Effects of MLN518, a dual FLT3 and KIT inhibitor, on normal and malignant hematopoiesis

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Internal tandem duplications (ITDs) of the FMS-like tyrosine kinase 3 (FLT3) receptor tyrosine kinase are found in approximately 30% of patients with acute myelogenous leukemia (AML) and are associated with a poor prognosis. FLT3 ITD mutations result in constitutive kinase activation and are thought to be pathogenetically relevant, implicating FLT3 as a plausible therapeutic target. MLN518 (formerly CT53518) is a small molecule inhibitor of the FLT3, KIT, and platelet-derived growth-factor receptor (PDGFR) tyrosine kinases with significant activity in murine models of FLT3 ITD-positive leukemia. Given the importance of FLT3 and KIT for normal hematopoietic progenitor cells, we analyzed the effect of MLN518 on murine hematopoiesis under steady-state conditions, after chemotherapy-induced myelosuppression, and during bone marrow transplantation. In these assays, we show that MLN518 has mild toxicity toward normal hematopoiesis at concentrations that are effective in treating FLT3 ITD-positive leukemia in mice. We also demonstrate that MLN518 preferentially inhibits the growth of blast colonies from FLT3 ITD-positive compared with ITD-negative patients with AML, at concentrations that do not significantly affect colony formation by normal human progenitor cells. In analogy to imatinib mesylate in BCR-ABL–positive acute leukemia, MLN518-induced remissions may not be durable. Our studies provide the basis for integrating this compound into chemotherapy and transplantation protocols.

Introduction

The FMS-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase family that includes KIT, FMS, and the platelet-derived growth factor receptors (PDGFRs). Class III tyrosine kinases consist of 5 immunoglobulin-like extracellular domains, a transmembrane domain, a juxtamembrane domain, an intracellular split kinase domain, and a C-terminal domain.1,2 Mutations of FLT3 occur in 25% to 30% of patients with acute myelogenous leukemia (AML).3,7 The most common mutations are internal tandem duplications (ITDs) in the juxtamembrane domain. These duplications lead to constitutive receptor dimerization and activation. Less common are point mutations in the activation loop of the kinase domain. In both cases, activation of the kinase leads to the stimulation of multiple signaling pathways, including the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3 (P3) kinase pathways.8 Most studies indicate that the presence of FLT3 mutations confers a poor prognosis, with shorter progression-free survival (PFS), overall survival (OS), or both.3,7

Expression of a constitutively activated FLT3 mutant in factor-dependent cell lines such as 32D leads to factor-independent growth.9,10 Transduction of mouse bone marrow stem cells with mutant FLT3 prior to bone marrow transplantation (BMT) causes a fatal myeloproliferative syndrome,11 whereas the transformation to acute myeloid leukemia may require an additional mutation that blocks differentiation. This was recently demonstrated for FLT3 ITD and promyelocyte–retinoic acid receptor α (PML-RARα), which cooperate to induce a disease resembling acute promyelocytic leukemia in mice.12

Given the role of FLT3 in AML, activated FLT3 is a plausible therapeutic target. Several small molecule inhibitors of FLT3 have been developed, among them MLN518 (formerly CT53518). A piperazinyl quinazoline, MLN518 inhibits the autophosphorylation of FLT3, PDGFR, and KIT tyrosine kinases with IC50 (concentration that inhibits 50%) values of approximately 100 to 200 nM in intact cells.13 In proliferation assays of FLT3 ITD-positive cell lines, the IC50 of MLN518 was in the range of 30 nM; significant apoptosis was induced at 1 μM, whereas ITD-negative lines were unaffected.14 In addition, MLN518 was shown to be effective in murine models of FLT3-positive AML.

Targeted disruption of the gene encoding FLT3 or the FLT3 ligand results in hematopoietic defects that affect various lineages, including myeloid progenitors, T and B cells, as well as natural killer (NK) and dendritic cells.15,16 Mice deficient for FLT3 and KIT exhibit a more severe phenotype with large decreases in hematopoietic cell numbers and postnatal lethality.16 Given that...
MLN518 and other FLT3 inhibitors also inhibit KIT and PDGFR,\textsuperscript{17} we hypothesized that exposure of normal hematopoietic progenitors to this compound might result in significant toxicity, particularly if administered simultaneously with chemotherapy or during stem cell transplantation.

