

An Innovative Phase I Clinical Study Demonstrates Inhibition of FLT3 Phosphorylation by SU11248 in Acute Myeloid Leukemia Patients

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ABSTRACT

Purpose: Obtaining direct and rapid proof of molecular activity in early clinical trials is critical for optimal clinical development of novel targeted therapies. SU11248 is an oral multitargeted kinase inhibitor with selectivity for fms-related tyrosine kinase 3/Flk2 (FLT3), platelet-derived growth factor receptor α/β , vascular endothelial growth factor receptor 1/2, and KIT receptor tyrosine kinases. FLT3 is a promising candidate for targeted therapy in acute myeloid leukemia (AML), because activating mutations occur in up to 30% of patients. We conducted an innovative single-dose clinical study with a primary objective to demonstrate inhibition of FLT3 phosphorylation by SU11248 in AML.

Experimental Design: Twenty-nine AML patients each received a single dose of SU11248, escalated from 50 to 350 mg, in increments of 50 mg and cohorts of three to six patients. FLT3 phosphorylation and plasma pharmacokinetics were evaluated at seven time points over 48 h after SU11248 administration, and FLT3 genotype was deter-

mined. Study drug-related adverse events occurred in 31% of patients, mainly grade 1 or 2 diarrhea and nausea, at higher dose levels.

Results: Inhibition of FLT3 phosphorylation was apparent in 50% of FLT3-wild-type (WT) patients and in 100% of FLT3-mutant patients. FLT3 internal tandem duplication (ITD) mutants showed increased sensitivity relative to FLT3-WT, consistent with preclinical predictions. The primary end point, strong inhibition of FLT3 phosphorylation in >50% patients, was reached in 200 mg and higher dose cohorts. Downstream signaling pathways were also inhibited; signal transducer and activator of transcription 5 (STAT5) was reduced primarily in internal tandem duplication patients and at late time points in FLT3-WT patients, whereas extracellular signal-regulated kinase (ERK) activity was reduced in the majority of patients, independent of FLT3 inhibition.

Conclusions: This novel translational study bridges preclinical models to the patient setting and provides the first evidence of anti-FLT3 activity in patients. Proof of target inhibition accomplishes a crucial milestone in the development of novel oncology therapeutics.

INTRODUCTION

The use of targeted agents such as kinase inhibitors in oncology has the exciting potential to treat patients based on the molecular pathology of disease. This poses both an opportunity and a challenge to gain more information from early clinical trials, to help guide dosing regimens and patient selection. The use of PD readouts, to address whether a drug inhibits its target, and establishment of PK/PD⁹ relationships in early clinical trials is a key step in this process.

FLT3 is a RTK expressed and activated in the majority of AML patients and also expressed in some normal hematopoietic cell types (1–3). Two classes of FLT3 mutation have been identified in AML: ITD mutations in the juxtamembrane region domain in 23–25% of patients and point or short length muta-

Received 4/1/03; revised 7/15/03; accepted 7/29/03.

Grant support: Veterans Affairs Merit Review Program (to M. C. H.) and the Doris Duke Charitable Foundation (to M. C. H.).

A-M. O. and J. F. contributed equally to this work.

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⁹ The abbreviations used are: PK, pharmacokinetic; PD, pharmacodynamic; AML, acute myelogenous leukemia; FLT3, fms-related tyrosine kinase 3/Flk2; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; IP/W, immunoprecipitation/Western blot; P-, phosphorylated; STAT, signal transducer and activator of transcription; RTK, receptor tyrosine kinase; ITD, internal tandem duplication; WT, wild type; AUC, area under the plasma concentration-time curve; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein/ERK kinase; PBMC, peripheral blood mononuclear cell; ULN, upper limit of normal.

tions in the activation loop of the kinase domain in ~7% (4). Both classes of mutation result in constitutive FLT3 kinase activity and transform hematopoietic cell lines (5–8). In mouse models, FLT3-ITD alone is sufficient to induce myeloproliferative disease and cooperates with other molecular lesions to induce leukemia (9–11). Clinically, FLT3-ITD is an important independent negative prognostic factor in AML (12–14) and is associated with increased blast count, increased relapse rate, and poor overall survival. The catalytic D835 point mutation is also associated with leukocytosis, although the clinical significance of this mutation is unclear (15). FLT3 is, therefore, an attractive target for the treatment of AML.

SU11248 is an oral multitargeted kinase inhibitor that inhibits FLT3 as well as PDGFR α , PDGFR β , VEGFR1, VEGFR2, and KIT (16). In preclinical models, we have recently shown that SU11248 inhibits FLT3-ITD and FLT3-D835, in addition to FLT3-WT (8). *In vivo* SU11248 dramatically regresses s.c. xenograft FLT3-ITD tumors and increases survival in a FLT3-ITD bone marrow engraftment model (8). PK/PD analyses in xenograft models showed that target plasma concentrations of 50–100 ng/ml and 30–50 ng/ml correlated with robust inhibition of VEGFR2, PDGFR β or FLT3-WT, and FLT3-ITD, respectively (16). As one of the first steps in exploratory clinical development of SU11248 in AML, a single-dose clinical study was performed to directly evaluate target activity and establish a PK/PD relationship in humans. AML provides an ideal indication for such analysis, because peripheral blood can be sampled at multiple time points relatively noninvasively and FLT3 phosphorylation can be measured. Analysis of target modulation in solid tumors is technically and logistically more difficult, particularly at multiple time points.

This study clearly showed that a single dose of SU11248 inhibits phosphorylation of wild-type and mutant FLT3 in the clinical setting. The inhibition was PK, genotype, and time dependent. Signaling downstream of FLT3, via STAT5 and ERK pathways was also inhibited, and decreased blast counts were observed in several patients. This study provides clinical proof of target modulation for SU11248. The data generated can be translated to other SU11248 targets and will help guide clinical development of SU11248.

MATERIALS AND METHODS

Patients and Treatment Regimen

A single-arm, single-dose Phase I study of oral SU11248 in AML was conducted to explore safety and target modulation. Written informed consent was obtained from all patients before they were enrolled in the study. Patients with all AML French-American-British classification types (except AML M3), a Karnofsky performance status of $\geq 60\%$, and at least 1 million KIT-positive peripheral blood blasts per milliliter were eligible. Patients were required to have adequate hepatic function (defined as serum transaminases $< 2.5 \times$ ULN or bilirubin $< 2 \times$ ULN) and adequate renal function (defined as serum creatinine $\leq 1.5 \times$ ULN or calculated creatinine clearance > 40 ml/min) at study entry. Patients could not be taking medications that were known to have a significant effect on the P450 CYP3A4 pathway.

Twenty-nine patients were enrolled at five clinical sites

that were located in the United States and Germany. Patients were administered a single dose of SU11248 L-malate capsules before food ingestion. The starting dose of 50 mg was escalated in 50-mg increments, with a highest dose of 350 mg. SU11248 was supplied as 50-, 75-, and 200-mg capsules. Interpatient dose escalation to the next level occurred only if none of three patients experienced unacceptable toxicity. Whole blood was harvested into EDTA and heparin anticoagulant vacutainers for PK and PD (phosphorylation) analysis, respectively, before and at 4, 6, 8, 10, 12, 24, and 48 h after SU11248 administration. Five milliliters of peripheral blood collected (EDTA anticoagulant) within 14 days of study enrollment were used for FLT3 and KIT genotyping.

