ABT-869 a Multi-Targeted Receptor Tyrosine Kinase Inhibitor: Inhibition of FLT3 Phosphorylation and Signaling in Acute Myeloid Leukemia

Running Title: ABT-869 Receptor Tyrosine Kinase Inhibitor in AML

Deepta B. Shankar1,7, Junling Li2,7, Paul Tapang2, J. Owen McCall2, Lori J. Pease2, Yujia Dai2, Ru-Qi Wei2, Daniel H. Albert2, Jennifer J. Bouska2, Donald J. Osterling2, Jun Guo2, Patrick A. Marcotte2, Eric F Johnson2, Niru Soni2, Kresna Hartandi2, Michael R. Michaelides2, Steven K. Davidsen2, Saul J. Priceman3, Jenny C. Chang1, Katrin Rhodes1, Neil Shah6, Theodore B. Moore1, Kathleen M. Sakamoto1,3,4,5, and Keith B. Glaser2*

1Division of Hematology/Oncology, Department of Pediatrics, Gwynne Hazen Cherry Memorial Laboratories, and Mattel Children’s Hospital Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, 10833 LeConte Avenue, Los Angeles, CA 90095-1752

2Cancer Research, R47J-AP9, Global Pharmaceutical Research and Development Abbott Laboratories, Abbott Park, IL 60064-6121

3Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, 10833 LeConte Avenue, Los Angeles, CA 90095-1752

4Molecular Biology Institute, UCLA, Los Angeles, CA 90095

5Division of Biology, California Institute of Technology, Pasadena, CA 91125

6Division of Hematology-Oncology, Department of Medicine, University of California at San Francisco, San Francisco, CA

7These authors contributed equally to the content and preparation of this manuscript.

*Correspondence: Dr. Keith B. Glaser, Cancer Research, R47J-AP9, Abbott Laboratories, Abbott Park, IL 60064-6121, Phone (847) 937-1558, Fax (847) 935-3622, e-mail: keith.glaser@abbott.com

^D.B.S. is supported by the Hamburger endowment from the UCLA Jonsson Comprehensive Cancer Center. K.M.S. is a Scholar of the Lymphoma and Leukemia Society and is supported by the NIH (CA108545, HL75826, RHL083077A), the Department of Defense and the Diamond-Blackfan Anemia Foundation. Both T.B.M. and K.M.S. are funded by the UCLA Jonsson Comprehensive Cancer Center.

BLOOD Scientific Heading: Neoplasia
Contributions of authors:
DBS and JL – designed research, performed research, collected data, analyzed data, and wrote the paper
PT – performed research, analyzed data
OM - performed research, analyzed data
LJP - performed research, analyzed data
YD – contributed vital new reagent
R-QW - performed research, analyzed data
DHA – analyzed data and wrote the paper
JB – performed research and analyzed data
DO - performed research, analyzed data
JG - performed research, analyzed data
PAM - performed research, analyzed data
EFJ - performed research, analyzed data
NS – performed research
KH- contributed vital new reagent
MM – contributed vital new agent and analyzed data
SKD – analyzed data and contributed to the organization and editing of manuscript
SP – performed research, analyzed data
JC – performed research, analyzed data
KR- performed research, analyzed data
NS – contributed to study design
TM – contributed to study design
KMS – designed research, analyzed data, wrote the paper
KBG – designed research, analyzed data, wrote the paper

JL, PT, JOM, LJP, YD, R-QW, DHA, JJB, DJO, JG, PAM, EFJ, NS, KH, MRM, SKD, and KBG are all employees of Abbott Laboratories. All other authors received no financial support from Abbott Laboratories for the studies presented herein.
Abstract

In 15-30% of acute myeloid leukemia (AML) patients aberrant proliferation is a consequence of a juxtamembrane mutation in the \textit{FLT3} gene (FLT3-ITD) causing constitutive kinase activity. ABT-869 (a multi-targeted receptor tyrosine kinase inhibitor) inhibited the phosphorylation of FLT3, STAT5 and ERK, and Pim-1 expression in MV-4-11 and MOLM-13 cells (IC$_{50}$ ~ 1-10 nM) harboring the FLT3-ITD. ABT-869 inhibited the proliferation of these cells (IC$_{50}$ = 4 and 6 nM, respectively) through the induction of apoptosis (increased sub G0/G1 phase, caspase activation and PARP cleavage), whereas cells harboring wt-FLT3 were less sensitive. In normal human blood spiked with AML cells, ABT-869 inhibited phosphorylation of FLT3 (IC$_{50}$ ~100 nM), STAT5 and ERK, and decreased Pim-1 expression. In methylcellulose-based colony-forming assays, ABT-869 had no significant effect up to 1,000 nM on normal hematopoietic progenitor cells; whereas in AML patient samples harboring both FLT3-ITD and wt-FLT3, ABT-869 inhibited colony formation (IC$_{50}$ = 100 and 1000 nM, respectively). ABT-869 dose-dependently inhibited MV-4-11 and MOLM-13 flank tumor growth, prevented tumor formation, regressed established MV-4-11 xenografts, and increased survival by 20 weeks in an MV-4-11 engraftment model. In tumors, ABT-869 inhibited FLT3 phosphorylation, induced apoptosis (TUNEL) and decreased proliferation (Ki67). ABT-869 is under clinical development for AML.
Introduction

Acute myeloid leukemia (AML) is an aggressive, heterogeneous disease with numerous cytogenetic abnormalities and mutations within key signaling pathways involved in cell differentiation, proliferation and survival. One of these key signaling molecules is FLT3 (FMS-like tyrosine kinase 3), a receptor tyrosine kinase (RTK) expressed and activated in the majority of AML patients and also expressed in some normal hematopoietic cell types. Despite the success of initial chemotherapy, there is a relatively high relapse rate for AML patients. These patients ultimately become refractory to traditional chemotherapies and succumb to the disease. The failure of initial chemotherapy or the refractory nature of AML has been associated with the acquisition of mutations that constitutively activate kinases essential for AML cell survival: ITD (internal tandem duplication) mutations in the juxtamembrane domain (15-30% of patients) and point or short length mutations in the activation loop of the kinase domain (about 7% of patients), and various similar mutations. The FLT3-ITD induces ligand-independent dimerization, autophosphorylation and constitutive activation of these receptors, and is able to transform hematopoietic cells. Generation of a constitutively active FLT3 also activates downstream phosphorylation events, e.g. STAT5, Akt, and ERK, which regulate the FLT3 dependent survival of these cells. The ITD effectively activates STAT5 phosphorylation and the induction of STAT5 target genes, e.g. CIS and Pim-2, whereas the D835 mutations behave similarly to the wt-FLT3 with only a weak activation of STAT5 phosphorylation and no induction of STAT5 target genes. Clinically, the FLT3-ITD is an important independent negative prognostic factor in AML.
and is associated with increased blast count, increased relapse rate, and poor overall survival. Inhibition of FLT3, especially the mutant forms responsible for the refractory nature of this disease, has made this an attractive target for the treatment of AML.