Stimulated by the success of imatinib mesylate for the treatment of chronic myeloid leukemia (CML), several FLT3 inhibitors, including MLN518, are being tested in clinical trials. From the experience with imatinib mesylate in blast crisis CML,\textsuperscript{18} it may be expected that FLT3 inhibitors would have only limited antileukemic activity when used as single agents.\textsuperscript{19} Thus, to exploit their full potential, it may be necessary to integrate compounds like MLN518 into conventional chemotherapy regimens, stem cell transplantation, or both. To evaluate the effects of MLN518 on normal hematopoietic progenitor cells, we monitored the peripheral blood white cell count and white cell differential count in Balb/c mice treated with therapeutically relevant doses of MLN518 in 3 settings: under steady-state conditions, after cytotoxic chemotherapy, and during BMT from syngeneic donor mice. We also studied hematopoietic colony formation by normal human progenitor cells in the presence of graded concentrations of MLN518 and tested the activity of this compound in blast colony assays from patients with AML with and without FLT3 ITD.

**Materials and methods**

**FLT3 Inhibitor**

MLN518 was kindly provided by Dr Jin-Chen Yu (Millennium Pharmaceuticals, San Francisco, CA). For the animal studies, MLN518 was dissolved in 0.5% methylcellulose (MC) before use. For the in vitro studies, a stock solution in dimethyl sulfoxide (DMSO) was prepared and diluted appropriately before each experiment.

**Genotyping of AML samples**

Mutational analysis of FLT3, KIT, and RAS was performed in all AML samples by denaturing high-power liquid chromatography, as described.\textsuperscript{20-22} Mutations were confirmed by sequencing.

**Colony-forming assays of normal human hematopoietic progenitors**

Bone marrow mononuclear cells (5 × 10\textsuperscript{5}) obtained either commercially (n = 3) (AliCells, San Diego, CA) or from a volunteer donor (n = 1) were plated in methylcellulose media containing 50 ng/mL recombinant human (rh) stem cell factor, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), and 10 ng/mL rh interleukin 3 (IL-3) (MethoCult GF H4534; StemCell, Vancouver, BC) for culture of granulocyte-macrophage colony-forming units (CFU-GMs) or additionally 3 U/mL rh erythropoietin (MethoCult GF H4534; StemCell) for culture of erythocyte burst forming units (BFU-Es). MLN518 was dissolved in DMSO and Iscoves modified Dulbecco media (IMDM; Gibco BRL, Grand Island, NY). The concentration of DMSO at the highest drug concentration was 0.02%. At this concentration, DMSO had no effect on colony growth as compared with controls with 0.01% DMSO. CFU-GM and BFU-E colonies were counted after 14 days. Only clusters with more than 50 cells were counted. Results were calculated as the percentage of growth versus control.

**AML blast colony assays**

AML blast colony formation was assayed as described by Buick et al\textsuperscript{23} and Minden et al,\textsuperscript{24} with minor modifications. Cryopreserved or freshly obtained mononuclear cells from patients with AML were incubated in plastic tissue-culture dishes with 2% fetal bovine serum containing IMDM for 1 hour (37°C, 5% CO\textsubscript{2}). Nonadherent cells were depleted of T cells by immunomagnetic separation using a CD3\textsuperscript{+} selection cocktail (StemCell). Finally, 2 × 10\textsuperscript{5} nonadherent, T-cell-depleted cells were plated in 1 mL methylcellulose media containing 50 ng/mL rh stem cell factor, 10 ng/mL GM-CSF, and 10 ng/mL rh IL-3 (MethoCult GF H4534; StemCell). Blast colony formation was scored after 7 to 14 days of culture at 37°C, 5% CO\textsubscript{2}. MLN518 DMSO dilutions were performed as described in “Colony-forming assays of normal human hematopoietic progenitors.” For assays with imatinib mesylate, the drug was treated exactly as MLN518. A blast colony was defined as a cluster of 10 or more cells. Results were calculated as colony survival, with the control defined as 100%. Approval for this study was obtained from the Institutional Review Board (IRB) at Oregon Health and Science University. Informed consent was obtained in accordance with the Declaration of Helsinki.