Bioanalytical Methods and PK Analysis

SU11248 has an active metabolite, SU12662. Analysis of SU11248 and SU12662 was conducted by liquid chromatography-mass spectroscopy-mass spectroscopy by BAS Analytics (West Lafayette, IN). Internal standards were generated by extracting human plasma with methanol containing 1 ng/ml [$^2\text{H}_{10}$]SU11248. Calibration curves were constructed by plotting the ratio of the area of the compounds and the internal standard against analyte concentration. A weighted linear regression function was used to calculate concentrations of SU11248 and its active metabolite SU12662 in plasma samples. The primary PK parameters evaluated for SU11248 were C_{\max} (peak plasma concentration was identified by inspection of individual subject concentration-time curves), T_{\max} (time of observed peak plasma concentration was identified by inspection of individual subject concentration-time curves), and AUC. $\text{AUC}_{0-48\text{ h}}$ was calculated by noncompartmental methods using Kinetica (version 3.1; InnaPhase Corporation). Here, drug plasma concentration refers to the combined values for SU11248 and SU12662, because they exhibit similar activity.

Correlative Laboratory Analyses

Analysis of FLT3 and STAT5 Phosphorylation. Control experiments were performed to define the optimal processing method for patient peripheral blood to measure RTK phosphorylation. The addition of peripheral blood to lysis buffer, without enrichment for blasts, was optimal for detection of RTK phosphorylation by IP/W analysis, as described below.

Freshly harvested peripheral blood was added to a $2 \times$ volume of lysis buffer [20 mM Tris (pH 7.5), 137 mM NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 , 2 mM Pefabloc, 2 $\mu\text{g/ml}$ aprotinin, 3.5 $\mu\text{g/ml}$ bestatin, 0.5 $\mu\text{g/ml}$ E-64, 0.5 $\mu\text{g/ml}$ leupeptin, and 0.7 $\mu\text{g/ml}$ pepstatin A]. Lysates were transported on dry ice, stored at -70°C , thawed at 4°C , and cleared by centrifugation. Protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, IL). Approximately 35 mg of lysate from each sample were immunoprecipitated for FLT3 or STAT5, as described previously (8). Phosphorylated FLT3 and total FLT3 were detected by Western blot with 4G10 (Upstate Biotechnology, Lake Placid, NY) and total FLT3 (sc-490; Santa Cruz Biotechnology, Santa Cruz, CA). Data from several groups on FLT3 signaling suggest that phosphotyrosine analysis of FLT3 using 4G10 is a surrogate for FLT3 kinase activity (8, 17, 18). In addition,

in vitro experiments using a recently available phospho-specific FLT3 antibody, Y591 (Cell Signaling Technologies, Beverly, MA), have shown similar results as phosphotyrosine (4G10).¹⁰ Phospho-STAT5 (Y694/Y699) and STAT5 antibodies were from Upstate Biotechnology and BD Biosciences (Mountain View, CA), respectively.

Analysis of blood from normal donors by IP/W did not show significant levels of FLT3 expression, most likely because FLT3 expression is restricted to dendritic cells, natural killer cells, and monocytes in normal peripheral blood, as shown by RT-PCR and fluorescence-activated cell-sorting analysis (4). A number of quality control steps were used for analysis of FLT3 inhibition. First, negative (healthy donor blood) and positive (FLT3-expressing leukemia cell line spiked into healthy donor blood before lysis) controls were processed and analyzed in parallel with samples from each patient and provided a reference for interpatient variation. In all cases, controls showed anticipated results; phosphorylated FLT3 was not detectable in normal blood, whereas a strong signal was apparent in the positive control. Second, inhibition of FLT3 phosphorylation was assessed by visual analysis of blots and also by densitometric quantitation of films to facilitate accurate comparison between patients. Similar results were apparent between methods. When IP/W analysis was performed in triplicate for patient samples, <10% variation in phosphorylated FLT3 relative to total was apparent. Therefore, inhibition was classified as weak or strong (21–50% and 51–100% decrease in phosphorylation, respectively). Finally, to verify that data are within the linear range of enhanced chemiluminescence, validation was performed by comparison of enhanced chemiluminescence with a high-dynamic range infrared Western blot detection system (Licor Odyssey System), which has a large dynamic linear range.

Analysis of ERK and MEK Phosphorylation. For analysis of ERK1/2 and MEK1/2 activity, PBMCs were enriched by Ficoll purification as described (19). Activated ERK1/2 and MEK1/2 were assessed by Western blot analysis using phospho-ERK1/2 (Thr 202/Tyr 204) and phospho-MEK1/2 (Ser 217/221). Phospho-specific and control antibodies were from Cell Signaling Technologies.

Genotype Analysis. Peripheral blood samples harvested within 2 weeks of study initiation were genotyped for FLT3 and KIT mutations by denaturing high-precision liquid chromatography (WAVE), as described previously (7).

The primers used for D835 mutation detected were 5'-gcactccaggataatacacatca-3' (forward) and 5'-aacgacacacacaaatagccg-3' (reverse), with a WAVE melting temp of 59.1°C. All mutations were confirmed by direct DNA sequencing using ABI310 sequencer and labeled Big Dye dideoxy-chain terminators.

RESULTS

Study Design and Patient Demographics. Data from preclinical models show that SU11248 is a potent inhibitor of wild-type and mutant FLT3 (8). For translation to clinical de-

Table 1 Patient characteristics

Patient	Age	Gender	FAB ^a	AML disease
1	72	M	M4	Refractory
2	27	F	NC	Secondary
3	73	F	M2	Newly diagnosed
4	70	F	NC	Secondary
5	73	M	M5	Secondary
6	19	M	M2	First relapse
7	54	M	NC	Secondary
8	81	F	M4	Secondary
9	61	F	M0	Newly diagnosed
10	74	M	M0	Secondary
11	63	M	M4	Refractory
12	36	F	M1	Newly diagnosed
13	74	F	M4	Refractory
14	67	M	NC	Secondary
15	82	M	M1	Newly diagnosed
16	52	M	M7	Secondary
17	34	F	M1	Refractory
18	40	F	M2	Newly diagnosed
19	45	M	M5	Newly diagnosed
20	77	M	M2	First relapse
21	76	F	M0	Secondary
22	76	M	NC	Refractory
23	41	M	M4	Newly diagnosed
24	41	M	NC	Refractory
25	60	M	M2	First relapse
26	68	M	NC	Secondary
27	67	F	M2	Secondary
28	63	M	M4	Newly diagnosed
29	72	F	M5	Newly diagnosed

^a FAB, French-American-British classification; NC, not classifiable.

velopment, a single-dose Phase I clinical study was performed in AML patients to address whether SU11248 can modulate target activity in humans at drug levels that are safely achievable. The primary objective was to assess the degree and duration of FLT3 inhibition by SU11248 in AML patients, with a secondary objective to assess safety, tolerability, and PK of a single SU11248 dose. Because this was a single-dose study, determination of clinical response was not a study objective and has been assessed in separate repeat dose studies.