ABT-869 (Table 1) is a structurally novel multi-targeted receptor tyrosine kinase inhibitor that potently inhibits all members of the VEGF and PDGF receptor families, but has much less activity (IC₅₀ values >1 µM) against unrelated receptor tyrosine kinases, cytoplasmic tyrosine kinases or Ser/Thr kinases. The ability of ABT-869 to inhibit RTKs is also evident in cellular assays of RTK phosphorylation and VEGF-induced endothelial cell proliferation; however, ABT-869 is not a general anti-proliferative agent since, in most cells, > 1,000-fold higher concentrations of ABT-869 are required to inhibit proliferation. In preclinical tumor growth studies, ABT-869 exhibits efficacy in human fibrosarcoma, breast, colon, and small cell lung carcinoma xenograft models, as well as in orthotopic breast, prostate and glioma models.

Herein, we report the characterization of ABT-869 against AML cell lines harboring receptor tyrosine kinase mutations that result in constitutively activated RTKs or signaling pathways; these cells appear to be more sensitive to the effects of ABT-869. These results demonstrate the efficacy of ABT-869 in both in vitro, spiked blood model, and in vivo leukemia models and that phosphorylation of FLT3 and STAT5 appear to be feasible biomarkers for the assessment of clinical activity of ABT-869 in AML.
Materials and Methods

IRB approval for human bone marrow and blood samples were obtained at both UCLA and Abbott Laboratories to perform this work. All animal studies were completed under IACUC approved protocols for animal welfare.

Cell Culture and Reagents

Cell culture media were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone (defined, heat inactivated) or from Invitrogen (Carlsbad, CA). MV-4-11, RS4;11, Kasumi-1, KG-1, U937, K562, NB 4, SUP-B15, HL60 and Jurkat human cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). MOLM-13 cells were purchased from DSMZ (Braunschweig, Germany). All cells were cultured according to ATCC or DSMZ guidelines.

Viability and Cell Proliferation Assays

For cell lines treated with ABT-869, live and dead cells were counted 24, 48 and 72 hr after treatment using Trypan blue exclusion assay. All experiments were performed in triplicate. Percent viability was calculated and compared to the control cells treated with DMSO (0.1%). Cell proliferation was assessed with alamarBlue (Biosource, Camarillo, CA; final solution 10%) as described in 16. Data represents two separate experiments with each data point carried out in duplicate in each experiment.

Colony Formation Assays with Normal and AML Patient Samples.

Normal and AML patient human bone marrow cells were resuspended in a volume of 0.3 mL of Iscoves with 2% fetal bovine serum at 5 X 10^5 cells/mL and mixed with varying concentrations of the drug (10 pM to 10 μM suspended in 0.1% DMSO final) or DMSO control. This was then added to 3 mL of methyl cellulose-containing
growth factors IL-3, IL-6, GM-CSF, G-CSF, erythropoietin, and Stem Cell Factor (Methocult GF-H4434, Stem cell technology, Vancouver, Canada). The mixture of 1.1 mL was plated in culture dishes. The colonies were observed each day and counted on day 14. The total number of colonies and types of colonies were scored. For the Normal samples, total colony numbers and the number of CFU-GM, CFU-GEMM, BFU-E, CFU-E and CFU-Ms were analyzed. For the AML samples, the colonies were less differentiated and ranged from nice clusters with lots of cells to small clusters of 8-10 cells. Since we were starting with a heterogeneous population of cells, we also observed some CFU-GM and rarely BFU-E colonies. We counted all the colonies, the undifferentiated colonies, clusters and the differentiated colonies in our study.

**Electrophoresis and Western Blotting**

SDS-PAGE samples were electrophoresed, transferred to PVDF membranes (Invitrogen, Carlsbad, CA) and visualized by enhanced chemiluminescence with the Pierce Dura SuperSignal substrate (Pierce, Rockford, IL) according to 16.

**Blood Spike Model and Bone Marrow for FLT3 Phosphorylation**

MV-4-11 cells, 1 x 10^7 cells, were treated with compound (final 0.1% DMSO), immediately added to 1 mL healthy normal human donor blood, incubated at 37°C for 2 hr with gentle shaking, then added to 2 volumes of lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 10% glycerol, protease inhibitor cocktail, phosphatase inhibitor cocktails (Sigma, St. Louis, MO)). The sample was pre-cleared then immunoprecipitated with anti-FLT3 (SC480; Santa Cruz Biotechnology, Santa Cruz, CA, and 50 µL of Protein G-agarose), incubated overnight, and washed 5 times with 1 mL of PBS containing 1 mM vanadate and 1x protease inhibitor cocktail (16,000 x g for 2 min). Phosphorylated FLT3
was detected with 4G10 (Upstate Biotechnology, Lake Placid, NY) and blots re-probed for total FLT3 (sc-480; Santa Cruz Biotechnology, Santa Cruz, CA). Each experiment was carried out in duplicate and a representative result from one of these studies is presented.