**Animal studies**

Balb/c mice were purchased from Charles River Laboratories, Wilmington, MA, and were kept under standard conditions at the Oregon Health and Science University animal care facility. To establish an effective dose to achieve clinically relevant trough plasma drug concentrations 4 mice each were treated with a single dose of MLN518 at 60, 120, 180, and 300 mg/kg. Plasma was collected 12 hours after dosing for pharmacokinetic analysis. For assessment of MLN518 effects on steady-state hematopoiesis, 14 mice were treated with MLN518 (180 mg/kg once daily) or carrier (methylcellulose) by oral gavage for 21 days. For the determination of MLN518 trough plasma concentrations 4 mice were killed on day 1, 3, mice on day 7 with the remaining 6 mice killed on day 21.

For assessment of MLN518 effects on recovery from chemotherapy-induced myelosuppression mice were treated with MLN518 (180 mg/kg twice daily; n = 14) or carrier only (n = 8) for 21 days. On day 3, 200 mg/kg cyclophosphamide (Bristol-Myers Squibb, Princeton, NJ) was injected intraperitoneally. In pilot experiments, this dose of cyclophosphamide (200 mg/kg) had been shown to lead to a 90% ± 5.5% reduction of peripheral blood white cells 3 days after injection. For the determination of MLN518 trough plasma concentrations 4 mice were killed on days 1 and 7 with the remaining 5 mice killed on day 21.

For the determination of white blood cell (WBC) counts and 3-part differential, 10 μL peripheral blood was drawn and added to an equal volume of 10 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Samples were analyzed with a VET ABC blood analyzer (VET Anemometer, Fort Collins, CO).

Mice were killed at 12 hours after treatment to determine the trough drug plasma concentrations of MLN518. Pharmacokinetic (PK) analysis was carried out as previously described.\textsuperscript{14}

**Stem cell transplantation**

Eight male donor Balb/c-mice (8 weeks old) were administered 3 mg 5-fluorouracil (5-FU; ICN, Humacao, Puerto Rico) by tail vein injection 5 days prior to bone marrow harvest. Bone marrow cells were flushed from murine tibiae and femurs by using a 25-gauge needle into 0.073% NH\textsubscript{4}Cl buffer to lyse red blood cells. Two groups of female recipient Balb/c mice (aged 12-16 weeks) were lethally irradiated (90 Gy, divided into 2 doses 4 hours apart). Following irradiation, recipient mice were injected with 5 × 10\textsuperscript{6} bone marrow cells harvested from donor mice by retroorbital injection. MLN518 (180 mg/kg twice daily) or carrier only was administered by oral gavage from day −3 prior to transplantation to day 28. Bone marrow engraftment was monitored by analyzing peripheral blood as described earlier. For the determination of MLN518 trough plasma concentrations 4 mice each were killed on days 1 and 7 with the remaining 6 mice killed on day 28. On day 28, bone marrow was harvested from 6 recipient mice that received transplants in the presence and 6 mice in the absence of MLN518, using the same procedure as described earlier. Approximately 5 × 10\textsuperscript{6} cells were harvested per mouse. Bone marrow from individual mice was divided for use in colony-forming assays and fluorescence-activated cell sorting (FACS) analysis, with the remaining bone marrow pooled for transplantation into secondary recipients. Cells (1 × 10\textsuperscript{7}) for each of the 6 donor mice from each group were analyzed by FACS for expression of B-cell (B220), T-cell (Thy1.2), myeloid (Gr-1),
early myeloid (Mac-1), and erythroid (Ter-119) markers. All antibodies were purchased from BD Bioscience (San Jose, CA). Cells (5 \times 10^3) were plated in duplicate in methylcellulose media (MethoCult GF M3231; StemCell) supplemented with 50 ng/mL recombinant murine (rm) stem cell factor (StemCell), 10 ng/mL rm IL-6 (StemCell), and 10 ng/mL rm IL-3 (StemCell). CFU-GM colonies were counted after 7 and 14 days. Only clusters with more than 50 cells were counted. The remaining cells from each group were pooled and transplanted into lethally irradiated secondary recipients, following the same procedures as described earlier. The WBC counts of these mice were followed at 4- to 5-day intervals as described in “Animal studies.”

