

# SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo

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**FLT3 (fms-related tyrosine kinase/Flk2/Stk-2) is a receptor tyrosine kinase (RTK) primarily expressed on hematopoietic cells. In blasts from acute myelogenous leukemia (AML) patients, 2 classes of FLT3 activating mutations have been identified: internal tandem duplication (ITD) mutations in the juxtamembrane domain (25%-30% of patients) and point mutations in the kinase domain activation loop (7%-8% of patients). FLT3-ITD mutations are the most common molecular defect identified in AML and have been shown to be an independent prognostic factor for decreased survival. FLT3-ITD is therefore an attractive molecular target for therapy.**

**SU11248 is a recently described selective inhibitor with selectivity for split kinase domain RTKs, including platelet-derived growth factor receptors, vascular endothelial growth factor receptors, and KIT. We show that SU11248 also has potent activity against wild-type FLT3 (FLT3-WT), FLT3-ITD, and FLT3 activation loop (FLT3-Asp835) mutants in phosphorylation assays. SU11248 inhibits FLT3-driven phosphorylation and induces apoptosis in vitro. In addition, SU11248 inhibits FLT3-induced VEGF production. The in vivo efficacy of SU11248 was investigated in 2 FLT3-ITD models: a subcutaneous tumor xenograft model and a bone marrow en-**

**graftment model. We show that SU11248 (20 mg/kg/d) dramatically regresses FLT3-ITD tumors in the subcutaneous tumor xenograft model and prolongs survival in the bone marrow engraftment model. Pharmacokinetic and pharmacodynamic analysis in subcutaneous tumors showed that a single administration of an efficacious drug dose potently inhibits FLT3-ITD phosphorylation for up to 16 hours following a single dose. These results suggest that further exploration of SU11248 activity in AML patients is warranted. (Blood. 2003;101:3597-3605)**

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## Introduction

Signaling via receptor tyrosine kinases (RTKs) is frequently dysregulated in disease. FLT3 (fms-related tyrosine kinase/Flk2/Stk-2) belongs to the type III split-kinase domain family of RTKs, and is primarily expressed on immature hematopoietic progenitors and also on some mature myeloid and lymphoid cells.<sup>1-3</sup> FLT3 is activated following binding of FLT3 ligand (FL), which causes receptor dimerization leading to increased kinase activity and activation of downstream signaling pathways including Stat5, Ras, and PI3'kinase.<sup>4-6</sup> FLT3 normally regulates survival and proliferation of hematopoietic progenitor cells, in particular by synergy with other RTKs and cytokine receptors.<sup>7-9</sup> FLT3 is also expressed on acute myelogenous leukemia (AML) cells from the majority of patients and stimulates survival and proliferation of leukemic blasts.<sup>10-12</sup>

Two classes of activating FLT3 mutations have been identified in AML patients: internal tandem duplication (ITD) mutations in the juxtamembrane region expressed in 25% to 30% of AML patients, and point mutations in the activation loop of the kinase domain found in approximately 7% of patients (for review, see Gilliland and Griffin<sup>13</sup>). Both classes of mutation result in constitutive FLT3 tyrosine kinase activity and have been shown to transform hematopoietic cell lines in vitro and in vivo.<sup>5,14</sup> Recently Kelly et al<sup>15</sup> have reported that hematopoietic reconstitution with

primary bone marrow cells transduced with FLT3-ITD causes myeloproliferative disease in mice, with a latency period of 40 to 60 days. FLT3-ITD has also been shown to cooperate with promyelocytic leukemia-retinoic-acid receptor  $\alpha$  translocation to induce leukemia in a mouse model.<sup>16</sup>

FLT3-ITD is the most frequently observed molecular defect in AML and has been found in pediatric, adult, and elderly AML patients at frequencies of 10% to 16%, 21% to 27%, and 24% to 34%, respectively.<sup>17-20</sup> FLT3-ITD has been shown to be the single most significant poor prognosis factor in AML in several recent independent studies.<sup>21-23</sup> Clinically, FLT3-ITD is associated with increased leukocytosis, increased blast count, increased relapse rate, decreased disease-free survival, and poor overall survival. A recent study has shown that an increased ratio of FLT3-ITD relative to wild-type FLT3 (FLT3-WT) confers a poorer prognosis and that the FLT3-WT allele is absent in a minority of patients.<sup>24</sup> The catalytic Asp835 point mutation is also associated with leukocytosis and poor prognosis, though not as statistically significant as FLT3-ITD.<sup>25</sup> FLT3 therefore appears to be necessary for disease progression and is an attractive target for consideration in AML therapies.

Other split kinase RTKs such as vascular endothelial growth factor receptor 2 (VEGFR2; KDR) may play a role in the

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pathophysiology of AML by regulation of bone marrow angiogenesis. Increased bone marrow vascularity and increased cellular VEGF levels are evident in AML patients and predict a poor outcome.<sup>26-28</sup> Paracrine interactions between the bone marrow microenvironment and AML blasts likely play a role in increasing microvessel density and contributing to blast cell survival and proliferation (for review see Fiedler et al<sup>29</sup>). Agents targeting VEGFR2 may therefore also have clinical benefit in AML.

SU11248 is a small molecule that potently inhibits platelet-derived growth factor receptors (PDGFR)  $\alpha$  and  $\beta$ , VEGFR1, VEGFR2, and KIT,<sup>30</sup> and therefore has both direct antitumor and antiangiogenic properties. In this report we demonstrate that SU11248 also targets FLT3. SU11248 inhibits phosphorylation of wild-type FLT3 (FLT3-WT), FLT3-ITD, and FLT3-Asp835 in cell line models. In vivo SU11248 dramatically regresses subcutaneous FLT3-ITD tumors and increases survival in a FLT3-ITD bone marrow engraftment model. SU11248 is currently in AML and solid-tumor clinical trials.

## Materials and methods

### Cell lines

Cell culture media and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Gaithersburg, MD). RS4;11 and MV4;11, human leukemia cell lines that express FLT3-WT and a FLT3-ITD mutation, respectively,<sup>31</sup> were obtained from American Type Culture Collection (ATCC, Manassas, VA) (CRL-1873 and CRL-9591, respectively), and propagated as described.<sup>31</sup> OCI-AML5 cells (FLT3-WT) were obtained from Sreesha Srinivasa (Pharmacia, St Louis, MO) and maintained in RPMI 1640 with 10% FBS supplemented with 1 ng/mL each of recombinant human FL, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and KIT ligand (R&D Systems, Minneapolis, MN). Chinese hamster ovary (CHO) cells were maintained in F12 (HAM) media with 10% FBS.