**Analysis of ERK and STAT5 Phosphorylation and PIM-1 Expression in Human Blood Spiked Model**

PBMCs were isolated from spiked human blood in hypotonic buffer (155 mM NH₄Cl, 10 nM KHCO₃, 0.1 mM EDTA), centrifuged at 260 x g for 10 min at 20°C, washed 1 - 2 times in 10 mL of PBS (260 x g for 10 min at 20°C). Lysis buffer (M-PER, PIERCE, Rockford, IL), protease inhibitor cocktail, and phosphatase inhibitor cocktails (Sigma, St. Louis, MO) were added to the PBMC pellet, 3 volumes per cell pellet volume, and sonicated. Lysates were clarified (20,000 x g for 20 min at 4°C) and electrophoresed. Phospho-ERK and total ERK were detected using phospho-ERK (Thr 202/ Try 204, Upstate Biotechnology, Lake Placid, NY) and total ERK (p44/42, Cell Signaling Technologies, Beverly, MA). Phospho-specific STAT5 was from Upstate Biotechnology (Lake Placid, NY), and β-actin was used as the load control for STAT5 (Sigma, St. Louis, MO). Pim-1 antibody was from Bethyl Laboratories (Montgomery, TX)

**Analysis of Apoptosis in MV-4-11 Cells**

MV-4-11 cells treated for 72 hr with ABT-869 were fixed with 80% ethanol, washed with PBS, and incubated with propidium iodide (50 mg/mL). DNA content was determined by fluorescence cell analysis using a FACS Calibur (Becton Dickinson, San
Diego, CA) flow cytometer, and cell cycle distribution analyzed with CellQuest software. MV-4-11 or MOLM-13 cells (1 x 10^5 cells/mL) were treated with ABT-869 for 48 hours, and analyzed for apoptosis using Annexin V-FITC (Apo-alert apoptosis kit (BD Biosciences, San Diego, CA)) and propidium iodide. Flow cytometry was performed at the UCLA Flow Cytometry core facility. Cleavage of poly-ADP-ribose polymerase (PARP) or activation of caspase-3 and caspase 9 was determined from cell lysates made 48 hours after treatment. Western blot was performed as previously described 17,18. Anti-Caspase-3 was from Upstate Biotechnology (Lake Placid, NY), anti-PARP from Cell Signaling Technology, anti-β-Tubulin and anti-Caspase-9 were from Santa Cruz, Inc. (Santa Cruz, CA).

In Vivo Models

AML Cell Line Flank and Engraftment Model in SCID Mice

MV-4-11 or MOLM-13 cells (5 x 10^6 cells) were mixed with matrigel and injected subcutaneously into the hind flank of the mice on day 0. For MV-4-11 tumors, each cohort of mice (15 mice) was randomized into five groups and dosed with compound or vehicle. ABT-869 (5, 10, 20, and 40 mg/kg/day) dissolved in 100% pure refined corn oil was delivered orally by gavage. Tumor growth was measured every two days using vernier calipers. Tumor volume was calculated as the product of length x width x height. The tumors were allowed to grow to 400 mm^3 before the mice were grouped randomly after outliers were culled and treated with ABT-869.
**Histology**

The animals were euthanized, tumors and organs removed, fixed in formalin, and embedded in paraffin. Sections were prepared and stained with hematoxylin-eosin. Proliferation was assessed by immunostaining with Ki67 antisera (DAKO Laboratories, Denmark). Apoptosis was determined using TUNEL staining. These experiments were performed by the UCLA Immuno-Histochemistry Core facility, Department of Pathology.

**Analysis of Tumor FLT3 Phosphorylation**

MV-4-11 tumors (7 day) received a single 10 mg/kg dose of ABT-869 administered p.o., at 3 and 6 hr post-dose the mice were sacrificed and tumors resected and snap frozen in liquid nitrogen. Tumors were homogenized in ice-cold RIPA buffer using a Polytron (Westbury, NY). Phosphorylated proteins were immunoprecipitated overnight at 4°C using PY20-agarose beads (sc-508, Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitation from 2 mg of tumor lysate was loaded in each gel lane and visualized on Western blots using total FLT3 antibody sc-480 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Bone Marrow Engraftment Model**

NOD-SCID mice were pretreated with cyclophosphamide by intra-peritoneal injection of 150 mg/kg/day for 2 days, followed by 24 hr of rest prior to intravenous injection of 5 x 10⁶ MV-4-11 cells via the tail vein. Bone marrow cell suspensions were prepared by flushing mouse femurs with cold, sterile PBS; cell suspensions were fixed, stained with anti-human CD45 and analyzed by flow cytometry. ABT-869 or its vehicle (ABT-869 was dissolved in 2% ethanol, 5% Tween 80, 20% PEG 400, 73%
hydroxypropyl methylcellulose (0.2% HPMC)) was dosed orally QD by gavage (10 – 12 animals per dose of ABT-869). Survival was determined by observation when the animals demonstrated hind limb paralysis and became moribund (in control animals this occurred between days 42 and 50 after engraftment).

**Pharmacokinetic Analysis of ABT-869 in Mice**

ABT-869 was administered orally to non-tumor bearing mice at doses of 3 and 10 mg/kg, the vehicle used was 2.5% ethanol, 5% Tween 80, 25% PEG 400 in PBS, and for the 40 mg/kg dose pure refined corn oil. Blood was sampled at the appropriate times by either orbital bleed or cardiac puncture into heparinized tubes. Plasma was prepared and frozen until protein precipitation with acidified methanol and analyzed by LC-MS on an YMC-ODS-AQ column with a mobile phase of 45% acetonitrile and 0.1% acetic acid. ABT-869 was detected as m/z = 376.1 on a Finnigan LCQ Duo LC/MS.
Results

In Vitro Assessment of ABT-869

ABT-869 inhibits proliferation of cells expressing mutant, constitutively active RTKs

The activity of ABT-869 was evaluated in cell lines that harbored either wt-FLT3 (RS4;11, KG1), a mutated kinase or signaling pathway (MV-4-11, MOLM-13, Kasumi-1, KG1, K562) or were FLT3-null (Jurkat, HL-60). Cell lines dependent on mutated kinases, FLT3-ITD in MV-4-11 and MOLM-13 or c-KIT activating mutations in Kasumi-1 cells, for survival are more sensitive to the anti-proliferative effects of ABT-869 (IC50 values of 4, 6 and 16 nM, respectively) (Table 2A). Cell lines that are not dependent on these mutations harboring wt-FLT3 (RS4;11) or FLT3-null cells (Jurkat and HL-60) are far less sensitive to ABT-869 (Table 2A). Consistent with its kinase activity profile (Table 1), ABT-869 does not appear to affect the proliferation of KG-1 cells harboring an FGF-FGFR autocrine loop.

Cell viability assays demonstrated that MV-4-11 cells (FLT3-ITD) responded to ABT-869 in a concentration-dependent manner (IC50 ~10 nM) (Table 2A). Other myeloid leukemia cell lines, HL60, NB4, KG1 and K562, were not responsive to ABT-869 at concentrations less than 10 µM (Table 2A).