Twenty-nine patients were enrolled at five sites, three in the United States and two in Germany. The patient group comprised 17 males and 12 females, ranging in age from 19 to 82 years (median age, 67), 93% of who had at least one prior therapy (Table 1). Patients entered without prior therapy were considered by their treating physicians to be inappropriate candidates for chemotherapy. Each AML patient received a single dose of SU11248, with three initially recruited to each dose cohort. If inhibition of FLT3 phosphorylation was apparent in at least one patient of three, the cohort was expanded to six patients to enable more extensive phosphorylation analysis. The dose was escalated in 50-mg increments from 50 mg to a highest dose of 350 mg. In each cohort of 50, 100, 150, 200, 250, 300, and 350 mg, the number of patients enrolled was three, three, three, seven, six, six, and one, respectively.

Study drug-related adverse events occurred in 31% of patients. The only drug-related adverse events that were experienced by ≥10% patients were diarrhea and nausea (each 10%). In most cases, this was limited to grade 1 or grade 2 gastroin-

¹⁰ S. G. Louie and A-M. O'Farrell, unpublished data.

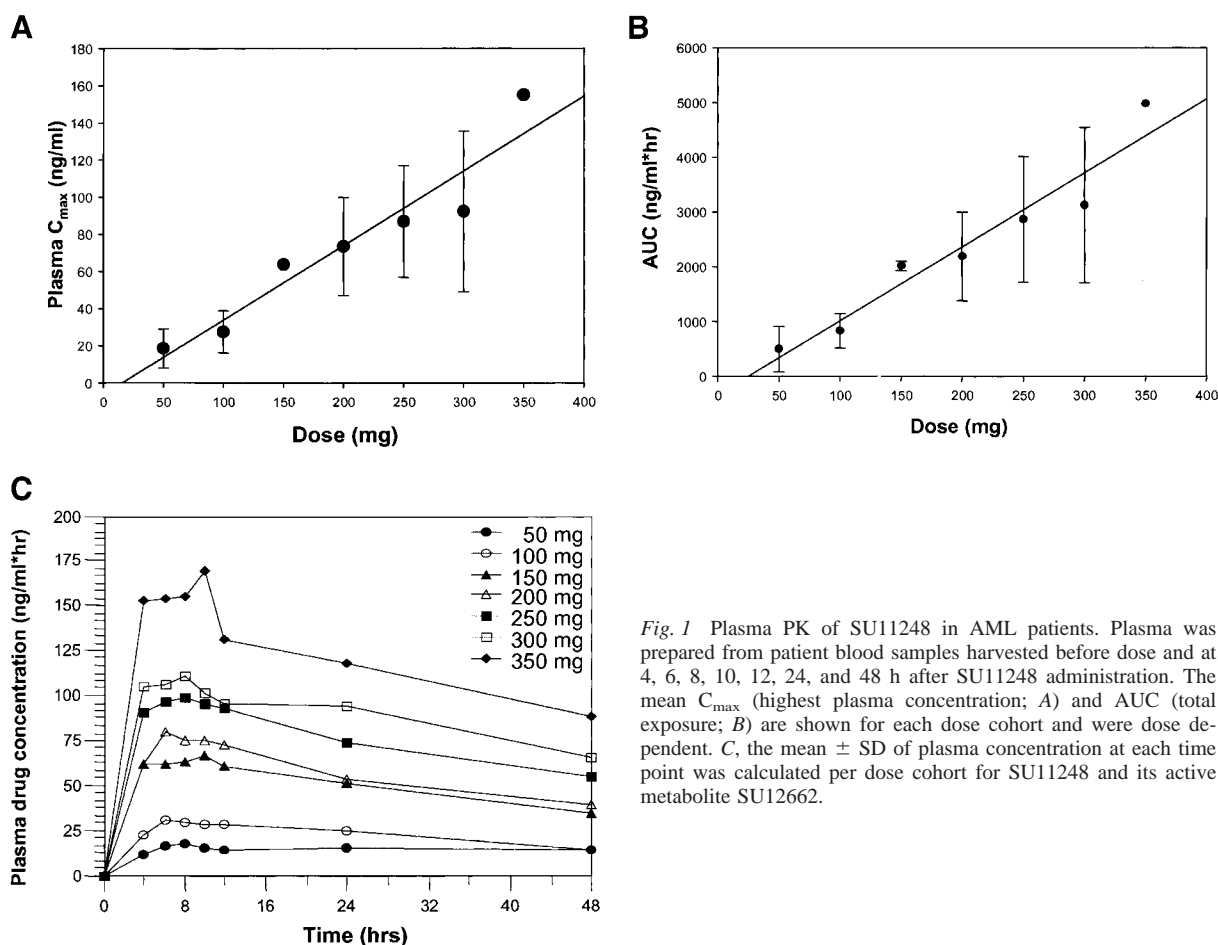


Fig. 1 Plasma PK of SU11248 in AML patients. Plasma was prepared from patient blood samples harvested before dose and at 4, 6, 8, 10, 12, 24, and 48 h after SU11248 administration. The mean C_{max} (highest plasma concentration; **A**) and AUC (total exposure; **B**) are shown for each dose cohort and were dose dependent. **C**, the mean \pm SD of plasma concentration at each time point was calculated per dose cohort for SU11248 and its active metabolite SU12662.

testinal disorders and only reported at the 250- to 350-mg dose levels. All other study drug-related adverse events were mild, infrequent, and dose independent.

Two patients had serious adverse events possibly related to study drug. One 82-year-old male patient with a history that included hypothyroidism, diabetes, prostate cancer, weight loss, thrombocytopenia, neutropenia, and anemia developed a short episode of grade 3 hypertension the day after receiving a single 200-mg dose of SU11248. This resolved the same day without sequelae. A second 68-year-old male patient with a history of hypertension, pericardial effusion with early tamponade, grade 1 atrioventricular block, myocardial infarction, coronary heart disease, congestive heart failure, dyspnea, pulmonary nodules, and coagulopathy, whose baseline signs and symptoms included edema, edema lower limb, and cardiac murmur (systolic), developed a transient asymptomatic increase in cardiac ectopy with a grade 1 ventricular tachycardia on his electrocardiogram on the same day he received a single 350-mg dose of SU11248. This rhythm change resolved the following day, and the patient was discharged from the hospital without sequelae. This event was classified as a serious adverse event because the patient was admitted to the hospital for observation. Serial electrocardiograms (screen, baseline, and 8, 24, and 48 h after SU11248 administration) and the changes from baseline for the resting

rate, PR, QRS, QT, and corrected QT intervals were assessed for all patients. No clinically relevant or consistent changes from baseline were observed. Overall, a single dose of SU11248 was well tolerated at all dose levels studied. After completing this study, patients had the option to enter a separate repetitive-dose Phase I study with SU11248 if they qualified for study entry (12 of the patients described entered this study).