### Site-directed mutagenesis and transfection

FLT3 cDNA was generously provided by Dr Olivier Rosnet (Molecular Oncology Unit, Institut National de la Santé et de la Recherche Médicale [INSERM], Marseille, France) and cloned into an internal ribosomal entry site-enhanced green fluorescent protein (IRES-EGFP) plasmid (Clontech, Palo Alto, CA). Site-directed mutagenesis was used to create ITD or activation loop mutations (QuickChange Kit, Stratagene, La Jolla, CA), and the mutations were confirmed by bidirectional sequencing. CHO cells were transfected with IRES-EGFP FLT3 using Lipofectamine as directed (Invitrogen, Carlsbad, CA).

### Cell proliferation, apoptosis, and enzyme-linked immunosorbent assays (ELISAs)

Cell lines were starved overnight in medium containing 0.1% FBS prior to addition of SU11248 and FL (50 ng/mL; FLT3-WT cells only). Proliferation was measured after 48 hours of culture using the Alamar Blue assay (Alamar Biosciences, Sacramento, CA) in triplicate for each condition, as described by the manufacturer. Trypan blue cell viability assays were performed in parallel and yielded similar results.

VEGF ELISAs on culture supernatants were performed at 72 hours of culture in triplicate for each condition, as directed by the manufacturer (QuantiGlo human VEGF Immunoassay kit, R&D Systems).

Apoptosis was measured 24 hours after compound addition by Western blotting to detect cleavage of poly (ADP-ribose) polymerase (PARP) or levels of caspase-3. Cells were lysed in lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane], pH 7.5; 137 mM NaCl; 10% glycerol; 1% nonidet P-40 [NP-40]; 0.1% sodium dodecyl sulfate [SDS]; 2 mM EDTA [ethylenediaminetetraacetic acid]) containing protease and phosphatase

inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM Pefabloc, 1.2  $\mu$ M aprotinin, 40  $\mu$ M bestatin, 5.6  $\mu$ M E-64, 4  $\mu$ M leupeptin, and 4  $\mu$ M pepstatin A). Equivalent amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred to nitrocellulose membranes. Membranes were probed with an anti-PARP antibody (Cell Signaling Technology, Beverly, MA) or caspase-3 (Upstate Biotechnology, Lake Placid, NY).

### Immunoprecipitation and Western blot (IP/W) analysis

For in vitro experiments, cells were treated with SU11248 for 2 hours in medium containing 0.1% FBS. Cells expressing FLT3-WT were stimulated with 150 ng/mL FL for 5 minutes. Cells were lysed as described above. Equivalent amounts of protein from each sample were immunoprecipitated overnight at 4°C with an agarose-conjugated anti-FLT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immune complexes were washed (150 mM NaCl; 1.5 mM MgCl<sub>2</sub>; 50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.5; 10% glycerol; 0.1% Triton X-100; and 1 mM EGTA [ethylene glycol tetraacetic acid]), and, following SDS-PAGE, proteins were transferred to nitrocellulose membranes. Membranes were probed with an antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, or Transduction Laboratories, Lexington, KY) and then stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL). Membranes were reprobed with an anti-FLT3 antibody (Santa Cruz Biotechnology). Stat5 antibodies for immunoprecipitation and Western blot analysis were from Upstate Biotechnology and Transduction Laboratories, respectively.

### In vivo models

Prior to implantation, cells were harvested during exponential growth, washed once with sterile phosphate-buffered saline (PBS), and, for subcutaneous injection, resuspended in Matrigel (BD Biosciences, Bedford, MA). All animal studies were carried out with the approval of the SUGEN Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited animal facility and were in accordance with the Institute of Laboratory Animal Research (National Institutes of Health, Bethesda, MD) *Guide for the Care and Use of Laboratory Animals*.<sup>32</sup> Female athymic nu/nu mice (8 to 12 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and female nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice from Jackson Laboratories (Bar Harbor, ME). All animals were maintained under clean room conditions in sterile microisolator cages (Allentown Caging Equipment, Allentown, NJ) with Sani-Chips (PJ Murphy, Forest Products, Montville, NJ) and were provided sterile rodent chow and water ad libitum.

**Subcutaneous model.** Athymic nu/nu mice received subcutaneous injections into the hind flank on day 0 with  $5 \times 10^6$  MV4;11 or RS4;11 cells. In vivo experiments were scheduled to evaluate the therapeutic effects of daily oral administration of SU11248 on pre-existing tumors (size 300-500 mm<sup>3</sup>) in all studies. Animals were randomized into treatment groups of 10 mice each for efficacy studies and 2 to 3 mice each for target modulation studies. A range of doses of SU11248 or its vehicle were administered, as indicated in figure legends. SU11248 was delivered orally (PO) in a citrate-buffered solution (pH 3.5) by gavage. Tumor growth was measured twice weekly using Vernier calipers (Fowler, Des Plaines, IL) for the duration of the treatment. Tumor volumes were calculated as the product of length  $\times$  width  $\times$  height.

**Target modulation.** Mice bearing tumors were administered a single oral dose of SU11248 at the indicated concentrations. Control animals received either no treatment (predose) or vehicle. At the indicated times after dosing, individual mice were killed, their tumors resected, and a blood sample taken by cardiac puncture using a syringe primed with heparin sulfate. Pharmacokinetic analysis of plasma and generation of tumor lysates were performed as described.<sup>30</sup> IP/W analysis was performed as described for in vitro experiments.

**Bone marrow model.** NOD-SCID mice were pretreated with cyclophosphamide (Neosar, Pharmacia, Kalamazoo, MI) by intraperitoneal injection of 150 mg/kg/d for 2 days,<sup>33</sup> followed by 24 hours of rest prior to

intravenous injection of  $5 \times 10^6$  cells via the tail vein. At experimental end points (within 90 days of implantation) mice were anesthetized, followed by terminal blood collection via intracardiac puncture. Bone marrow cell suspensions were prepared by flushing mouse femurs with cold, sterile PBS. A range of doses of SU11248 or its vehicle were orally administered once daily, as indicated in figure and table legends. For all studies, a paired Student *t* test was used to assess differences between treated and control groups ( $P < .05$  was considered significant).

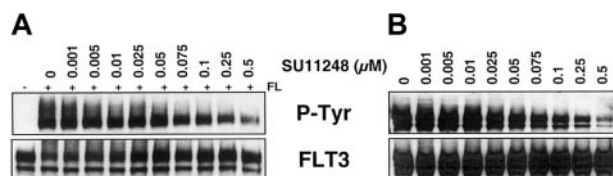
**Flow cytometric analysis.** For flow cytometric analysis of bone marrow samples, erythrocytes were lysed using Cal-Lyse (Caltag Laboratories, Burlingame, CA) following the manufacturer's protocol. The percentage of human cells in bone marrow was determined by staining with phycoerythrin-conjugated antihuman CD45 (Pharmingen, San Diego, CA) and isotype controls. Samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer. Data analysis was performed using CellQuest.

