

11. Cherrington JM, Strawn LM, Shawver LK. New paradigms for the treatment of cancer; the role of anti-angiogenesis agents. In: Klein G, Vande Woude GF, editors. Advances in cancer research. vol. 79. San Diego: Academic Press; 2000. p. 1 – 38.

12. Manley PW, Martiny-Baron G, Schlaeppi JM, Wood JM. Therapies directed at vascular endothelial growth factor. Expert Opin Investig Drugs 2002;11:1715 – 36.

13. Laird AD, Cherrington JM. Small molecule tyrosine kinase inhibitors: clinical development of anticancer agents. Expert Opin Investig Drugs 2003;21:51 – 64.

14. Cohen MH, Williams G, Johnson JR, et al. Approval summary for imatinib mesylate capsules in the treatment of chronic myelogenous leukemia. Clin Cancer Res 2002;8:935 – 42.

15. Dagher R, Cohen M, Williams G, et al. Approval summary: imatinib mesylate in the treatment of metastatic and/or resectable malignant gastrointestinal stromal tumors. Clin Cancer Res 2002;8:3034 – 8.

16. Laird AD, Vajkoczy P, Shawver LK, et al. SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. Cancer Res 2000;60:4152 – 60.

17. Laird AD, Christensen JG, Li G, et al. SU6668 inhibits Flk-1/KDR and PDGFRβ *in vivo*, resulting in rapid apoptosis of tumor vasculature and tumor regression in mice. FASEB J 2002;16:681 – 90.

18. Mendel DB, Laird AD, Xin X, et al. *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 2003;9:327 – 37.

19. Abrams TJ, Lee LB, Murray LJ, Pryer NK, Cherrington JM. SU11248 inhibits KIT and PDGFRβ in preclinical models of human small cell lung cancer. Mol Cancer Ther 2003;2:471 – 8.

20. O'Farrell AM, Abrams TJ, Yuen HA, et al. SU11248 is a novel FLT3

tyrosine kinase inhibitor with potent activity *in vitro* and *in vivo*. Blood 2003;101:3597 – 605.

21. Patel N, Sun L, Moshinsky D, et al. A selective and oral small molecule inhibitor of VEGFR-2 and VEGFR-1 inhibits neovascularization and vascular permeability. J Pharmacol Exp Ther 2003;306:838 – 45.

22. Capdeville R, Buchdunger E, Zimmermann J, Matter A. Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat Rev Drug Discov 2002;1:493 – 502.

23. Mauro MJ, O'Dwyer M, Heinrich MC, Druker BJ. STI-571: a paradigm of new agents for cancer therapeutics. J Clin Oncol 2002;20:325 – 34.

24. Buchdunger E, O'Reilly T, Wood J. Pharmacology of imatinib (STI571). Eur J Cancer 2002;38:S28 – 36.

25. Pindolia VK, Zarowitz BJ. Imatinib mesylate, the first molecularly targeted gene suppressor. Pharmacotherapy 2002;22:1249 – 65.

26. Buchdunger E, Cioffi CL, Law N, et al. 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 2000;295: 139 – 45.

27. Laird AD, Li G, Moss KG, et al. Src family kinase activity is required for signal tranducer and activator of transcription 3 and focal adhesion kinase phosphorylation and vascular endothelial growth factor signaling *in vivo* and for anchorage-dependent and -independent growth of human tumor cells. Mol Cancer Ther 2003;2:461 – 9.

28. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-over-expressing metastatic breast cancer. J Clin Oncol 2002;20:719 – 26.

29. Wedge SR, Ogilvie DJ, Dukes M, et al. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res 2002;62:4645 – 55.

30. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669 – 76.

31. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 2003;111:1287 – 95.

32. Lindahl P, Johansson BR, Leveen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 1997;277: 242 – 5.

33. Fukumura D, Xavier R, Sugiura T, et al. Tumor induction of VEGF promoter activity in stromal cells. Cell 1998;94:715 – 25.

34. Sjoblom T, Shimizu A, O'Brien KP, et al. Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. Cancer Res 2001;61:5778 – 83.

35. Yu JL, Rak JW, Coomber BL, Hicklin DJ, Kerbel RS. Effect of p53 status on tumor response to antiangiogenic therapy. Science 2002;295: 1526 – 8.

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