PK Analysis of Plasma Drug Levels. SU11248 has an active desethyl metabolite, SU12662, which has comparable activity with the parent compound in biochemical and ligand-dependent proliferation assays and in VEGF-driven vascular permeability assays *in vivo*.¹¹ The plasma concentrations of SU11248 and SU12662 were measured at a series of time points within 48 h after SU11248 administration. A dose-proportional increase in both C_{max} and AUC for SU11248 was observed with increasing doses from 50 to 350 mg (Fig. 1). Similar linearity was observed with the active metabolite SU12662 (10–15% of total; data not shown). The combined plasma concentration curves over time are shown in Fig. 1C. Across all dose levels, the T_{max} was generally observed at 4–8 h for both SU11248 and

¹¹ A. D. Laird and D. Mendel, unpublished data.

metabolite. The calculated half-life for SU11248 was 44 ± 18.6 h. Because of the short sampling period, the terminal half-life for SU12662 could not be determined in this study but appeared likely to exceed 80 h. Moderate inpatient variability was observed but, given oral administration, this is not unexpected. These results indicate that a single dose of SU11248 exhibits dose-dependent PK in humans.

Analysis of FLT3 Genotype and Phosphorylation in Predose Samples. FLT3 genotype analysis was performed in all patients on entry to the study. The observed incidence of FLT3-ITD (exon 14) mutations was 10.3% [3 of 29; VDFREYE (592–598), KYFYVDFRE (588–596), and DFREY (593–597) in patients 3, 4, and 10, respectively]. The incidence of FLT3-D835 (exon 20) mutations was 3.4% (1 of 29), observed in patient 1 (D835Y). In addition, a novel activation loop mutation, G846S (exon 20), was identified in patient 22. Patients were also genotyped for mutations in KIT, another SU11248 target. No mutations were identified in the KIT extracellular, juxtamembranous or activation loop region (exons 9, 11, 13, 17), consistent with the low reported incidence of KIT mutations in adult AML (20).

To measure predose FLT3 phosphorylation, AML peripheral blood samples were taken before SU11248 administration. Lysates from each patient were immunoprecipitated for FLT3 and analyzed by Western blot for phosphorylated and total FLT3 protein. Phosphorylated FLT3 was evident in predose samples from 72% of patients (21 of 29). Representative data are shown in Fig. 2 (lane 1 of each gel). Both the mature (cell surface) and immature (likely intracellular) forms of FLT3 protein, approximately M_r 160,000 and M_r 135,000, respectively (2), were detectable in WT and mutant genotypes. In most cases, both forms were phosphorylated in mutant patients, consistent with constitutive activity, whereas only the higher molecular weight species was phosphorylated in FLT3-WT, as observed in leukemia cell lines (8). It is noteworthy that for the patient with the FLT3-G846S mutation, both FLT3 species were phosphorylated, suggesting that G846S is an activating mutation, rather than a single nucleotide polymorphism. Additional *in vitro* studies will be needed to assess the transforming potential of this mutant. In the eight patients considered nonevaluable for FLT3 modulation, FLT3 protein was detectable but not phosphorylated ($n = 4$), not detectable ($n = 3$), or protein was degraded ($n = 1$). For samples in which FLT3 protein was not apparent by IP/W, FLT3 expression by flow cytometry staining on peripheral blood was either negative or low (data not shown). This analysis showed that phosphorylated FLT3 is detectable in peripheral blood samples from the majority of AML patients, and 21 were evaluable for analysis of FLT3 modulation.

SU11248 Treatment Inhibited FLT3 Phosphorylation.

Analysis of FLT3 phosphorylation in peripheral blood samples harvested after SU11248 administration showed strong (>50%) inhibition in both FLT3-WT and mutant patients, with strong inhibition in 12 of 21 evaluable patients. Inhibition was SU11248 dose dependent; 77% (10 of 13) of evaluable patients who received a 200-mg or higher dose exhibited strong FLT3 modulation, whereas only 25% (2 of 8) of evaluable patients who received <200 mg showed strong inhibition. FLT3 phosphorylation data for all patients is summarized in Table 2. The primary study end point of strong inhibition in at least 50% of

patients in a given cohort was reached at 200 mg, in which three of five evaluable patients showed strong inhibition, and was also evident in the higher 250-mg and 300-mg dose cohorts. Representative examples are shown in Fig. 2 and discussed below.

Eight of the 16 evaluable WT patients exhibited strong inhibition of FLT3 phosphorylation. These were primarily in higher-dose cohorts, and all evaluable FLT3-WT patients who received ≥ 250 mg showed modulation (Table 2). Data for two representative patients are shown (Fig. 2, A, B, and F), in which inhibition was sustained for at least 12 h and 48 h, respectively. FLT3 protein levels did not change over this time period. The increased phosphorylation at 48 h in patient 13 was also observed in several additional patients (data not shown) and may reflect FLT3 protein turnover and/or decreased plasma drug levels at later time points. FLT3 inhibition was generally more sustained in patients in the higher-dose cohorts. For example, the mean percentage of inhibition of FLT3 phosphorylation at 24 h for patients who received 100, 150, 200, and 250 mg of SU11248 was 15, 29, 39, and 64%, respectively (minimum, $n = 3$ per group).

All patients who exhibited strong FLT3 inhibition at doses <200 mg were FLT3 mutant. SU11248 inhibited FLT3 phosphorylation in all five FLT3 mutant cases. IP/W analysis of FLT3 phosphorylation in ITD patients 3 and 10 is shown in Fig. 2, C and D. For patient 3, decreased phosphorylation of both FLT3 species was apparent at 4–6 h. Inhibition of the lower, but not upper, FLT3 species was sustained to 24 h (Fig. 2F). This patient also exhibited decreased blast counts at 24 and 48 h, indicating that the observed inhibition translated to biological activity. A similar pattern was apparent in ITD patient 4 (data not shown), whereas the third ITD patient, 10, who received a higher dose (200 mg), exhibited comparable inhibition of both FLT3 species (Fig. 2D). The FLT3-G846S patient (22), received 200 mg of SU11248, and FLT3 phosphorylation was strongly inhibited for at least 12 h (Fig. 2, E and F). The D835 patient in this study (1) showed weak transient inhibition consistent with low plasma drug levels (C_{max} , 15 ng/ml; data not shown). These data clearly demonstrate that a single dose of SU11248 results in inhibition of FLT3 phosphorylation in a dose-dependent manner in AML patients, with inhibition in all genotypes.

Inhibition of FLT3 Phosphorylation Was PK-Dependent.