**Histopathology and IHC.** Sections were prepared from formalin-fixed, decalcified, and paraffin-embedded mouse tibias. General tissue morphology and microvessels were visualized using conventional hematoxylin and eosin (H&E) staining. Cell proliferation was visualized using the MIB-1 monoclonal antibody for Ki-67 (Immunotech, Westbrook, ME) and biotinylated polyclonal rabbit antimouse secondary antibody (Zymed, South San Francisco, CA), using a peroxidase-based immunostaining protocol (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA).

## Results

### SU11248 inhibits FLT3-WT, FLT3-ITD, and FLT3-Asp835 mutant phosphorylation

SU11248 was designed to have broad selectivity for the split kinase family of RTKs and potently inhibits PDGFR $\alpha$ , PDGFR $\beta$ , VEGFR2 (KDR), VEGFR1 (FLT1), and KIT, as recently described.<sup>30</sup> Given the sequence conservation between FLT3 and other members of the class III RTK family, we reasoned that SU11248 may also inhibit FLT3. To investigate SU11248 activity, cellular assays to detect changes in FLT3 phosphorylation were performed using leukemia cell lines RS4;11 (which expresses wild-type FLT3) and MV4;11 (which expresses FLT3-ITD mutant).<sup>31</sup> For both cell lines, IP/W analysis showed 2 forms of FLT3 protein, a higher ( $\approx 160$  kDa) and lower (135 kDa) molecular weight form that likely correspond to mature and immature forms of FLT3 expressed on the cell surface and intracellularly, respectively.<sup>2</sup> In RS4;11 cells (FLT3-WT), addition of FL was necessary to stimulate FLT3 phosphorylation, and, as predicted, primarily the higher molecular weight (likely extracellular) FLT3 species was phosphorylated. As shown in Figure 1A, treatment with SU11248 inhibited FLT3-WT phosphorylation in a dose-dependent manner with a 50% inhibitory concentration (IC<sub>50</sub>) of approximately 250 nM. Similar results were obtained in the OC1-AML5 human leukemia cell line, which also expresses FLT3-WT, as assessed by genotyping (data not shown).



**Figure 1. SU11248 inhibits FLT3-WT and FLT3-ITD phosphorylation.** (A) RS4;11 and (B) MV4;11 cells were incubated with SU11248 at the indicated concentrations for 2 hours. For RS4;11 only, cells were stimulated for 5 minutes with FL (150 ng/mL). Lysates were prepared and immunoprecipitated with an anti-FLT3 antibody. After SDS-PAGE and transfer to nitrocellulose, the blots were probed with an antiphosphotyrosine antibody (top) and subsequently stripped and reprobed with an anti-FLT3 antibody (bottom). Similar results were obtained in at least 4 independent experiments.

In MV4;11 cells that express FLT3-ITD, it is noteworthy that both high and low molecular weight forms of FLT3 are phosphorylated in the absence of FLT3 ligand (FL), consistent with expression of FLT3-ITD (Figure 1B). SU11248 inhibited FLT3-ITD phosphorylation in a dose-dependent manner with an IC<sub>50</sub> of 50 nM following a 2-hour treatment (Figure 1B). In 5 separate experiments, IC<sub>50</sub> values ranged from 10 to 60 nM for FLT3-ITD, with a mean of 50 nM (data not shown).

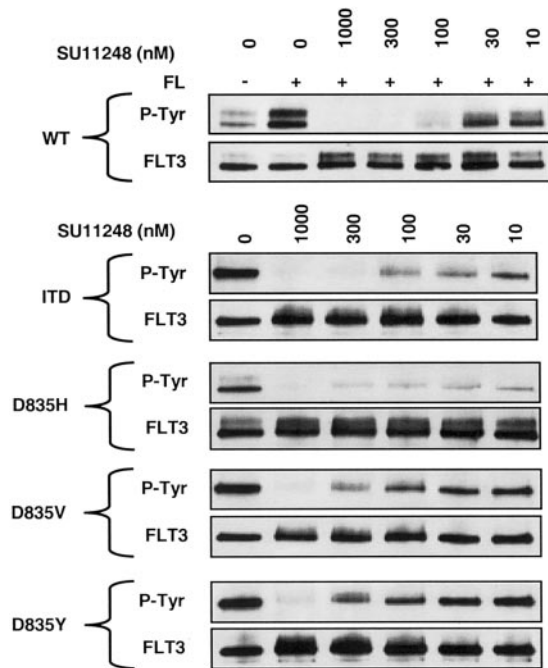
To address whether the apparent difference in sensitivity of FLT3-WT and FLT3-ITD to SU11248 could be attributed to use of these nonisogenic cell lines, a 32D myeloid cell line engineered to express FLT3-WT or FLT3-ITD<sup>5</sup> was tested. SU11248 inhibited 32D:FLT3-WT phosphorylation with an IC<sub>50</sub> of approximately 250 nM; while 32D:FLT3-ITD was inhibited with an IC<sub>50</sub> of 50 nM (data not shown). These cell lines showed similar levels of FLT3 cell surface staining when assessed by flow cytometry analysis. It therefore appears that FLT3-ITD has increased sensitivity to SU11248 relative to FLT3-WT.

We also investigated the activity of SU11248 against FLT3 activation loop mutations using transiently transfected CHO cells. For CHO cells expressing FLT3-WT, treatment with FLT3 ligand stimulated phosphorylation, and SU11248 inhibited phosphorylation with an IC<sub>50</sub> of approximately 30 nM (Figure 2). FLT3 was constitutively phosphorylated in all cells expressing mutant FLT3. SU11248 inhibited phosphorylation of FLT3-ITD with an IC<sub>50</sub> of less than 10 nM. Similarly, phosphorylation of activation loop mutants Asp835Tyr, Asp835Val, and Asp835His was inhibited by SU11248, with IC<sub>50</sub> values of 30 to 300 nM (Figure 2). Taken together these data demonstrate that SU11248 inhibits phosphorylation of FLT3-WT, FLT3-ITD, and FLT3-Asp835 mutant forms of FLT3.

### SU11248 inhibits proliferation induced by FLT3-ITD and FLT3-WT

Having demonstrated that SU11248 inhibits FLT3 phosphorylation, the biologic consequence of inhibition was tested in cell proliferation and apoptosis assays. For the FLT3-ITD cell line MV4;11, SU11248 dramatically inhibited cellular proliferation in a dose-dependent manner with an IC<sub>50</sub> of 1 to 10 nM (Figure 3A). We used the factor-dependent FLT3-WT cell line, OC1-AML5, to investigate the effect of SU11248 on proliferation driven by FLT3-WT. Addition of FL stimulated an approximately 3-fold increase in OC1-AML5 cell number over 48 hours (data not shown). SU11248 inhibited proliferation of OC1-AML5 with an IC<sub>50</sub> of approximately 10 nM in the presence of FLT3 ligand (Figure 3A). The mean IC<sub>50</sub> values for inhibition of proliferation of MV4;11 and OC1-AML5 cells at 48 hours were 8 nM and 14 nM, respectively, in at least 3 independent experiments. Similar results were apparent in trypan blue viable cell count assays, in which SU11248 inhibited expansion of MV4;11 and OC1-AML5 cell lines in a dose-dependent manner, with IC<sub>50</sub> values in the 10 to 50 nM range. RS4;11 cells (FLT3-WT) are not factor-dependent and addition of FLT3 ligand induced only a modest increase in proliferation. Accordingly SU11248 inhibited FLT3 ligand-stimulated proliferation in RS4;11 cells with an IC<sub>50</sub> of more than 500 nM (data not shown).