Cell cycle analysis

The effect of MLN518 on the cell cycle profile of hematopoietic progenitors was assessed by using bone marrow from mice treated for 3 days with MLN518 (n = 3) or carrier (n = 3). Bone marrow was harvested as described in “Stem cell transplantation.” Samples were enriched for hematopoietic progenitors by using StemSep murine progenitor negative selection cocktail (StemCell). Cells were washed with PBS and fixed with 50% ethanol in buffered saline. Cells were again washed in PBS, RNase was added at 0.05 mg/mL, and the samples were incubated for 30 minutes at 37°C. Cells were stained with propidium iodide (PI) at 4°C and analyzed with a Guava PCA (Guava Technologies, Hayward CA) to access DNA content. Data were fit to ascertain the percentage of cells in G1/G0, S, and G2/M phases (ModFit; Verify Software House, Topsham, ME).

Statistical analysis

Categorical variables were compared by 2-sided Fisher exact test. Noncategorical variables were compared by 2-tailed Student test. All calculations were done with the SPSS 11.0 software package (SPSS, Chicago, IL).

Results

Toxicity studies in mice

MLN518 has minimal effects on steady-state hematopoiesis. Our initial experiments evaluated the effect of therapeutically relevant doses of MLN518 on the peripheral blood white cell count under steady-state conditions. In a previously reported Balb/c mouse BMT model of FLT3 ITD-positive leukemia, a trough plasma concentration of 266 nM MLN518, near the IC90 for inhibition of proliferation of FLT3 in ITD-positive cell lines, led to prolonged survival of MLN518-treated mice.14 In our studies administration of 180 mg/kg twice daily was required to achieve these trough concentrations (Figure 1A). Balb/c mice (n = 14) were treated twice daily with 180 mg/kg MLN518 by oral gavage over 21 days. Four mice were killed on day 1, 3 mice on day 7, and the remaining 6 mice on day 21 for PK analysis. Average plasma drug concentrations were 229 nM on day 1, 389 nM on day 7, and 325 nM on day 21 (Figure 1B). The rise in plasma drug concentrations between days 1 and 7 suggests a slight buildup in trough drug concentrations that appeared to reach steady state by day 7. In all cases the trough plasma concentration was near the IC90 reported for inhibition of phosphorylation of FLT3 in phase 1 clinical trials.25 Similar plasma concentrations were observed in all of the animal studies (Figure 1C-D). We found that the white blood counts varied between the treatment group and the control group (n = 8) treated with carrier only, oscillating around 8 \times 10^6/L for both groups. When compared with the initial WBC counts, these differences were not significant with the exception of day 7 (P < .05) (Figure 2). One mouse in the treatment group died on day 4 immediately following gavage. Otherwise, the mice appeared healthy in both groups, and body weight was unchanged.