The PK/PD relationship in humans was assessed by correlation of FLT3 inhibition status with C_{max} plasma drug levels for each patient. The plasma C_{max} levels in FLT3-WT patients who attained strong inhibition of FLT3 were significantly different from those who did not show inhibition [mean \pm SD, 133 ± 15 ($n = 8$) and 70 ± 8 ($n = 6$) ng/ml, respectively]. Given the sustained drug plasma concentrations at values close to the C_{max} (Fig. 1C), inhibition analysis with AUC data was consistent with C_{max} observations (data not shown). Of the 16 evaluable FLT3-WT patients, 7 of 8 (87%) with a $C_{max} > 100$ ng/ml and only 1 of 8 (13%) with $C_{max} < 100$ ng/ml were inhibited (Fig. 3A). Five FLT3-WT patients had C_{max} levels from 50 to 100 ng/ml but only one patient showed strong modulation, suggesting that ≥ 100 ng/ml is required for strong modulation of FLT3-WT in most (87%) cases. In contrast, for FLT3-ITD patients, phosphorylation was inhibited at a C_{max} as low as 16 ng/ml, at a mean

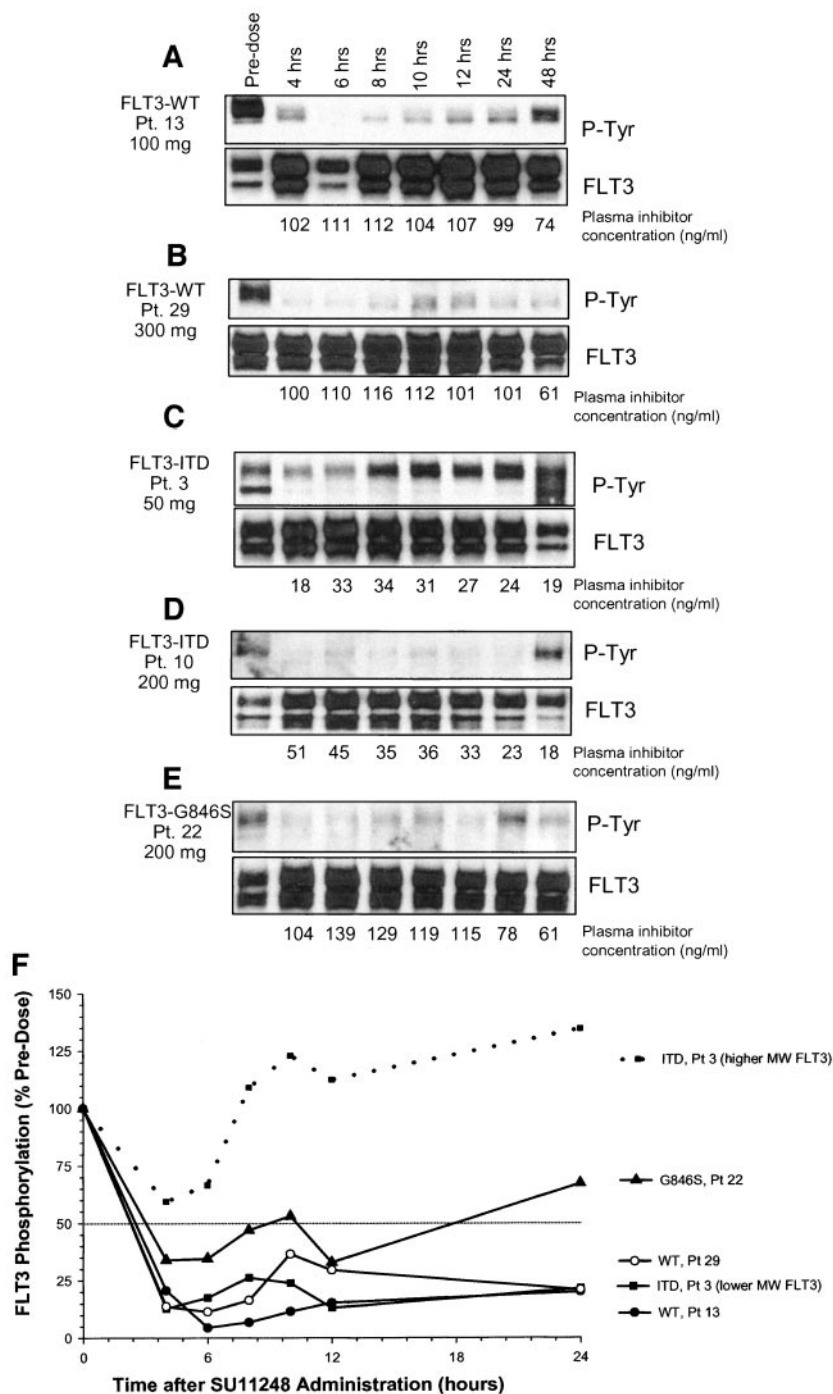


Fig. 2 Analysis of FLT3 phosphorylation in AML patients. **A–E**, FLT3 phosphorylation was assessed by IP/W analysis of peripheral blood harvested before dose and at the indicated time points after SU11248 administration. Lysates were prepared and immunoprecipitated with an anti-FLT3 antibody. After 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*). Inhibition of FLT3 phosphorylation was assessed by both visual analysis and densitometric quantitation of Western blots for each patient, and good correlation was apparent between these methods. **F**, Western blots were quantitated using Quantity One Software, and FLT3 phosphorylation was calculated as percentage of predose (%Pre-Dose), normalized for FLT3 protein, for each time point. Points below the *dotted line* indicate strong inhibition of FLT3 phosphorylation. Data for representative patients are shown.

C_{max} of 34 ± 17 ng/ml ($n = 3$). These data suggest that a target plasma concentration of 100 ng/ml is required for strong inhibition of FLT3-WT whereas less is required for inhibition of FLT3-ITD. These data are consistent with preclinical results in which the target plasma drug concentration for modulation of WT targets was higher (50–100 ng/ml; Ref. 16) than that required for FLT3-ITD (30–50 ng/ml; Ref. 8).

The plasma concentration required for sustained FLT3 inhi-

tion was also assessed. Inhibition of FLT3 phosphorylation was sustained for at least 8 h in all modulated FLT3-WT patients and for 24 h in seven of the eight cases. Seven of these patients maintained the target plasma concentration of at least 100 ng/ml for ≥ 12 h, whereas the eighth patient with sustained inhibition did not reach 100 ng/ml but maintained 50 ng/ml for 46 h (Fig. 3B). This meets or exceeds biological efficacy requirements defined in repeat dose experiments with preclinical models, in which inhibition of

Table 2 Summary of Inhibition of FLT3 Phosphorylation in all Patients

Patient	Dose (mg)	FLT3 genotype	FLT3 inhibition	C _{max} (ng/ml)	T _{max} (h)	Modulation (strong)
1	50	D835Y	Weak (25)	15	4	1/3
2		WT	Weak (49)	12	24	
3		ITD	Strong (61, 87*)	34	8	
4	100	ITD	Strong (40, 69*)	16	10	1/2
5		WT	NE	36	6	
6		WT	No	45	10	
7	150	WT	No	75	10	0/3
8		WT	No	68	4	
9		WT	No	71	10	
10	200	ITD	Strong (78, 78*)	51	4	3/5
11		WT	Strong (85)	60	12	
12		WT	No	104	4	
14	250	WT	No	55	10	3/3
15		WT	NE	102	12	
16		WT	NE	84	8	
22		G846S	Strong (67)	139	6	
13		WT	Strong (96)	111	8	
17		WT	Strong (70)	118	4	
18		WT	NE	60	4	
19		WT	NE	115	8	
20		WT	NE	80	10	
21		WT	Strong (80)	139	8	
23	300	WT	Strong (72)	201	8	3/4
24		WT	NE	76	8	
25		WT	NE	108	6	
27		WT	Weak (35)	48	10	
28		WT	Strong (71)	148	4	
29		WT	Strong (89)	116	8	
26	350	WT	Strong (70)	170	10	1/1

* Strong and weak inhibition are defined as 50–100% and 20–50% inhibition of predose phosphorylation, respectively. Numbers in parentheses denote maximum inhibition observed for each patient. For FLT3-ITD patients, inhibition of both upper and lower FLT3 species combined and lower band only* are shown. ITD sequences were VDFREYE (592–598), KYFYVDFRE (588–596); and DFREY (593–597) for patients 3, 4, and 10, respectively. NE, not evaluable.

target phosphorylation for 8–12 h in a 24-h dosing period was associated with tumor regression. These data indicate that maintaining a plasma concentration of ≥ 100 ng/ml is associated with sustained inhibition of FLT3-WT by SU11248.