Next the ability of SU11248 to induce apoptosis was assessed using measurement of PARP cleavage and caspase-3 levels as indicators. For FLT3-ITD-expressing cells (MV4;11) a low level of cleaved PARP was evident at baseline. Addition of SU11248 increased PARP cleavage (89-kDa and 24-kDa fragments) in a dose-dependent manner, evident at 10 nM SU11248, with a



**Figure 2. SU11248 inhibits phosphorylation of FLT3-Asp835.** CHO cells were transiently transfected with DNA encoding wild-type (top) or mutant (bottom) FLT3 and incubated with SU11248 at indicated concentrations for one hour. Following treatment, CHO cells transfected with FLT3-WT were stimulated with FL (100 ng/mL) for 10 minutes. Lysates were prepared and immunoprecipitated with an anti-FLT3 antibody. Following transfer to nitrocellulose, blots were initially probed with an antiphosphotyrosine antibody and subsequently stripped and reprobed with an anti-FLT3 antibody.

concomitant decrease in full-length PARP (Figure 3B). Similarly, a dose-dependent increase in levels of the proteolytic (active) fragment of caspase-3 was observed in the presence of SU11248 (Figure 3D). Similar observations were made using FACS analysis for active caspase-3 (data not shown). For OC1-AML5 cells, cleaved PARP and caspase-3 were evident within a similar dose range (Figure 3C,E), although the magnitude of cell death in cultures was less than MV4;11 cells. RS4;11 cells did not undergo apoptosis in the presence of SU11248, consistent with a lack of dependence on FLT3 for survival (data not shown). Taken together, these data suggest that SU11248 inhibits proliferation driven by both FLT3-WT and FLT3-ITD, resulting in apoptosis.

#### SU11248 causes dramatic regression of large subcutaneous FLT3-ITD tumors in athymic mice

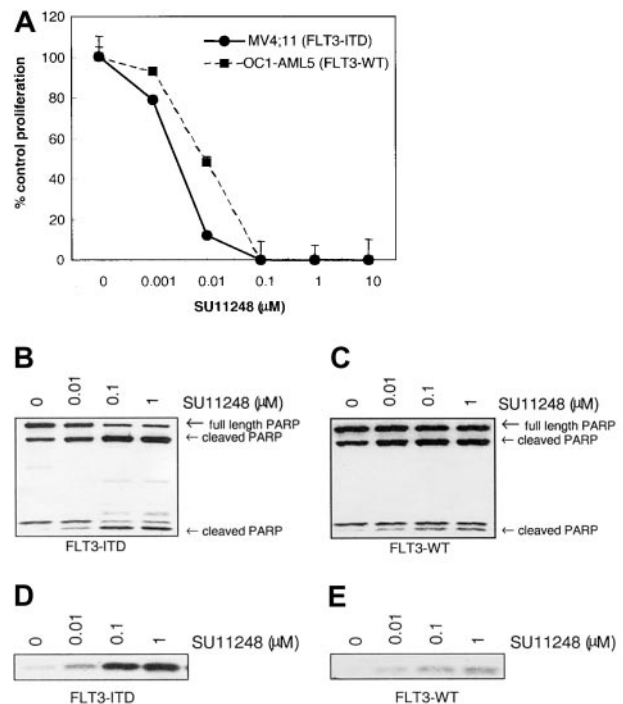
Having determined that SU11248 is a potent inhibitor of FLT3 *in vitro*, we evaluated the *in vivo* activity of this compound using a FLT3-ITD tumor model. Subcutaneous implantation of MV4;11 cells expressing FLT3-ITD into athymic mice resulted in growth of solid tumors of approximately 500 mm<sup>3</sup> within 4 weeks; tumors continued to increase in size until mice were killed. Immunohistochemical staining for Ki67 demonstrated a high proliferation index, consistent with the rapid tumor growth rate. In addition strong FLT3 expression on tumor cells was observed (data not shown).

We previously reported that SU11248 dosed orally at 40 mg/kg/d is fully efficacious in a number of subcutaneous tumor xenograft models, including SF763T and Colo205.<sup>30</sup> Therefore, the 40 mg/kg/d dose was initially tested for efficacy in mice bearing established MV4;11 tumors of approximately 300 to 500 mm<sup>3</sup>. SU11248 treatment resulted in a dramatic destruction of the tumor with visual disappearance 4 days after treatment in all mice

(n = 10) (Figure 4A). Daily administration of SU11248 was ceased 8 days after full regression was observed to determine if the effect of SU11248 was reversible. This resulted in eventual regrowth of 6 of 10 tumors, with no regrowth in the remaining 4 (2 of which were observed for 10 months after treatment stopped). Of the regrowing tumors, 4 were again treated with 40 mg/kg/d of SU11248 when they reached approximately 2000 mm<sup>3</sup>. All of these tumors responded dramatically to the second treatment with 40 mg/kg/d of daily SU11248, suggesting that tumors had not developed resistance (Figure 4B).

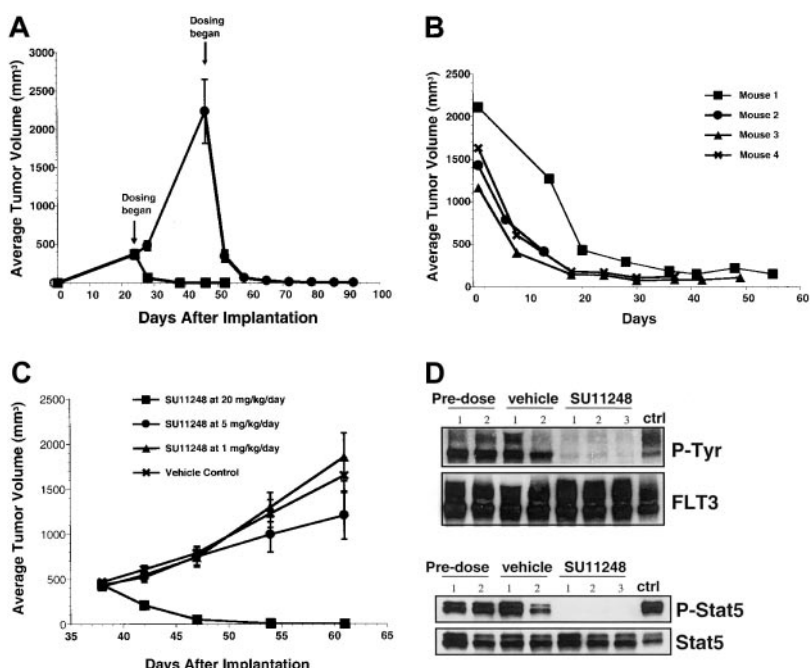
When tumors from the vehicle control group in Figure 4A reached approximately 2000 mm<sup>3</sup>, these mice were treated with SU11248, resulting in complete tumor regression. Dosing was ceased after an average of 23 days of treatment. Of 10 mice, 2 exhibited tumor regrowth, while 8 mice exhibited no regrowth after 2.5 months (2 of which are still viable and remain tumor-free after 9 months). Daily treatment with SU11248 was well tolerated, with no signs of gross toxicity. The rapid regression of the FLT3-ITD-expressing cell line MV4;11 by SU11248 is consistent with dependence on constitutive FLT3 signaling for survival.