Effects of MLN518 on recovery from chemotherapy-induced myelosuppression. In patients with AML, it may be desirable to administer MLN518 in conjunction with conventional cytotoxic chemotherapy. Given the importance of FLT3 and KIT in early hematopoiesis, we hypothesized that the inhibition of these 2 kinases might inhibit the recovery from chemotherapy-induced myelosuppression. In a first series of experiments, Balb/c mice were treated with increasing doses of intraperitoneal cytarabine. Doses of up to 5.4 g/kg failed to induce a reduction of WBC counts by more than 75%. However, at this dose there was 40% mortality (data not shown). In contrast, cytarabine at 200 mg/kg consistently reduced the white cell count by an average of 90% \pm 5.5%, 3 days after intraperitoneal injection without associated mortality. To assess the effect of MLN518 on the recovery from cytarabine-induced myelosuppression, Balb/c mice were treated twice daily with 180 mg/kg MLN518 (n = 14) or carrier (n = 8) by oral gavage, starting 3 days before and continuing for 18 days after chemotherapy. Four mice were killed on days 1 and 7 with the remaining 5 mice killed on day 21 for PK analysis. Plasma drug concentrations were 247 nM on day 1, 269 nM on day 7, and 353 nM on day 21. In both groups WBC counts fell from a baseline of 10 \times 10^9/L to a nadir of less than 1.1 \times 10^9/L on day 7. This was followed by a rapid recovery to 7.2 \times 10^9/L in the MLN518 group and 4.4 \times 10^9/L in the control group (P = .008) on day 11 (Figure 3A). The control group WBC count returned to 10 \times 10^9/L by day 17, whereas the treatment group returned to a slightly lower baseline of 8 \times 10^9/L. Examination of the 3-part differential revealed that in the MLN518 treatment group the number of granulocytes and monocytes had returned to normal by day 11 with the number of lymphocytes lagging behind (Figure 3B). All mice lost approximately 10% of body weight, and 1 mouse (1 of 14) in the MLN518 group died of gavage trauma.

Effects of MLN518 on the cell cycle profile of normal mouse hematopoietic progenitors. The lack of toxicity of MLN518 on hematopoietic recovery from cytarabine-induced myelosuppression raised the question whether the progenitor cells may be protected from toxicity because of MLN518-induced cell cycle arrest. To test this hypothesis, cell cycle analysis was performed on...
bone marrow enriched for hematopoietic progenitors. Bone marrow was harvested from mice treated with MLN518 (n = 3) or carrier (n = 3) for 3 days. Samples enriched for early progenitors were assessed for DNA content and fit to a model containing G0/G1, S, and G2/M phases. The population distribution of cells harvested from the MLN518-treated group was not significantly different from the control group (Table 1).

**MLN518 administered during stem cell transplantation does not compromise engraftment.** To assess the effects of MLN518 on stem cell engraftment, lethally irradiated mice were administered 180 mg/kg MLN518 (n = 14) twice daily by oral gavage or vehicle control (n = 8) beginning 3 days prior to and for 28 days following BMT. Animals in both groups lost approximately 10% of body weight. Plasma drug concentrations were 138 nM on day 0, 455 nM on day 7, and 408 nM on day 21. After irradiation the WBC count dropped from a baseline of 10 × 10^9/L to a nadir of less than 1 × 10^9/L on day 4 (Figure 4A). Repopulation of both treatment and control groups established a new baseline at 7 × 10^9/L by day 17. No difference in WBC counts between groups was observed until day 28, when the counts in the MLN518 treatment group were slightly lower (P = .015). The 3-part differential showed no appreciable difference in the numbers of lymphocytes, monocytes, or granulocytes in the peripheral blood throughout the experiment (P > .05) (Figure 4B).

One mouse in the treatment group (no. 356) began to accumulate fluid in the abdomen at approximately day 21. Concomitant with the buildup of fluid was a decline in the number of platelets and lymphocytes in the peripheral blood. Necropsy revealed only clear fluid in the abdomen with no other sign of disease. This mouse was excluded from further analysis.

Given that mice with combined deletions of the FLT3 receptor gene and the KIT gene have severe hematopoietic defects, we studied the expression of lineage-specific markers on bone marrow cells of mice that received transplants in the presence and absence of MLN518. Bone marrow cells from animals that received transplants in the presence of MLN518 had no significant difference in the expression of lineage-specific markers (Table 2). We also assessed their capacity to reconstitute secondary recipients and ability to form CFU-GM colonies in semisolid culture. Cells (5 × 10^6) from each mouse in both the MLN518 treatment group (n = 6) and the control group (n = 6) were seeded in cytokine-supplemented semisolid culture medium. The number of colonies counted on day 7 was not statistically different between treatment and control (P = 0.15) with the number of colonies being 70.0 ± 15.4 and 57.0 ± 10.3 in the treatment and control group, respectively. Similar results were seen on day 14 with the number of colonies being 80.9 ± 12.4 and 73.5 ± 9.1 for treatment and control, respectively (P = .3). Pooled cells from each group were used for transplantation into lethally irradiated secondary recipients (8 per group). One mouse in the MLN518 group suffered a prolapsed colon on day 1 after BMT and had to be killed. In the remaining mice there was no difference in the time to WBC recovery. Compared with primary recipients there was no prolonged leukopenia observed regardless of prior exposure to MLN518. The WBC count returned to normal ranges 4 weeks after BMT (Figure 4C). The surviving animals appear normal, without signs of disease.