SU11248 Decreased STAT5 Activity in a Subset of Patients. STAT5 is activated by several RTKs and fusion proteins associated with leukemogenesis, more strongly by FLT3-ITD than FLT3-WT (5, 21). Activated STAT5 alone is

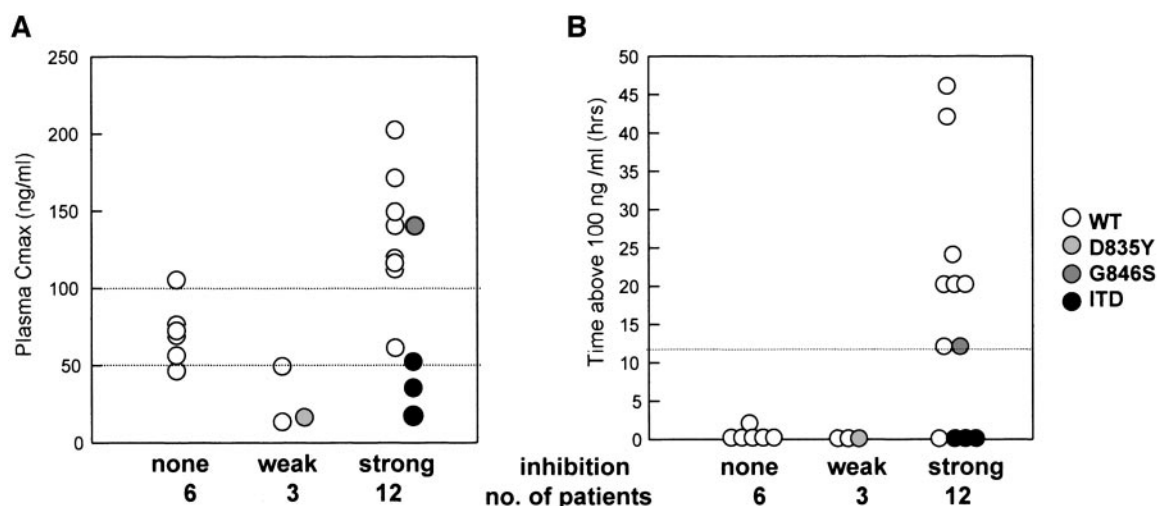


Fig. 3 PK/PD analysis of FLT3 phosphorylation. FLT3 phosphorylation was assessed as described for Fig. 1. Plasma C_{max} (combined SU11248 and SU12662; A) and time exceeding the target plasma concentration of 100 ng/ml (B) are shown for each patient, grouped according to degree of FLT3 inhibition, and color coded based on FLT3 genotype.

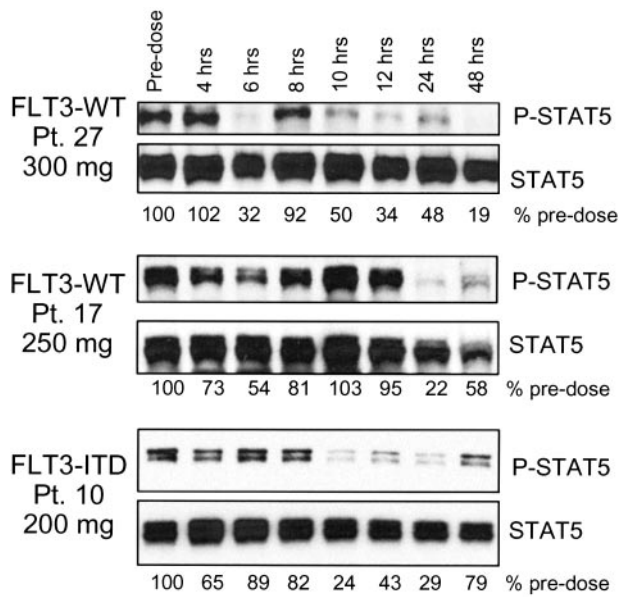


Fig. 4 Inhibition of STAT5 phosphorylation by SU11248. STAT5 phosphorylation was assessed by IP/W analysis of peripheral blood harvested before dose and at 4, 6, 8, 10, 12, 24, and 48 h after SU11248 administration. Lysates were prepared and immunoprecipitated with an anti-STAT5 antibody. After SDS-PAGE and transfer to nitrocellulose, the blots were probed with an antiphospho-STAT5 antibody (*top*) and subsequently stripped and reprobed with an anti-STAT5 antibody (*bottom*). Data for representative patients are shown.

sufficient to cause myeloproliferative disease (22) and is activated in AML blasts (23–25). We, therefore, assessed STAT5 as a downstream marker of FLT3 signaling. Baseline STAT5 tyrosine phosphorylation was detected by IP/W in peripheral blood samples from 26 of 29 (89%) evaluable patients. STAT5 protein was also detectable in peripheral blood from healthy donors, but levels of phosphorylation were undetectable or low relative to AML patients (data not shown).

SU11248 treatment was associated with decreased STAT5 activity at one or more time points in the majority of AML patients in this study. Several trends were observed; STAT5 phosphorylation was markedly decreased (>50% inhibition) at several time points after SU11248 administration in most (four of five) of the FLT3 mutant patients (*e.g.*, patient 10; Fig. 4, *top*). In FLT3-WT patients, two patterns of STAT5 inhibition were noted. In four cases, primarily at high drug exposure, inhibition was observed before 24 h and sustained at later time points (*e.g.*, patient 27; Fig. 4). In nine other WT patients, decreased STAT5 phosphorylation was apparent only at late time points (24 and/or 48 h) and, in several cases, was associated with decreased STAT5 protein expression (*e.g.*, patient 17; Fig. 4). A similar decrease in AKT phosphorylation and protein expression was apparent in several patients (data not shown). This may be a consequence of previous decreases in FLT3 activity or may reflect signaling downstream of other targets of SU11248. Two FLT3-WT patients who exhibited strong FLT3 inhibition did not show STAT5 inhibition at any time point, suggesting that these two pathways are not completely coupled in FLT3-WT patients.

To rule out the contribution of other RTKs and blood cell types to any observed STAT5 inhibition, STAT5 activity in peripheral blood from solid tumor cancer patients who received SU11248 in advanced malignancy Phase I trials was assessed. Inhibition of STAT5 phosphorylation was not observed ($n = 10$; data not shown). These data suggest that SU11248 impacts STAT5 signaling in AML blasts rather than normal blood cells, consistent with a role for STAT5 in mediating leukemogenic FLT3 signaling.