ABT-869 inhibits the phosphorylation of FLT3 and downstream signaling molecules

In MV-4-11 cells the FLT3 is constitutively phosphorylated (Fig. 1A). The ligand-independent phosphorylation of FLT3-ITD was inhibited by ABT-869 (IC50 ~1 nM) after a 2 hr exposure (Fig. 1A). ABT-869 did not modulate the expression of FLT3
protein during the 2 hr incubation period (data not shown, for *in vitro* cell culture β-actin was used as a load control).

Similar to its potency against phosphorylation of FLT3, the observed IC₅₀ was approximately 1 nM for inhibition of phosphorylation of STAT5 (Fig. 1B) and approximately 10 - 100 nM for phosphorylation of ERK (Fig. 1C). In MOLM-13 cells ABT-869 potently inhibited STAT5 phosphorylation (IC₅₀ 1 to 10 nM) and as expected decreased the expression of a STAT5 responsive gene, Pim-1 (IC₅₀ < 1 nM) (Fig. 1D). Consistent with the effect of ABT-869 on c-KIT and Kasumi-1 cell proliferation, ABT-869 also inhibited the phosphorylation of KIT and STAT5 in Kasumi-1 cells with an IC₅₀ of ~100 nM (data not shown).

**ABT-869 induces apoptosis in MV-4-11 and MOLM-13 cells.**

ABT-869 induced an apoptotic response in MV-4-11 cells as determined by an increase in the sub G0/G1 cell population upon FACS analysis (EC₅₀ ~30 nM) (Fig. 2A). Annexin-V staining demonstrated a concentration-dependent apoptosis of cells, with the greatest increase in Annexin-V staining occurring between 10 and 100 nM ABT-869 (Fig. 2B). With increasing concentrations of ABT-869 there was significant cleavage of PARP with decreasing amounts of pro-caspase 3 indicating that caspase 3 was activated, resulting in PARP cleavage (Fig. 2C). Decreases in pro-caspase 3, and cleavage of pro-caspase 9 and PARP were also observed for ABT-869 between 10 and 100 nM in MOLM-13 cells (Figure 2D).

**In vitro AML model: Human Blood Spiked with AML Cells**

Modulation of FLT3, STAT5 and ERK phosphorylation in the AML model
In normal human blood, the amount of FLT3 protein was too low to be detected by Western blot (data not shown). Therefore, an *ex vivo* model was developed where MV-4-11 or MOLM-13 cells were spiked into normal human blood to mimic the clinical setting. ABT-869 inhibited the phosphorylation of FLT3 in a concentration-dependent manner (IC$_{50}$ ~100 nM, Fig. 3A). *In vitro*, the IC$_{50}$ value for ABT-869 was 1 nM in MV-4-11 cells; significantly lower than the concentration needed to inhibit phosphorylation in the blood model. This result suggested a differential of 100 fold relative to low protein conditions, consistent with the fact that ABT-869 is >99% protein bound in human plasma$^{15}$. ABT-869 also inhibited phosphorylation of STAT5 (IC$_{50}$ between 1 and 10 nM) and ERK (IC$_{50}$ between 1 and 10 µM) (Fig 3B and C, respectively). Inhibition of STAT5 phosphorylation occurs before inhibition of FLT3, while inhibition of MAPK phosphorylation occurs much after FLT3 inhibition. This effect is probably due to differential effects on other signaling pathways that affect the phosphorylation of downstream targets of FLT3. This is also evident in MOLM-13 cell spiked blood by the inhibition of STAT5 phosphorylation and expression of the STAT5 responsive gene, Pim-1 (Fig. 3D). As the effect of ABT-869 was weaker (~ 100 nM) in Kasumi-1 cells in culture, when spiked into blood ABT-869 inhibited KIT phosphorylation between 1 and 10 µM, also consistent with a 100-fold shift in potency relative to low protein conditions (data not shown).

As demonstrated in Fig. 3E, at efficacious doses of ABT-869 (10 mg/kg/d in 2.5% ethanol, 5% Tween 80, 25% PEG 400 in PBS vehicle or 40 mg/kg/d in pure refined corn oil vehicle) plasma concentration exceeded 1 µM for up to 8 hrs after dosing. These
levels are sufficient to cover the IC₅₀ values for FLT3 and downstream signaling phosphorylation events observed in the in vitro spiked blood model.

**ABT-869 does not inhibit growth of normal human bone marrow progenitor cells in vitro at concentrations less than 1µM**

FLT3 is expressed on immature hematopoietic progenitors and also on some mature myeloid and lymphoid cells. ABT-869 demonstrated a concentration-dependent inhibition of FLT3 ligand (FL) stimulated FLT3 phosphorylation (estimated IC₅₀ value of 10 nM) with complete inhibition being observed at 1 µM ABT-869 in normal human bone marrow (data not shown).

Colony-forming assays in methylcellulose containing IL-3, IL-6, and SCF, demonstrate that ABT-869 does not have a significant effect on the proliferation and differentiation of human bone marrow cells up to a concentration of 1 µM (26% and 43% inhibition at 0.1 and 1.0 µM, respectively) (Fig. 4A). Above 1 µM there was significant decrease in the numbers of CFU-GM colonies (92% inhibition at 10 µM). These results indicate that ABT-869 is not toxic to normal human bone marrow, cell proliferation and differentiation, at the concentrations effectively inhibiting growth of AML cells in vitro.

**ABT-869 effectively inhibits primary AML bone marrow cells in vitro.**

To examine the effect of ABT 869 on primary AML patient samples, we performed in vitro colony assays with methylcellulose containing IL-3, IL-6, GM-CSF, G-CSF, erythropoietin, and Stem Cell Factor on bone marrow samples from AML patients that expressed either the FLT3-ITD or no FLT3 mutation. Our results demonstrated that ABT-869 inhibits colony formation of FLT3-ITD positive cells at an IC₅₀ of 100 nM (Fig. 4B). ABT-869 inhibited colony formation of AML cells without
FLT3 mutations at an IC$_{50}$ of 1µM (Fig. 4C), suggesting that the drug may be inhibiting growth by targeting additional pathways. The effect of ABT-869 on CFU-E and CFU-GM in AML cells without FLT3 mutations (Supp. Figure B) is similar to that shown in Fig. 4C where significant inhibition is observed between 100 nM and 1 µM.