Since 40 mg/kg/d of SU11248 caused profound tumor regression, lower daily doses of SU11248 (20, 5, and 1 mg/kg/d) were evaluated to determine the lowest efficacious dose. In mice receiving 20 mg/kg/d of SU11248, complete tumor regression was apparent although not as rapidly as observed with the 40 mg/kg/d dose (Figure 4C). For the lower dose of 5 mg/kg/d, a marginal but insignificant effect on tumor growth was apparent, and 1 mg/kg/d had no effect. In a separate study a 10 mg/kg dose induced tumor growth inhibition but not regression (data not shown). These efficacy studies confirm dose-dependent inhibition of tumor growth



**Figure 3. SU11248 inhibits cellular proliferation and induces apoptosis.** (A) Cells were serum starved overnight and then cultured in the presence of SU11248 or vehicle control for 48 hours (with addition of FL for OC1-AML5 [FLT3-WT] cells). Proliferation was assessed in triplicate for each condition using the Alamar blue assay, and the mean  $\pm$  SD is shown for each condition. (B,D) MV4;11 cells (FLT3-ITD) and (C,E) OC1-AML5 treated with SU11248, or vehicle control, in the presence of FL for OC1-AML5 cells only. Apoptosis was assessed after 24 hours of incubation by Western analysis for PARP cleavage (B-C) or caspase-3 (D-E). Similar results were obtained in at least 3 independent experiments.

**Figure 4. SU11248 exhibited dose-dependent efficacy and regressed large established subcutaneous FLT3-ITD tumors when administered at 40 and 20 mg/kg/d in athymic mice.** Daily oral administration of (A) SU11248 at 40 mg/kg/d or (C) a dose response of 20, 5, and 1 mg/kg/d was initiated when MV4;11 tumors reached an average of 400 mm<sup>3</sup> volume. (A) Additionally, SU11248 (■) was administered to mice bearing large tumors (2000 mm<sup>3</sup>) from the original vehicle-treated control group (●). (B) In mice with fully regressed tumors, dosing of SU11248 at 40 mg/kg/d was ceased and tumor regrowth occurred in some animals. When tumor volume exceeded 1000 mm<sup>3</sup>, SU11248 administration at 40 mg/kg/d recommenced to evaluate any alteration in sensitivity to the compound (4 mice). Tumor volume was measured on the indicated days, with the mean tumor volume ± SEM indicated for each group, each of which consisted of 10 mice (excluding panel B). (D) Athymic mice bearing established MV4;11 tumor xenografts were given a single oral dose of SU11248 (40 mg/kg) or citrate buffer vehicle; predose animals received no treatment. FLT3 (top panel) or Stat5 (bottom panel) were immunoprecipitated from tumor lysates, and Western blots were probed for phosphotyrosine or phospho-Stat5, respectively. Blots were reprobed for total FLT3 or Stat5. Each lane represents a separate animal. Lane labeled "ctrl" is positive control cell lysate for FLT3 immunoprecipitation.



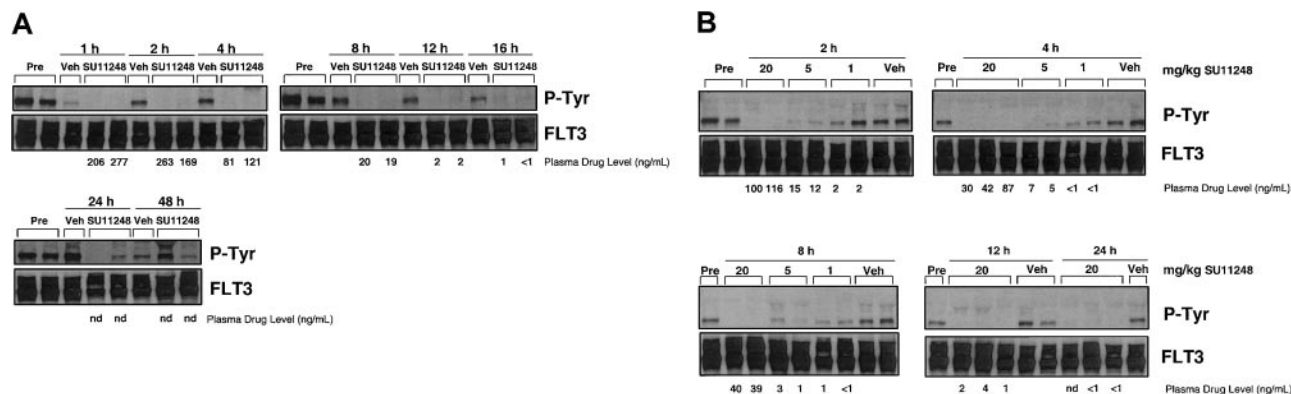
by SU11248 and demonstrate that 20 mg/kg/d is the minimal efficacious dose to induce regression.

To assess FLT3 phosphorylation in tumor cells, tumor (MV4;11)-bearing mice were treated with vehicle control or a single dose of SU11248 (40 mg/kg). Analysis of FLT3 phosphorylation by IP/W showed high levels of phosphorylation in tumor lysates from predose (untreated) or vehicle-treated animals (n = 3 per group). FLT3 protein levels were not altered by SU11248 but FLT3 phosphorylation was dramatically suppressed in all SU11248-treated animals within 4 hours (Figure 4D). Analysis of a downstream FLT3-ITD signaling protein, Stat5, showed similarly decreased phosphorylation; Stat5 was highly activated at baseline in both vehicle and untreated animals and markedly inhibited by a single administration of SU11248 (Figure 4D).