ERK1/2 and MEK1/2 Phosphorylation Were Inhibited after SU11248 Treatment. FLT3 signaling also leads to phosphorylation and activation of ERK1 and ERK2 mitogen-activated protein kinases (3, 5, 7, 23, 24). ERK phosphorylation has been used previously as a marker of RTK signaling in human skin samples from a clinical trial of the EGFR inhibitor ZD1839 in advanced malignancy patients (25). To evaluate ERK1/2 as a potential marker of target modulation by SU11248, blasts were enriched from patient blood samples before dose and at 24 and 48 h after SU11248 administration. ERK1/2 activation was assayed by Western blot analysis using a phospho-specific antibody that detects activated ERK1 and ERK2. Phosphorylated ERK1/2 was evident in predose samples from the majority of patients (20 of 29, 69%). At 24 or 48 h after SU11248 administration, ERK1/2 activity was decreased in 16 of the 20 evaluable patients, of which 10 were inhibited at both time points. Examples from two patients with sustained inhibition of ERK1/2 are shown in Fig. 5. No obvious correlation was apparent between ERK1/2 inhibition and FLT3 modulation, FLT3 genotype, or PK.

To further define the ERK1/2 signaling pathway, activation of MEK1/2, which directly phosphorylates and activates ERK1/2, was investigated. Western blot analysis with a phospho-specific MEK1/2 antibody showed that activated MEK1/2 was detectable in the majority of cases and was inhibited after

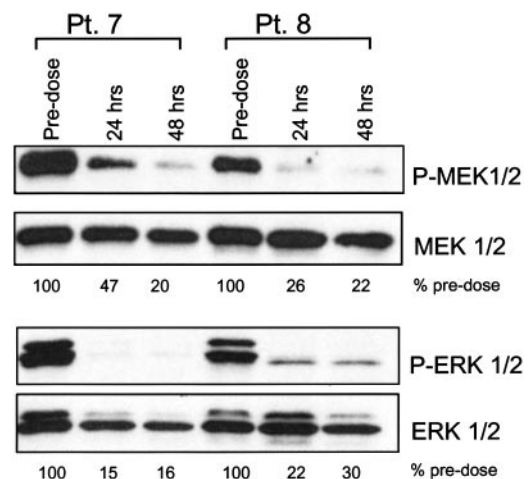


Fig. 5 Inhibition of the MEK-ERK pathway by SU11248. Blasts were enriched by ficoll purification from patient blood harvested before and at 24 and 48 h after SU11248 administration. Lysates were prepared and analyzed by Western blot for P-ERK and P-MEK and subsequently stripped and reprobed for total ERK and total MEK. Data for representative patients are shown. The patients shown were both FLT3-WT, and each received 150 mg of SU11248.

SU11248 administration in 7 of 18 evaluable patients. In most patients, P-MEK and P-ERK showed similar trends, as shown in Fig. 5, in which inhibition of both MEK1/2 and ERK1/2 was detected. Accordingly, a moderate correlation was observed between changes in MEK1/2 and ERK1/2 phosphorylation at 24 and 48 h relative to before dose ($r^2 = 0.39$ and 0.46 , respectively; $n = 19$; data not shown). These data suggest that signaling through the RAS-RAF-MEK-ERK pathway is decreased after SU11248 treatment.

Decreased Blast Counts Were Apparent in Several Patients after SU11248 Treatment. Given that a single dose of SU11248 inhibits phosphorylation of FLT3 and downstream pathways, patient peripheral blood blast counts were analyzed to investigate potential biological activity. In most patients, no significant change in blast counts was apparent at 24 or 48 h after administration of a single dose of SU11248 (mean, 1.19 and 1.08-fold change at 24 and 48 h, respectively, relative to pre-dose; Table 3). However, five patients exhibited large decreases in blast count (patients 3, 5, 8, 10, 29). Of these five, two were FLT3-ITD patients, and both exhibited inhibition of FLT3 phosphorylation (Table 2). Three were FLT3-WT patients, one who exhibited strong FLT3 inhibition (patient 29); the second was not evaluable for FLT3 phosphorylation (patient 5), whereas the third did not show inhibition but had a weak baseline FLT3 phosphorylation signal. These data suggest that FLT3 inhibition by a single dose of

SU11248 can translate to reduced blast cell counts and may be most common in FLT3-ITD patients. These data link molecular activity to a biological readout.

DISCUSSION

FLT3 is a promising target for AML treatment and is potentially inhibited by SU11248 in preclinical models. We conducted an innovative single-dose clinical study to determine the PK/PD properties of SU11248 in AML patients. This study was unique in that a PD readout, investigation of target (FLT3) modulation and PK/PD analysis, was the primary end point. This study extends preclinical observations and demonstrates inhibition of the FLT3 kinase for the first time in humans. The magnitude and kinetics of FLT3 inhibition were dependent on genotype, dose, and plasma drug levels. Overall, inhibition was seen in 54% of patients, including 87% of FLT3-WT patients who achieved a plasma drug concentration of at least 100 ng/ml. Inhibition of major FLT3 downstream signaling pathways was also apparent; STAT5 was most strongly inhibited in FLT3-ITD patients, whereas ERK signaling was modulated in the majority of patients independent of FLT3 genotype or modulation. In addition, decreased blast counts were evident in several patients, the majority of whom exhibited inhibition of FLT3 phosphorylation, providing preliminary evidence of biological activity of SU11248 in AML.

This is the first comprehensive analysis of inhibition of RTK signaling in a large group of AML patients. Successful collaboration between clinical investigators, the clinical study team, and the correlative analysis team enabled logistical and technical challenges in conducting this study to be overcome. Dose-escalation decisions were made based on rapid analysis of phosphorylation state. Acceptable quality lysates were generated from 28 of 29 patients from five international sites, and most were evaluable for FLT3 inhibition. Baseline levels of RTK phosphorylation have not been well described in uncultured human AML samples. We demonstrated that FLT3 is phosphorylated in the majority of AML patients, irrespective of genotype, consistent with previous data.¹² This may be a consequence of high levels of circulating FLT3 ligand or potentially membrane-associated FLT3 ligand acting in an autocrine manner on AML blasts, consistent with the role of FLT3 ligand in maintaining blast cell survival (26, 27). This study shows that PD studies are feasible and can help guide and accelerate clinical development of new targeted agents.