**In Vivo Assessment of ABT-869**

**ABT-869 inhibits growth of MV-4-11 tumor xenografts**

MV-4-11 cells were subcutaneously implanted into SCID mice and solid tumors of approximately 400 mm$^3$ grew within three weeks. Histology of the tumors showed a large number of mitotic figures indicative of active proliferation (see Fig. 5D). Immunohistochemical staining for Ki67 demonstrated a high proliferative index consistent with rapid tumor growth, in contrast to treated tumors, which showed no mitotic activity (Fig. 5E).

Mice treated with 40 mg/kg/day ABT-869 showed complete regression of the tumors within two weeks of treatment (Fig. 5A). Histology of the regressed tumors showed necrosis and a number of inflammatory cells. TUNEL staining of the sections from treated tumors indicated a number of apoptotic bodies (Figs. 5D & E). Tumors treated with 20 mg/kg/day showed complete regression within a month of treatment (Fig. 5A). Tumors treated with 10 mg/kg/day stopped growing and showed minimal regression (Fig. 5A). Ki67 staining of these tumors indicates that there is no mitotic activity in these treated tumors (data not shown). These results indicated that ABT-869 causes regression of the tumors in a dose-dependent manner by inhibiting proliferation and inducing apoptosis. In established MV-4-11 tumors (~1 g), ABT-869 demonstrated a
dose-dependent inhibition of FLT3 phosphorylation in the MV-4-11 tumors at 3 and 6 hr after receiving a dose of ABT-869, 10 mg/kg (Fig. 5C).

**ABT-869 prevents tumor formation using MV-4-11 xenograft**

To determine whether treatment of ABT-869 prevents MV-4-11 cells from forming tumors, treatment was started 24 hours after tumor cell injection with 40 mg/kg/day ABT-869. The group receiving ABT-869 did not develop tumors even after three months, in contrast to the control group in which tumors grew rapidly (Figure 5B). Daily dosing of ABT-869 was well tolerated with no signs of toxicity.

**ABT-869 Inhibits and Prevents Tumor Growth in MOLM-13 Xenograft Models**

In MOLM-13 xenograft tumors, representative studies demonstrate that ABT-869 was less efficacious than in MV-4-11 xenograft tumors (Fig. 6 A&B5), but still produced profound effects on MOLM-13 tumor formation and growth. ABT-869 slowed the growth of MOLM-13 tumors from approximately day 18 onward (Fig. 6A). ABT-869 did not produce frank regressions in the MOLM-13 xenograft model. In the tumor prevention model, ABT-869 was able to prevent MOLM-13 tumor growth up to day 26 after which tumor growth recommenced but at a slower rate than control untreated tumor growth for the 40 mg/kg group (Fig. 6B).

**ABT-869 Enhances Survival in a Bone Marrow Engraftment Model of Leukemia**

In the engraftment model of MV-4-11 cells in mice with their endogenous bone marrow ablated by pretreatment with cyclophosphamide, human cells (7% of total cells) were detected by CD45 expression in bone marrow 25 days after cell inoculation. Within 42 to 50 days the bone marrow content of CD45-positive cells from vehicle-treated animals had increased to 22% and clinically the mice exhibited hind limb
paralysis, ruff coat, and decreased activity. Treatment with ABT-869 (1, 3, and 10 mg/kg/d), initiated 25 days after MV-4-11 cell inoculation, decreased the proportion of CD45-positive cells detected in bone marrow 40 days after inoculation (16, 16, and 0.7%, respectively). Continued treatment resulted in a dose-dependent prolongation in survival (Fig. 6C) from 3 weeks up to 20 weeks at 1 and 10 mg/kg/d, respectively. Preliminary flow cytometric analyses of the blood and bone marrow from these engraftment mice demonstrated a reduction in the amount of phosphorylated STAT5 and ERK in the MV-4-11 positive cells at 10 mg/kg/d (data not shown) and further supports the mechanism of ABT-869 in vivo.

**Comparison of ABT-869 to Other FLT3 and VEGFR/PDGFR Inhibitors**

Comparison of the kinase inhibition profile of ABT-869 and other FLT3 inhibitors (MLN 518, CHIR 258, sunitinib (SU-11248) and sorafenib (BAY 43,9006)) is shown in the heatmap of $K_i$ values determined for 79 different kinases (Supplemental Fig. A) and provides one method of evaluating the potential clinical merit of new RTK inhibitors. MLN 518 is the apparently the most selective FLT3 inhibitor but is less potent against FLT3 than the VEGFR/PDGFR family inhibitors, including ABT-869. The IC$_{50}$ values against FLT3 at 1 mM ATP are 4 nM for ABT-869, 98 nM for MLN 518, 1 nM for CHIR 258, 4 nM for sunitinib, and 46 nM for sorafenib. These IC$_{50}$ values are consistent with the antiproliferative properties of these inhibitors in MV-4-11, MOLM-13, RS4;11, SUP-B15 and Kasumi-1 cells in vitro (Table 2B).

**Diverse Effects of ABT-869 on Activation Loop Mutants of FLT3**

ABT-869 inhibits the catalytic domain of wt-FLT3 with an IC$_{50}$ value of 4 nM. (Table 1) Different activation loop mutations of the FLT3 gene (D835H, D835Q,
D835V, and D835Y) were expressed as catalytic domains and demonstrated diverse responses to ABT-869. The D835H and D835Q mutations were approximately 10 fold less sensitive to ABT-869 (IC_{50} values of 52 and 64 nM, respectively). The D835Y mutation was >1000 fold less sensitive to ABT-869 (IC_{50} value of 4,600 nM) and the least sensitive mutant was D835V with an IC_{50} value of 19,800 nM. The effect of ABT-869 on the growth of Baf3 cells transfected with these mutations is being investigated both in vitro and in vivo.
Discussion

In AML cell lines harboring mutated kinases, RTKIs of the VEGFR/PDGFR families inhibit proliferation and survival subsequent to the inhibition of FLT3 phosphorylation and phosphorylation of downstream targets of FLT3. In several instances this has also been translated into clinical samples from AML patients\(^{10,11,25,26}\). Strong inhibition of FLT3 phosphorylation by SU-11248 (sunitinib) was evident in all five mutant FLT3 patients (three ITDs, one D835Y and one G846S) suggesting that AML patients with these mutations may be more sensitive to RTKIs\(^{11,25}\). Similar results were also obtained for CEP-701\(^{10}\) and PTK412\(^{26}\) in AML patients.