**Establishment of pharmacokinetic and pharmacodynamic (PK/PD) relationship for FLT3-ITD inhibition in vivo**

Establishment of a PK/PD relationship is an important aspect for translation of preclinical data to clinical trials. We have reported

that SU11248 exhibits predictable and dose-dependent pharmacokinetics in mice.<sup>30</sup> To define the PK/PD relationship for SU11248 in modulation of FLT3-ITD in vivo, we first addressed the kinetics of inhibition of FLT3 phosphorylation at 20 mg/kg SU11248, the lowest dose inducing tumor regression. After a single dose of SU11248, tumors were removed at various time points for analysis of FLT3 phosphorylation, and plasma was simultaneously collected for analysis of drug levels. The single SU11248 administration completely inhibited FLT3 phosphorylation within 2 hours, for at least 12 to 16 hours (Figure 5A). At 24 and 48 hours, phosphorylation increased, although levels remained lower than those observed in tumors from untreated animals. Plasma inhibitor levels are indicated in Figure 5 and showed expected concentrations and kinetics: a dose of 20 mg/kg produced a maximal concentration (C<sub>max</sub>) of more than 100 ng/mL (≈ 250 nM) within 2 hours and plasma drug levels were less than 1 ng/mL at 16 hours after dosing. The observed inhibition of FLT3 phosphorylation at 12 and 16 hours, when plasma drug levels were less than 1 ng/mL, may reflect the duration of FLT3 inhibition once target plasma concentration is attained (below).



**Figure 5. Inhibition of FLT3-ITD phosphorylation by SU11248 is time- and dose-dependent.** (A) Athymic mice carrying established MV4;11 tumor xenografts were given a single oral dose of SU11248 (20 mg/kg) or citrate buffer vehicle; predose animals received no treatment. Tumors were harvested at the indicated times, and FLT3 was analyzed by IP/W for phosphotyrosine, followed by total FLT3. Each lane represents a separate animal, and plasma drug concentrations are indicated below for each. ND indicates not detectable. (B) Experiment performed as in panel A except that mice received SU11248 at either 20, 5, or 1 mg/kg for the indicated times.

Next, the effects of subefficacious (5 mg/kg) and nonefficacious (1 mg/kg) doses of SU11248 were examined at 2, 4, and 8 hours after administration, as shown in Figure 5B. A single dose of SU11248 at 5 mg/kg inhibited FLT3 phosphorylation at 2 hours with less inhibition at 4 and 8 hours. Inhibition was weaker and more transient than that observed with 20 mg/kg (Figure 5B). The nonefficacious dose of 1 mg/kg marginally inhibited FLT3 phosphorylation. Quantitation of Western blots using Quantity One software (BioRad, Hercules, CA) supported these observations (data not shown). It therefore appears that strong (> 50%) inhibition of FLT3-ITD phosphorylation maintained for 8 to 16 hours correlates with regression in this model. Additional PK/PD analysis comparing different drug doses suggested that a target plasma concentration of 30 to 50 ng/mL for at least 8 hours correlated with robust sustained inhibition of FLT3-ITD, attained with 20 mg/kg and not with 5 mg/kg. This is slightly lower than, but in a similar range to, that predicted for PDGFR and Flk-1 (50-100 ng/mL).<sup>30</sup> These data show that inhibition of FLT3-ITD phosphorylation by SU11248 is dose-dependent, and the magnitude and extent of FLT3 inhibition at 1, 5, and 20 mg/kg correlate with results of the efficacy experiments.

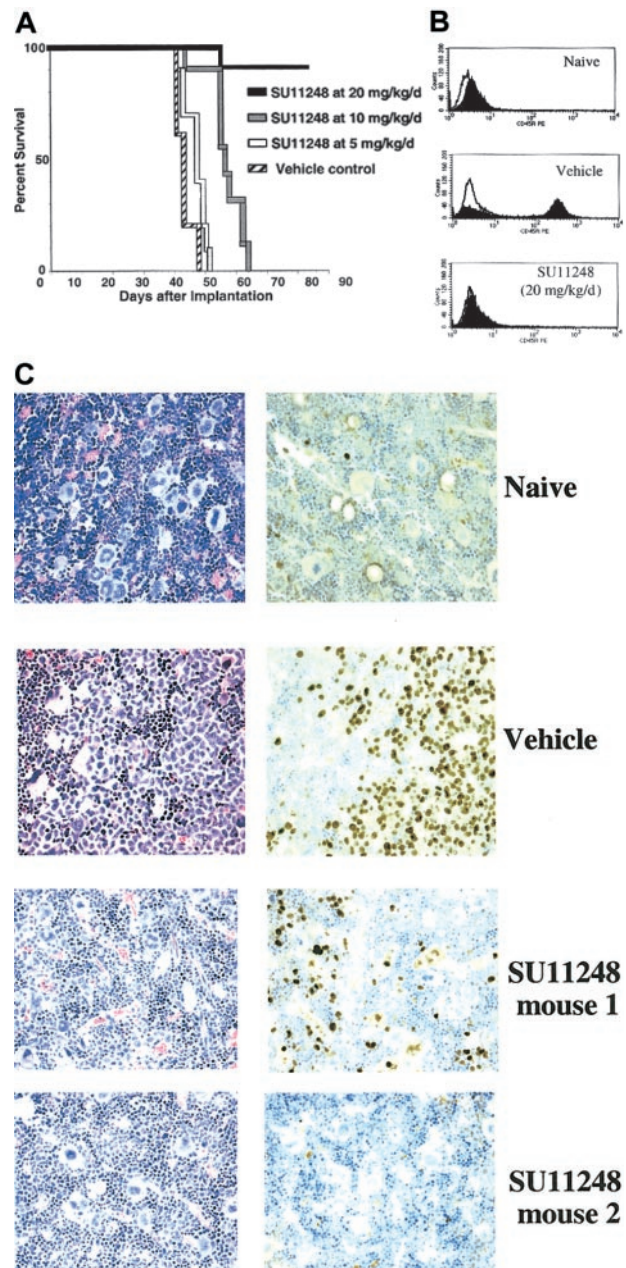
#### SU11248 enhances survival in a bone marrow engraftment model

Having shown that SU11248 regresses FLT3-ITD tumors in a subcutaneous tumor model, we investigated the effects of SU11248 in a more physiologically relevant leukemia model in which cells engraft in the bone marrow, established using MV4;11 cells. Mice were sublethally pretreated with cyclophosphamide<sup>33</sup> to reduce the endogenous bone marrow cell population and facilitate engraftment before intravenous injection of  $5 \times 10^6$  MV4;11 cells. Flow cytometric analysis of human CD45 expression in bone marrow was performed to provide evidence of disease. Human cells were detectable in bone marrow approximately 4 weeks after cell inoculation. Clinically, within 40 to 50 days mice exhibited hind limb paralysis, ruffled fur, and decreased spontaneous activity (grooming and ambulation).