The PK/PD requirements for SU11248 inhibition of FLT3, PDGFR β , and VEGFR2 in preclinical tumor xenograft models have been described recently (8, 16). Robust inhibition of FLT3-ITD requires a drug plasma concentration of 30–50 ng/ml, and wild-type FLT3 requires 50–100 ng/ml,¹¹ similar to PDGF and VEGF receptors. FLT3-ITD also shows increased susceptibility to inhibition by SU11248 relative to FLT3-WT cellular phosphorylation assays (8). The difference in plasma concentration required to inhibit ITD (<50 ng/ml) and WT (>100 ng/ml) is

Table 3 Peripheral blood blast counts

Patient	Absolute blasts		
	Pre-dose $\times 10^9$ /liter	24 h/pre-dose	48 h/pre-dose
1	41.3	1.7	1.76
2	7.5	1.26	0.81
3	32.6	0.59	0.15
4	31.8	0.73	1.11
5	61.5	0.57	0.39
6	40.4	1.34	1.15
7	5.4	1.43	0.63
8	0.41	nd ^a	nd
9	1.2	1.17	0.54
10	36.9	0.83	0.42
11	7.5	2.12	2.52
12	3.8	0.83	0.91
13	47.4	1.91	1.64
14	42.6	0.99	1.58
15	0.4	1.47	1.26
16	0.99	0.72	1.78
17	25.4	1.71	0.84
18	2.7	1.19	1.31
19	1.6	1.1	0.78
20	11.5	1.35	1.96
21	24.5	1.08	1.18
22	14.4	1.45	1.19
23	30.2	1.16	0.48
24	2.5	0.74	0.76
25	41.4	1.18	1.34
26	3.96	1.15	1.55
27	4.1	1.43	0.89
28	16.4	1.26	1.16
29	5.5	0.85	0.12
Mean	18.8	1.19	1.08
Median	11.5	1.18	1.13
SD	18	0.39	0.57

^a nd, no blasts detectable.

¹² O'Farrell *et al.* Effects of SU5416, a small molecule tyrosine kinase inhibitor on FLT3 expression and phosphorylation in patients with refractory acute myeloid leukemia, submitted for publication.

consistent with these preclinical predictions. It should be noted that the lower limit for inhibition in ITD patients was not reached, although most ITD patients were enrolled in lower-dose cohorts. Strong inhibition of FLT3-WT phosphorylation was apparent overall in 50% of patients, in 87% with $C_{\max} > 100$ ng/ml, and not in patients with $C_{\max} < 50$ ng/ml. One FLT3-WT patient with a C_{\max} of 60 ng/ml exhibited strong inhibition of FLT3 phosphorylation, sustained for 16 h. Interestingly this patient had very high levels of baseline phosphorylation, raising the possibility that higher levels of FLT3 activity may enhance susceptibility to inhibition or may be technically easier to assay. Recording baseline levels of RTK activity as well as genotype will be important in future studies.

In preclinical xenograft models for PDGFR and VEGFR, daily dosing of SU11248 target inhibition for 12 h is sufficient for full biological efficacy. In AML patients, we observed that FLT3 modulation was sustained for longer than 24 h in most patients with a $C_{\max} > 100$ ng/ml. It would be predicted, therefore, that a SU11248 dosing regimen that results in a minimal concentration > 100 ng/ml would elicit sustained FLT3 inhibition. By extrapolation from preclinical models, this PK/PD relationship should also translate to target modulation requirements for PDGFR and VEGFR in patients with solid tumors.

Several FLT3 inhibitors reduce STAT5 activity in preclinical models (7, 8, 24, 28), but this has not been previously shown in patients. STAT5 induces myeloproliferative disease and is activated downstream of many oncogenes associated with hematopoietic disorders. The levels of STAT5 phosphorylation in whole blood lysates were higher in AML patients than in healthy donors or patients with advanced solid malignancies, consistent with recent observations that STAT5 is active in peripheral blood blasts from the majority of AML patients (29, 30). Data from several groups suggest that STAT5 is strongly activated by FLT3-ITD but not by FLT3-WT (5, 7, 23, 24). We observed that stimulation of FLT3-WT cells (the OC1-AML5 cell line or PBMCs isolated from healthy volunteer blood) with FLT3 ligand weakly activates STAT5 tyrosine phosphorylation and DNA binding activity,¹³ whereas FLT3-ITD strongly activates STAT5. Analysis of STAT5 activity after SU11248 administration revealed inhibition in both WT and mutant patients but most significantly in ITD patients. The inhibition in WT patients was only at late time points in most cases and may, therefore, be indirect, reflect weak association of STAT5 signaling with FLT3-WT, or may be downstream of other SU11248 targets such as KIT or VEGFRs, which are also expressed on AML blasts. In future clinical studies with FLT3 inhibitors, STAT5 may provide an additional readout of FLT3-ITD activity, analogous to phosphorylation of CRKL as a readout of BCR-ABL signaling in CML (31), and may have application in analysis of resistance that may arise during treatment with FLT3 inhibitors.

The ERK pathway was also modulated by SU11248. Because ERK is widely expressed and activated by many stimuli in

addition to FLT3, activity was measured in PBMCs rather than peripheral blood used for FLT3 analysis. We noted that ERK was inhibited in three patients in whom strong FLT3 inhibition was not detected (data not shown), and in FLT3-modulated patients, a correlation between FLT3 and ERK inhibition was not apparent. There are several possibilities to account for this; because of signal amplification that occurs between FLT3 and ERK activation, modest undetectable changes in FLT3 phosphorylation may result in larger changes in P-ERK, or the reagents for analysis of P-ERK may be more sensitive than phosphorylated FLT3. ERK may also be activated downstream of other SU11248 targets such as KIT or VEGFRs, which are also expressed on AML blasts, and is part of many other signaling pathways that may be indirectly modulated in patients. In a number of patients, increases in P-ERK were apparent after SU11248 administration (data not shown), which may be associated with sample processing *ex vivo*. N-RAS, which acts upstream of ERK, is mutated in up to 15% of AML patients (32). Because RAS signaling may impact sensitivity to FLT3 inhibitors, patients were genotyped for mutations in N-RAS (exon 1). Mutations were identified in two patients (6.9% incidence, G12D and G12A, respectively; data not shown). Correlation with ERK activity was not feasible with this small sample size.

ERK activity has also been used as a downstream biomarker of target inhibition in patient PBMC samples from a farnesyl transferase inhibitor Phase I trial in AML (33) and in skin biopsies from advanced malignancy patients treated with ZD1839 (25, 34). Taken together, it seems that ERK may be a general readout of targeted therapies that impact proliferation signaling in clinical samples. Additional studies are needed to investigate ERK as a specific biomarker of FLT3 signaling.

This clinical study provided rapid and direct evidence of FLT3 target modulation by SU11248 in cancer patients. Evidence of inhibition of downstream signaling through the STAT5 and ERK pathways was also clear but was more difficult to interpret, most likely because of redundancy among signaling pathways. Therefore, in this study, direct analysis of FLT3 provided the most valuable correlative information. The PK/PD relationship was consistent with preclinical data and can be applied to other solid tumor targets of SU11248. This proof of target inhibition in a clinical setting validates and guides additional clinical development of SU11248, which is currently in Phase I and II clinical trials.

ACKNOWLEDGMENTS

We are extremely grateful to the patients who generously contributed to this study. We also thank Tab Burkman, Cindy Paige, Maribeth Hohenstein, Mary Mailliard, Lu Caniglia, Gabi Vohwinkel, Bulient Sargin, Derya Omaldi-Mohr, and Barb Langsdon for assistance with sample collection and Laura McGreevey and Andrea Haley for genotyping analysis. We are grateful to Alyssa Morimoto, Beverly Smolich, Douglas Laird, Suzanne Coberley, Patricia Aouan, and Dirk Mendel for helpful discussions during this work.

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¹³ A-M. O'Farrell and H. M. Yuen, unpublished data.

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