In hematological malignancies the angiogenesis component has not been fully assessed; therefore, it would be essential for a potential new therapeutic agent not only to have an anti-angiogenic component but to also have a direct anti-tumor effect as demonstrated herein with ABT-869. Mutations in the growth factor signaling pathways that make survival dependent on that kinase activity provides this avenue for the RTKIs\(^{3}\).

Described herein, in AML cell lines harboring the FLT3-ITD mutation (MV-4-11 cells\(^{27}\) and MOLM-13\(^{28}\)), ABT-869 inhibited proliferation by induction of apoptosis as well as phosphorylation of FLT3 and downstream effectors, STAT5 and ERK as well as expression of the STAT5 responsive gene Pim-1. Kasumi-1 cells harboring the AML-ETO fusion gene, an autocrine SCF-KIT mutation\(^{20}\), and an activating c-KIT N822K mutation\(^{21,22}\), were also potently inhibited by ABT-869. These anti-proliferative results are consistent with the in vitro enzyme profile of ABT-869 (Table 1). In wt-FLT3 cells, RS4;11, and FLT3 negative Jurkat cells, the anti-proliferative effect of ABT-869 was
approximately 1,000 fold less potent. The differential between mutated and wt-FLT3 is also consistent with the lack of effect of ABT-869 (> 1,000 nM) on normal bone marrow progenitor cells in a colony-forming assay even though ABT-869 inhibits ligand-induced FLT3 phosphorylation with an IC₅₀ of approximately 10 nM (data not shown). Our data suggests that ABT-869 is significantly more active against cell lines that contain mutated or constitutively active RTKs targeted by ABT-869. The AML clinical studies with SU11248, PKC412 and CEP-701 suggest that this may also be the case in AML patients. Based on these results, the anti-leukemic activity of ABT-869 is dependent on (1) the expression of a mutated kinase or altered signaling pathway that is essential for cell survival and (2) the intrinsic potency of ABT-869 against that receptor tyrosine kinase.

Comparison of ABT-869 with other FLT3 inhibitors (Table 2B) suggests that ABT-869 is superior to MLN 518 at the enzyme and cellular level and is essentially equivalent to the more non-selective inhibitors sunitinib, sorafenib and CHIR 258. In a mechanism-based in vivo model, Albert et al. have demonstrated that ABT-869 is more potent than other RTKIs, e.g. sunitinib (SU-11248), sorafenib (BAY 43,9006), AG013736 and ZD6474, in blocking VEGF-induced edema. These data along with the overall in vivo anti-tumor assessment of ABT-869 suggest that ABT-869 is a novel RTKI that has potential in the treatment of AML through direct effects on mutated kinases and as an anti-angiogenic compound. The activity of ABT-869 on primary AML patient samples also demonstrates a selectivity for patients with the FLT3-ITD; however, the activity observed against wt-
FLT3 AML patient samples also suggests that ABT-869 may be acting through additional pathways that mediate AML cell survival.

In flank tumors, ABT-869 is able to cause the complete regression of established tumors (data herein) and in mice with large, established tumors (~2g) ABT-869 can also generate a significant tumor regression. When administered at the time of MV-4-11 cell inoculation, ABT-869 can prevent the outgrowth of a palpable tumor for greater than three months and for approximately 26 days in MOLM-13 tumors. The inhibition of MV-4-11 flank tumor growth is associated with a decrease in FLT3 phosphorylation, an increase in apoptotic cells and a decrease in proliferation index (Ki-67 staining) consistent with the in vitro effects of ABT-869 on MV-4-11 cells. To minimize the unnatural state of a flank leukemia tumor, the bone marrow engraftment model with MV-4-11 cells was evaluated with ABT-869. ABT-869 effectively increased survival in this orthotopic model of leukemia. Disease in these animals was evident when human CD45 positive cells were observed in the bone marrow and correlated with disease progression. At 10 mg/kg/d the survival of these animals was prolonged almost 20 weeks demonstrating that ABT-869 is a potent and potentially useful therapeutic for AML in this xenograft model of disease. Preliminary analysis of MV-4-11 cells from the blood of these animals demonstrated inhibition of STAT5 and ERK phosphorylation, consistent with mechanism observed in vitro. These results are consistent with the direct effect of ABT-869 on leukemia cell growth but do not exclude an anti-angiogenic component in both models.

The ex vivo AML model is another model for the evaluation of ABT-869. The IC₅₀ of approximately 100 nM for ABT-869 against FLT3 phosphorylation is approximately 100
fold greater than its IC$_{50}$ estimated in MV-4-11 cells in the absence of protein (IC$_{50}$ ~1 nM) and 25 fold greater than its effect on in vitro proliferation in the presence of 10% fetal bovine serum. These results correlate well with the fact that ABT-869 is highly protein bound, > 99%, in human plasma $^{15}$, suggesting that this model can be potentially used preclinically to define plasma drug concentrations. SU11248, CEP-701, PKC412, and MLN518 are currently in clinical studies as FLT3 inhibitors in AML patients $^{1,10,19,25,26}$. These compounds also demonstrate a 100-150 fold shift in their respective IC$_{50}$ values in similar normal blood models spiked with different AML cell lines or engineered cell lines $^{10,29}$.

In conclusion, ABT-869 has potent activity against AML cell lines with mutated kinases while being less effective against leukemia cell lines with wt-FLT3 at doses that do not affect normal bone marrow progenitor cells. Enzyme data would suggest variable activity against different activation loop mutations of FLT3. ABT-869 also has potent in vivo activity in MV-4-11 xenograft and engraftment tumor models demonstrating tumor regressions, tumor cell apoptosis and a decrease in proliferation index, and a 20-week prolongation of survival in the engraftment model. These attributes provide the basis for the clinical development of ABT-869 in AML. The in vitro activity of ABT-869 against AML patient samples with the FLT3-ITD and wt-FLT3 adds value to the development of ABT-869 in the treatment of AML.
References


Table 1. Summary of Kinase Inhibitory Activities of ABT-869

![ABT-869 Structure]

**ABT-869**

\(N\text-[4-(3-amino-1H-indazol-4-yl)phenyl]-N'-(2-fluoro-5-methylphenyl)urea\)