To study the effect of SU11248 on survival, once-daily treatment with either SU11248 or vehicle was initiated 3 weeks after MV4;11 cell implantation. Animals were continued on the assigned SU11248 or vehicle treatment regimen until sufficient clinical evidence of symptomatic disease progression (eg, hind limb paralysis or morbidity) was present to warrant humane killing. All vehicle control group mice died within 39 to 50 days, with a mean survival time of 41 days (Figure 6A). Mice on the SU11248 arm demonstrated prolonged dose-dependent survival. With 5, 10, and 20 mg/kg/d of orally administered SU11248, the mean survival time was significantly prolonged to 46, 56, and at least 83 days, respectively ( $P = .002$ ,  $P < .0001$ ,  $P < .0001$ , respectively). Accompanying the increase in survival time, SU11248-treated mice demonstrated a marked lack of hind limb paralysis, a healthier appearing coat, and more normal levels of physical activity, while the vehicle-treated mice had succumbed to disease. FACS analysis showed that vehicle-treated animals had an average of 49% human CD45<sup>+</sup> cells in bone marrow (range, 19%-73%) while SU11248-treated mice had an average of 2.3% (Figure 6B). In control mice pretreated with cyclophosphamide but not inoculated with MV4;11 cells, less than 1% human CD45<sup>+</sup> cells were detected in the bone marrow.

Morphologic examination of H&E-stained bone marrow sections from untreated or vehicle-treated animals showed large cells with pale nuclei and abundant mitotic figures in locally abundant

regions as well as diffuse infiltration (Figure 6C). MV4;11 cells had strong nuclear immunoreactivity for Ki67, while other cells stained weakly. Bone marrow from MV4;11-inoculated mice also appeared highly vascularized (data not shown). Treatment with SU11248 at 20 mg/kg/d resulted in reduced numbers of bone marrow MV4;11 cells when examined on day 50. In some cases, only a few leukemic cells distributed throughout the bone marrow remained, while other samples exhibited no evidence of leukemic cells, which correlated with lack of overt disease symptoms (Figure 6C). These data show



**Figure 6. SU11248 demonstrated a dose-dependent increase in survival in a FLT3-ITD (MV4;11) bone marrow engraftment model.** (A) Kaplan-Meier plot of survival. At 3 weeks after intravenous MV4;11 cell implantation in cyclophosphamide-pretreated NOD-SCID mice, daily oral administration of SU11248 at 20, 10, or 5 mg/kg/d, or vehicle, was initiated and continued through the end of the experiment (10 mice per group). Mice exhibiting hind limb paralysis or signs of morbidity were humanely killed. (B) Bone marrow was collected for flow cytometric analysis of human CD45 expression as a marker for MV4;11 cells from naive controls and MV4;11-inoculated mice treated with vehicle or SU11248 (20 mg/kg/d). (C) Paraffin sections of bone marrow from vehicle-treated mice and SU11248-treated mice (20 mg/kg/d) were stained with H&E (left panels) or Ki67 (right panels). Representative results are shown.

that SU11248 has efficacy in a FLT3-ITD model of lethal bone marrow disease, with similar dose-dependence to the subcutaneous model.

### SU11248 decreases VEGF levels following FLT3 signaling

Increased microvessel density and increased VEGFR2 expression<sup>26,27</sup> has been reported in bone marrow of AML patients, relative to healthy donors, and it has been proposed that paracrine growth stimulatory interactions occur between blasts and endothelial cells.<sup>29</sup> VEGF binding to its receptor KDR (VEGFR2, Flk-1) is one of the best-characterized positive inducers of tumor neovascularization by the stimulation of endothelial cell proliferation and migration<sup>34</sup> (for reviews see Ferrara and Gerber<sup>35</sup>; Albitar<sup>36</sup>; and Rosen<sup>37</sup>). To assess the effects of FLT3-ITD signaling on VEGF production, we measured VEGF levels in tissue culture supernatants from cell lines. MV4;11 (FLT3-ITD) cells constitutively produced relatively high levels of VEGF, ranging from 35 to 92 pg/mL per 10<sup>5</sup> cells. Culture in the presence of SU11248 inhibited VEGF production in a dose-dependent manner with an IC<sub>50</sub> of approximately 10 nM (Table 1). When VEGF levels were normalized for viable cell number the inhibitory effect was still apparent, suggesting that the reduced VEGF levels are not simply a reflection of decreased cell number in the presence of SU11248, but more likely due to decreased signaling in viable cells. To assess if VEGF production is specific to leukemia cell lines, similar experiments were performed in 32D:FLT3-ITD cells and murine VEGF was measured. We observed that 32D:FLT3-ITD cells constitutively produced VEGF (data not shown).

Analysis of FLT3-WT cells showed that VEGF was barely detectable in 72-hour culture supernatants from RS4;11 cells (< 1 pg/mL per 10<sup>5</sup> cells), while OC1-AML5 cells produced low levels (32 pg/mL per 10<sup>5</sup> cells). However addition of FLT3 ligand increased VEGF production by 3- to 4-fold in each cell line, and this effect was inhibited by SU11248 (Table 1). We also measured human VEGF levels in plasma from a survival study in the MV4;11 bone marrow engraftment model. In naive mice (n = 4) VEGF was not detected. In MV4;11-inoculated mice with hind limb paralysis treated with vehicle, VEGF was detectable in 9 of 9 cases (mean 49 pg/mL, range). VEGF was not detected in any of 6 apparently healthy MV4;11-inoculated mice treated with SU11248 at 20 mg/kg daily. These data suggest that signaling downstream of both FLT3-WT and FLT3-ITD results in VEGF production, which is inhibited by SU11248. VEGF is a potential marker for FLT3-ITD *in vivo*.

## Discussion

AML is a malignant disorder of hematopoietic progenitor cells and constitutes approximately 90% of adult acute leukemia. As the vast

majority of adults who develop AML eventually succumb to their disease or associated cytotoxic therapy, there is an urgent need for new therapies. The FLT3-ITD mutation is the most common molecular defect in AML and confers poor prognosis. In this report we have characterized SU11248 as a FLT3 inhibitor. We show that SU11248 inhibits phosphorylation of FLT3-ITD, FLT3-Asp835, as well as FLT3-WT, which results in inhibition of proliferation and induction of apoptosis *in vitro*. Consistent with these observations, SU11248 had dramatic efficacy in a FLT3-ITD xenograft model and also in a bone marrow engraftment model. Analysis of SU11248 effects on FLT3-ITD phosphorylation in tumor xenografts showed a time- and dose-dependent inhibition of phosphorylation, and a PK/PD relationship was established. Finally, SU11248 treatment can abrogate VEGF production, secondary to FLT3 signaling.

In cellular assays, SU11248 potently inhibited FLT3-ITD phosphorylation with an IC<sub>50</sub> of 50 nM, similar to those reported for other RTK targets of SU11248.<sup>30</sup> SU11248 exhibited a relative increase in potency for inhibition of FLT3-ITD phosphorylation relative to FLT3-WT when assayed in different cell lines that express either endogenous or overexpressed FLT3 and also when assayed in transiently transfected CHO cells. There are several possibilities to account for this effect: SU11248 may bind to FLT3-ITD with increased affinity due to differences in conformation between WT and ITD; accessibility to drug may be influenced by differences in cellular localization; or levels of ligand necessary to stimulate phosphorylation of FLT3-WT *in vitro* may be nonphysiologic. Irrespective of the mechanism, our data demonstrate that SU11248 inhibits phosphorylation of both mutant FLT3 and FLT3-WT, and may have an increased potency for FLT3-ITD relative to FLT3-WT. SU11248 also inhibited phosphorylation of Asp835 point mutations, Asp835Val, Asp835His, and Asp835Tyr.