**Effects of MLN518 on normal human hematopoietic progenitor cells**

Having established that MLN518 does not exhibit serious toxicity toward murine bone marrow, we assessed its effect on colony formation by mononuclear cells (MNCs) from healthy human bone marrow donors. MNCs were seeded in cytokine-supplemented semisolid culture medium in the presence of graded concentrations of MLN518. The compound inhibited the growth of BFU-E and CFU-GM with IC_{50} values of 500 and 800 nM, respectively (Figure 3A).
5). These values are much higher than the IC_{50} in proliferation assays of FLT3 ITD-positive cell lines, which have been reported in the range of 30 nM.\textsuperscript{14}

**MLN518 preferentially inhibits human AML cells harboring FLT3-ITD**

To determine whether a differential effect would be observed on AML blasts with or without FLT3 ITD, nonadherent and T-cell–depleted AML cells were cultured in the continuous presence of graded concentrations of MLN518. The FLT3 genotype was determined prior to the start of the experiments (Table 3). Figure 6 shows colony survival in 4 FLT3 ITD-positive and 4 ITD-negative samples. The 4 samples harboring FLT3-ITD showed IC_{50} values between 75 and 400 nM, below the value for normal BFU-E and CFU-GM. Two of the ITD-negative samples were completely resistant to MLN518. The remaining 2 samples showed IC_{50} values of 200 and 425 nM, respectively (Figure 6). Because some FLT3 ITD-negative samples were inhibited by MLN518 raised the question whether MLN518 effects in the primary cells might be mediated by inhibition of KIT, which is mutated in some patients with AML.\textsuperscript{26} However, mutational analysis of KIT by denaturing high-performance liquid chromatography (D-HPLC) was negative. To test whether the effects of MLN518 might be mediated by the inhibition of signaling from wild-type KIT or PDGFR, we assessed the sensitivity of FLT3 ITD-positive and -negative AML samples to imatinib mesylate, an inhibitor of KIT and PDGFR. Because of limited material availability, only 6 samples could be tested. ITD-positive samples 1, 2, and 4 and ITD-negative samples 5, 7, and 8 were cultured in the continuous presence of graded concentrations of imatinib mesylate. The ITD-positive samples had a large variation in IC_{50}, between 0.85 and more than 10 \mu M. This was in line with the ITD-negative samples, which ranged between 0.85 and 3.5 \mu M (Table 4). There was no apparent correlation between the sensitivity to imatinib mesylate and the sensitivity to MLN518, suggesting that the effects of MLN518 are due to the inhibition of FLT3. Altogether, our findings suggest that MLN518 preferentially but not exclusively inhibits AML blasts that express FLT3 ITD.

### Discussion

Seventy percent to 80% of patients with AML achieve complete remission with induction chemotherapy. However, with the exception of cases with favorable cytogenetics, most patients eventually relapse and die of their disease. Allogeneic transplantation, if feasible, reduces the risk of relapse but is associated with a 20% to 25% rate of transplant-related mortality that largely offsets this benefit.\textsuperscript{23} Approximately 30% of patients with AML harbor activating mutations of the FLT3 receptor tyrosine kinase,\textsuperscript{3-7} raising hopes that targeting this kinase with specific small molecule inhibitors may improve the outcome for this subgroup of patients.