<table>
<thead>
<tr>
<th>Structurally Related RTKs</th>
<th>Non-related TKs</th>
<th>Ser/Thr Kinases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kinase</strong></td>
<td><strong>IC(_{50}) (nM)(^b)</strong></td>
<td><strong>Kinase</strong></td>
</tr>
<tr>
<td>KDR</td>
<td>4</td>
<td>SRC</td>
</tr>
<tr>
<td>Flt1</td>
<td>3</td>
<td>IGFR</td>
</tr>
<tr>
<td>Flt4</td>
<td>190</td>
<td>INSR</td>
</tr>
<tr>
<td>PDGFR-(\beta)</td>
<td>66</td>
<td>LCK</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>3</td>
<td>EGFR</td>
</tr>
<tr>
<td>KIT</td>
<td>14</td>
<td>HCK</td>
</tr>
<tr>
<td>Flt3</td>
<td>4</td>
<td>CMET</td>
</tr>
<tr>
<td>TIE2</td>
<td>179</td>
<td>LYN</td>
</tr>
<tr>
<td>RET</td>
<td>1,900</td>
<td>FYN</td>
</tr>
<tr>
<td>FGFR</td>
<td>&gt;12,500</td>
<td>FGF</td>
</tr>
</tbody>
</table>

\(^a\) Methods and data can be found in Albert et al. 15.

\(^b\) IC\(_{50}\) values determined at an ATP concentration of 1 mM.

\(^c\) IC\(_{50}\) values determined at an ATP concentration of 5-10 \(\mu\)M.
Table 2.
A. Anti-proliferative effect of ABT-869 on various leukemia cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Designation/Mutation</th>
<th>IC₅₀ (nM)</th>
<th>alamarBlue (MTT)a</th>
<th>Viability (TB)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV-4-11</td>
<td>AML – FLT3-ITD</td>
<td></td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Kasumi-1</td>
<td>AML – ETO (SCF-KIT loop; KIT N822K)</td>
<td>16</td>
<td>ndc</td>
<td></td>
</tr>
<tr>
<td>RS4;11</td>
<td>ALL – wt-FLT3</td>
<td>4,100</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Jurkat</td>
<td>ALL – FLT3 negative</td>
<td>4,200</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>APL – FLT3 negative</td>
<td>~8,000</td>
<td>&gt; 10,000d</td>
<td></td>
</tr>
<tr>
<td>NB4</td>
<td>APL - PML-RARα</td>
<td>nd</td>
<td>&gt; 10,000d</td>
<td></td>
</tr>
<tr>
<td>KG-1</td>
<td>AML – wt-FLT3 (FGF-FGFR loop)</td>
<td>&gt; 100</td>
<td>&gt; 10,000</td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td>CML – Bcr-Abl FLT3 negative</td>
<td>&gt;5,000</td>
<td>&gt; 10,000</td>
<td></td>
</tr>
<tr>
<td>MOLM-13</td>
<td>AML – FLT3-ITD</td>
<td>6</td>
<td>10-100</td>
<td></td>
</tr>
</tbody>
</table>

a – alamarBlue assay determines viability of cells by mitochondrial metabolism of MTT-like dye after 72 hr exposure to compounds.
b – Viability (TB) determined by the trypan blue exclusion assay after 48 hr compound exposure.
c – nd = not determined for this cell line.
d – concentration is the maximum tested.

B. Comparison of ABT-869 to other FLT3 and VEGFR/PDGFR inhibitors.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Proliferation IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABT-869</td>
</tr>
<tr>
<td>MV-4-11a</td>
<td>4</td>
</tr>
<tr>
<td>RS4;11b</td>
<td>2,100</td>
</tr>
<tr>
<td>MOLM-13a</td>
<td>6</td>
</tr>
<tr>
<td>SUP-B15b</td>
<td>5,000</td>
</tr>
<tr>
<td>Kasumi-1c</td>
<td>16</td>
</tr>
</tbody>
</table>

a) AML cell line with FLT3-ITD
b) ALL cell line with wt-FLT3
c) AML cell line with KIT N822K mutation
d) nd – not determined for this cell line
e) sunitinib = SU-11248
f) sorafenib = BAY 43,9006
Figure legends

**Figure 1: ABT-869 inhibits phosphorylation of FLT3, STAT5 and ERK in MV-411 cells.**
MV-4-11 cells were cultured in the presence of varying concentrations of ABT-869 or vehicle control (0.1% DMSO) for 2 hr. Cells were lysed and lysates analyzed by Western blot using (A) anti-phospho-FLT3, (B) phospho-STAT5 and (C) phospho-ERK for MV-4-11 cells and (D) anti-phospho STAT5 and Pim-1 for MOLM-13 cells. For cell culture, β-actin was used as the load control for FLT3 and STAT5, whereas total ERK was used for ERK. The results from at least two independent experiments demonstrate similar inhibition of FLT3, STAT5 and ERK phosphorylation with ABT-869, a representative blot is shown.

**Figure 2: ABT-869 induces apoptosis of MV-411 cells.**
(A) MV-4-11 cells were treated for 72 hr with ABT-869, stained with propidium iodide (50 mg/mL) and analyzed on a FACS Calibur flow cytometer. Sub-G0/G1 population was determined by CellQuest software and designated as apoptotic cells. (B) MV-4-11 cells (1 x 10⁶) were harvested at 48 hr after treatment with various drug concentrations. Cells were stained with anti-Annexin V-FITC antibody and propidium iodide and analyzed by flow cytometry. Increase in apoptosis was observed with increasing concentrations of the ABT-869. (C) Western blot analysis for PARP cleavage and caspase-3 to assess apoptosis. (D) Representative Western blot analysis of caspase 3 and caspase 9 activation and PARP cleavage in MOLM-13 cells treated with ABT-869. The
results from three independent experiments show decrease in Pro-caspase-3 levels and cleavage of PARP with increasing concentrations of ABT-869.