As anticipated, the biologic consequences of FLT3 inhibition were most profound in MV4;11 cells where FLT3-ITD appears to drive proliferation. Proliferation was inhibited by SU11248 with an IC<sub>50</sub> of 1 to 10 nM, resulting in apoptosis. SU11248 also inhibited FL-driven proliferation in the FLT3-WT cell line OC1-AML5, most likely because this cell line undergoes apoptosis in the absence of added cytokines and FL maintains survival. It is unlikely that the effects of SU11248 on MV4;11 cells are mediated by targeting other RTKs, as MV4;11 cells do not appear to express PDGFR; and while weak expression of KIT was detected, no phosphorylation was apparent in IP/W experiments (data not shown). SU11248 inhibited FLT3 phosphorylation in RS4;11 cells, but this cell line is more than 100-fold less sensitive to SU11248 than MV4;11 in biologic assays. This can be attributed to the lack of requirement for FLT3 signaling for survival or proliferation in RS4;11, and also demonstrates that SU11248 does not have nonspecific effects. These data are consistent with observations of

**Table 1. MV4;11 cells were cultured in the absence of FL with SU11248 as indicated**

SU11248, uM	MV4;11		OC1-AML5		RS4;11	
	Mean ± SD	% control	Mean ± SD	% control	Mean ± SD	% control
0	346.7 ± 5.6	100.0	100.8 ± 0.5	100.0	31.03 ± 6.1	100.0
0.001	287.8 ± 4.0	83.0	92.5 ± 13.1	91.8	32.82 ± 1.9	105.8
0.01	65.4 ± 3.6	18.9	35.6 ± 2.7	35.3	9.62 ± 0.5	31.0
0.1	31.2 ± 2.4	9.0	33.6 ± 2.8	33.3	1.24 ± 0.4	4.0
1	30.5 ± 3.9	8.8	28.3 ± 1.7	28.0	2.3 ± 0.5	7.4
10	23.3 ± 4.6	6.7	15.6 ± 2.4	15.5	2.94 ± 1.0	9.5

OC1-AML5 and RS4;11 cultures contained FL (50 ng/mL). VEGF levels in tissue culture supernatants were measured by ELISA in triplicate for each condition, and units are pg/mL. One of 3 experiments that yielded similar results is shown.

Levis et al<sup>38</sup> who reported that FLT3 phosphorylation was inhibited by CEP-701, a different FLT3 inhibitor, in the BV173 cell line, but cells did not undergo apoptosis. It is likely that inhibition of additional signaling pathways is necessary to elicit a cytotoxic response in RS4;11.

A subcutaneous tumor xenograft model was used to assess the effects of SU11248 *in vivo* and to help define the PK/PD relationship for FLT3-ITD, important for the translation of this compound to clinical testing. We found that SU11248 dramatically regressed FLT3-ITD xenografts in a dose-dependent manner, with a minimum fully efficacious dose of 20 mg/kg/d most likely acting as a direct antitumor agent. This dose is lower than that identified for inhibition of other human tumor cell line xenografts such as SF763T and Colo205 (40 mg/kg/d). In these models SU11248 may act primarily as an antiangiogenic agent,<sup>30</sup> targeting VEGFR2/KDR and PDGFR expressed in tumor endothelium and/or stroma. Since SU11248 does not have increased potency against FLT3 in cellular phosphorylation assays relative to PDGFR or VEGFR2 (IC<sub>50</sub> approximately 10 nM for PDGFR $\alpha$ , PDGFR $\beta$ , and VEGFR2<sup>30</sup>), the increased sensitivity *in vivo* may reflect the high level of constitutive FLT3 activity and/or causative role of FLT3-ITD in maintaining MV4;11 cell survival and proliferation, such that FLT3 inhibition has catastrophic effects on tumor survival. In tumor models in which VEGFR2 and PDGFR were assessed, a plasma concentration of 50 to 100 ng/mL sustained for 12 hours led to durable inhibition of phosphorylation by SU11248.<sup>30</sup> For FLT3-ITD we show that the plasma drug concentration that corresponds to strong and durable inhibition is lower (approximately 30 ng/mL), consistent with efficacy in the FLT3-ITD model at a lower dose. In patients, we would predict that SU11248 doses that attain plasma concentrations of more than 30 ng/mL would be sufficient to inhibit FLT3-ITD phosphorylation. In the bone marrow engraftment model, a more representative model of leukemia, SU11248 prolonged survival in 90% of animals at the same dose (20 mg/kg) that sustained inhibition of FLT3-ITD phosphorylation and was fully efficacious in the subcutaneous model.

The target genes of FLT3 signaling pathways that function in oncogenesis have not been identified. We report the novel finding

that FLT3 signaling leads to secretion of VEGF *in vitro*, most notably in FLT3-ITD cell lines. It seems likely that VEGF is a direct rather than indirect target of FLT3 signal transduction pathways, given that this effect was apparent in several cell lines and also apparent following FLT3-WT stimulation. A mechanism may exist in AML by which FLT3-induced VEGF contributes to bone marrow angiogenesis in a paracrine fashion and has autocrine action on blast cells. It will be of significance to determine whether blasts from FLT3-ITD-positive AML patients express higher levels of VEGF than those of FLT3-WT patients.

The prognostic significance of FLT3-ITD mutations in clinical studies suggests that FLT3 plays a driving role in AML. Recent evidence in mouse models suggests that FLT3-ITD mutations alone induce myeloproliferative disease, whereas additional mutations are needed to induce AML.<sup>15,16</sup> In addition to SU11248, several other small-molecule FLT3 inhibitors have been recently described, including AG1295,<sup>39</sup> PKC412,<sup>40</sup> CT53518,<sup>41</sup> CEP701,<sup>38</sup> SU5416,<sup>31</sup> and MLN518.<sup>42</sup> Each has a unique spectrum of activity for other kinases including VEGFR2, PDGFR, KIT, protein kinase C (PKC), and tyrosine kinase (TRK). Additional strategies to target FLT3 have also been defined, such as use of HSP90 inhibitors.<sup>43</sup> SU11248 is orally bioavailable, can exert direct tumor inhibition by targeting FLT3 on blasts, and also has potent activity against PDGFR and VEGFR, which regulate angiogenesis, associated with disease progression. The demonstration that SU11248 exhibits sustained target inhibition and efficacy in FLT3-ITD models suggests that this compound may have biologic activity in AML. SU11248 is currently in phase 1 AML clinical trials.

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