MLN518 has potent activity against FLT3 but also against KIT and PDGFR. Both FLT3 and KIT play important roles in early hematopoiesis. In conjunction with other cytokines, FLT3 ligand stimulates the expansion of CD34\textsuperscript{+} hematopoietic cells\textsuperscript{27} and primitive B-cell progenitors.\textsuperscript{28} Mice with targeted disruption of the FLT3 gene are healthy with normal mature hematopoiesis, but they exhibit defects in early B cells and a reduced capacity to reconstitute T cells and myeloid cells after stem cell transplantation.\textsuperscript{16} Given that KIT has similar functions in early hematopoiesis,

#### Table 2. Percentage of cells expressing specific lineage-specific markers in control and MLN518-treated bone marrow

<table>
<thead>
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<th>Marker</th>
<th>MLN518</th>
<th>Control</th>
<th>(P)</th>
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<tr>
<td>Ly6C/G</td>
<td>44.6 ± 6.0</td>
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<td>CD11b</td>
<td>43.1 ± 6.2</td>
<td>41.2 ± 5.5</td>
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<td>Ter119</td>
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<td>B220</td>
<td>27.3 ± 1.4</td>
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<td>Thy1.2</td>
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</table>

Data are expressed as mean ± SD.

**Figure 5. Effect of MLN518 on hematopoietic colony formation by mononuclear cells from healthy individuals.** MNCs were plated in methylcellulose in the presence of cytokines and graded concentration of MLN518, and colonies were counted after 14 days. Results are plotted as a function of MLN518 concentration versus the percentage of inhibition of colony formation. Data points represent means of 4 bone marrow samples ± standard deviation. Assays were performed in duplicate.
it is not surprising that the combined inactivation of FLT3 and KIT in mice results in severe hematopoietic defects and lethality.\(^{16}\) One obvious concern about a combined FLT3/KIT inhibitor is that it might severely compromise normal hematopoiesis. Under steady-state conditions we found no significant decrease in the WBC counts of mice treated with therapeutically active concentrations of MLN518. Although we cannot exclude that more severe toxicity might be induced by prolonged treatment with MLN518, these findings suggest that toxicity to steady-state hematopoiesis is minimal at the doses used in our study. It is possible that in contrast to knock-out mice, the activity of the 2 kinases is not completely suppressed in hematopoietic progenitors with the MLN518 concentrations achieved in our experiments, with median trough concentrations of 350 nM. Because the IC\(_{50}\) in cellular assays of FLT3 and KIT phosphorylation is 220 and 170 nM, respectively, residual kinase activity is likely present in the mice treated with the schedule of MLN518 used in this study.\(^{14}\) Thus, it is possible that more severe suppression of normal hematopoiesis would be seen at higher doses. However, the trough concentration of 350 nM is similar to that observed in a phase 1 trial of MLN518.

By analogy to the results of imatinib mesylate on CML in blast crisis,\(^{29}\) MLN518-induced remissions are unlikely to be durable and there may be primary resistance. Thus, the compound may be administered together with or after conventional cytotoxic agents. This raises the issue of whether MLN518 might adversely affect the recovery from chemotherapy-induced myelosuppression. Because cytarabine, even at very high doses, failed to induce significant leukopenia in Balb/c mice and resulted in high mortality, we chose cyclophosphamide-induced leukopenia as our model. Both the MLN518 treatment and the control group initially had robust recoveries in the WBC count. This finding suggests that MLN518 could be safely added to chemotherapy for patients with AML, but this addition to chemotherapy will need to be confirmed in clinical trials.

Engraftment was not delayed in mice treated with MLN518 during the course of BMT. On the basis of the analysis of lineage-specific cell surface markers, MLN518 did not affect the composition of the recipient bone marrow after recovery of counts. Nor was there any effect on the ability to form colonies in semisolid media. Of clinical importance, these mice appeared healthy, and the reconstitution of secondary recipients was not different from controls.