**Figure 3: ABT-869 inhibits phosphorylation of FLT3, STAT5 and ERK in the human blood AML model.**

MV-4-11 cells were spiked into normal human blood, 1 x 10^7 cells/mL, and treated with varying concentrations of ABT-869 for 2 hr. For FLT3 phosphorylation (A), blood was lysed and the FLT3 immunoprecipitated from 1 mL of lysate with anti-FLT3 (sc-480, 10 mg/mL) and analyzed by Western blot. The results from at least three independent experiments demonstrate similar inhibition of phosphorylation of FLT3 by ABT-869, a representative blot is shown. (B) STAT5 and (C) ERK phosphorylation was analyzed from PBMCs prepared from blood treated as described above. Phospho-STAT5 and –ERK were determined directly in cell lysates. (D) Phospho-STAT5 and Pim-1 expression analyzed from PBMCs prepared from blood treated as described above with the exception of being spiked with MOLM-13 cells. The results from two independent experiments demonstrate similar inhibition of the phosphorylation of STAT5 and ERK and decreased Pim-1 expression by ABT-869, a representative blot is shown. (E) Pharmacokinetic analysis of ABT-869 plasma concentration from orally dosed mice with 3 and 10 mg/kg/d in 2.5% ethanol, 5% Tween 80, 25% PEG 400 in PBS, and the 40 mg/kg dose in pure refined corn oil. ABT-869 was analyzed from plasma that had been protein precipitated with acidified methanol and concentration determined using LC-MS as described in the Methods.
Figure 4: Effect of ABT-869 on proliferation and differentiation of normal human bone marrow progenitors and AML patient samples.

(A) Methylcellulose based colony assay was performed with normal human bone marrow cells treated with varying concentrations of ABT-869 and analyzed 14 days after treatment. The experiment is an average of 5 samples with triplicate platings. The inhibitor was not toxic to the bone marrow cells up to a concentration of 1 µM. (B) Methylcellulose based colony assay performed on AML patient samples containing the FLT3-ITD gene. The experiment is an average of two patients with triplicate platings. (C) Methylcellulose based colony assay performed on FLT3-ITD negative AML patient samples. The experiment is an average of 5 samples with triplicate platings. All human samples were obtained through an approved protocol from the UCLA IRB and uphold the tenets of the Helsinki protocol.

Figure 5: ABT-869 exhibits dose dependent efficacy and regression of established subcutaneous MV-4-11 tumors.

(A) Daily oral administration of ABT-869 at concentrations of 5, 10, 20 and 40 mg/kg/day was initiated when the MV-411 tumors reached an average of 400 mm³ volume. The graph shows the dosage response to the inhibitor in comparison to tumors treated with vehicle control (three mice per group). (B) Mice injected with MV-411 cells were treated with 40 mg/kg/day of ABT-869 or vehicle the day after injection of cells. The graph shows that the mice receiving treatment never developed tumors in contrast to the vehicle control (three mice per group). (C) After a single administration of ABT-869 (10 mg/kg), tumors were harvested 3 and 6 hours later, snap frozen in liquid
N₂, homogenized in lysis buffer, immunoprecipitated with anti-PY20 and analyzed by Western blot with anti-FLT3.  (D) H&E sections of tumors from day 12 of dosing treated with vehicle or 40 mg/kg/day of ABT-869 at low (10X) and high (40X) power.  Vehicle control treated tumors show increased mitotic activity (arrows, lower left panel).  Treated tumors show areas of cell death and inflammatory cells (arrows, lower right panel).  (E) Ki67 and TUNEL staining of tumor sections from day 12 of dosing were used to evaluate proliferation and apoptosis, respectively.

Figure 6.  ABT-869 inhibits the growth of and prevents MOLM-13 tumor formation.

(A) Daily oral administration of ABT-869 at concentrations of 5, 10, 20 and 40 mg/kg/day was initiated when the MOLM-13 tumors reached an average of 400 mm³ volume (one mouse per dosage group).  The graph shows the dosage response to the inhibitor in comparison to tumors treated with vehicle control.  (B).  Mice injected with MOLM-13 cells were treated with 40 mg/kg/day of ABT-869 or vehicle the day after injection of cells (two mice per dosage group).  The graph shows that the mice receiving treatment developed tumors after 26 days of treatment in contrast to the vehicle control.  (C) ABT-869 prolongs survival in MV-4-11 bone engraftment model.  NOD-SCID mice were injected with 5 x 10⁶ MV-4-11 cells after bone marrow ablation with cyclophosphamide.  Treatment with ABT-869 began on day 40 after injection and continued for another 20 weeks.  Survival was determined by observation when the animal demonstrated hind limb paralysis or became moribund.
Figure 1

A. p-FLT3

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10</th>
<th>1</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-869 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. p-STAT5

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10</th>
<th>1</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-869 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. p-ERK

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10,000</th>
<th>1,000</th>
<th>100</th>
<th>10</th>
<th>1</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-869 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D. P-STAT5

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100</th>
<th>10</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-869 (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

A. 

B. 

EC₅₀ ~30 nM

Annexin V

0.1% DMSO

0.6%

6.66%

6.68%

8.83%

0.55%

100pM ABT-869

0.7%

10nM ABT-869

1.67%

100nM ABT-869

5.09%

1nM ABT-869

4.19%
Figure 2

C.

Procaspe-3
β-tubulin

0 0.1 1 10 100 1000 ABT-869 (nM)

PARP Full-Length
PARP Cleavage

0 0.1 1 10 100 1000 ABT-869 (nM)
Figure 2

D.

Caspase 3
β-Tubulin
Caspase 9
β-Tubulin
Parp
β-Tubulin

1000 100 10 0 Untreat ABT-869 (nM)
Figure 3

A. ABT-869  -  10  1  0.1  0.01  0.001 μM
   p-FLT3
   FLT3

B. ABT-869  10  1  0.1  0.01  0.001 - μM
   p-STAT5
   β-actin

C. ABT-869  10  1  0.1  0.01  0.001 - μM
   p-ERK
   ERK
Figure 3
Figure 3
Figure 4

A.

Number of colonies vs. Concentration (N=5)

Legend:
- Normal
- 0.1% DMSO
- 10pM
- 100pM
- 1nM
- 10nM
- 100nM
- 1uM
- 10uM
Figure 4
Figure 4
Figure 5
Figure 5

D. Vehicle (40 mg/kg/day)

10X

40X
E. 

<table>
<thead>
<tr>
<th>Ki67</th>
<th>Vehicle</th>
<th>ABT-869 (40 mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5
Figure 6
Figure 6
ABT-869 a multi-targeted receptor tyrosine kinase inhibitor: inhibition of FLT3 phosphorylation and signaling in acute myeloid leukemia