In line with the results in mice, MLN518 had only moderate toxicity in colony-forming assays of human MNCs. At 350 nM, a dose comparable to the steady-state trough concentrations in mice, colony formation was reduced by approximately 30%, and the IC\(_{50}\) values for inhibition of CFU-GM and BFU-E were 800 and 500 nM, respectively. These values are high compared with the IC\(_{50}\) of 30 nM that was reported for FLT3 ITD-positive cell lines\(^{14}\) and suggest a large therapeutic window. Because our culture medium contains stem cell factor (SCF), it is possible that the inhibition of colony formation is mediated by suppression of KIT signaling\(^{14}\) and might be more pronounced if FLT3 ligand was also present.

Four of 4 samples that were negative for FLT3 ITDs were considerably more sensitive to the compound than normal CFU-GMs and BFU-Es. Somewhat surprisingly, the IC\(_{50}\) of most samples was considerably higher than the reported values for FLT3 ITD-positive samples. Similar observations were made with CEP-701, another FLT3 inhibitor.\(^{10}\) Possible explanations include expression of FLT3 by the leukemic blasts that simulates growth by an autocrine loop\(^{31}\) and autoactivation as a result of overexpression. The latter has been observed in SEMK2-M1 cells, a cell line that expresses high levels of FLT3 as a result of gene amplification.\(^{32}\) As a consequence, only a fraction of blasts would be affected by MLN518. Alternatively, drug efflux in AML cells may play a role. We also found strong inhibition of blast colony formation in 2 of 4 ITD-negative AML samples. Similar observations were made with CEP-701, another FLT3 inhibitor.\(^{10}\) Possible explanations include expression of FLT3 by the leukemic blasts that simulates growth by an autocrine loop\(^{31}\) and autoactivation as a result of overexpression. The latter has been observed in SEMK2-M1 cells, a cell line that expresses high levels of FLT3 as a result of gene amplification.\(^{32}\) Alternatively, MLN518 may inhibit growth stimulation by SCF that is present in the culture medium, or the compound may affect another yet unknown target that is essential for the proliferation of the leukemic blasts. To eliminate the possibility of inhibition of SCF-stimulated KIT signaling being responsible for the inhibition of proliferation, 3 of the 4 ITD-positive samples and 3 of the 4 ITD-negative samples were treated with imatinib mesylate. The

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**Table 3. Morphologic (FAB) and molecular classification of samples from patients with AML.**

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<tr>
<th>Patient ID</th>
<th>FAB</th>
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<td>WT</td>
<td>N-Ras G12D</td>
</tr>
<tr>
<td>8</td>
<td>M5</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

FAB indicates French-American-British; ITD, internal tandem duplication; WT, wild-type.

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**Table 4. IC\(_{50}\) values for MLN518 and imatinib mesylate on samples from patients with AML and normal bone marrow.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>AML ITD-positive</th>
<th>AML ITD-negative</th>
<th>Normal bone marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLN518, nM</td>
<td>Imatinib mesylate, nM</td>
<td>MLN518, nM</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>850</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>950</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>10,000</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>200</td>
<td>850</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>10,000</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>425</td>
<td>3400</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>10,000</td>
<td>2500</td>
</tr>
<tr>
<td>CFU-GM</td>
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<td>800</td>
</tr>
<tr>
<td>BFU-E</td>
<td>—</td>
<td>—</td>
<td>500</td>
</tr>
</tbody>
</table>

— indicates not applicable.

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**Figure 6. Inhibitory activity of MLN518 on colony formation by AML blasts.** AML cells expressing (A) FLT3 ITD or (B) wild-type FLT3 were plated in methylcellulose in the presence of cytokines, and graded concentration of MLN518 and colonies were counted after 7 to 14 days. Results are plotted as a function of MLN518 concentration versus the percentage of inhibition of colony formation. Data points represent means of individual patient samples. Assays were performed in duplicate.
effect of imatinib mesylate was similar in both the ITD-positive and ITD-negative samples, suggesting that inhibition of KIT signaling was not responsible for the sensitivity to MLN518 seen in the ITD-negative samples. Whatever the precise mechanism, our observations suggest that MLN518 may also induce responses in patients with AML without FLT3 ITD.

MLN518 is currently being tested in a phase 1 clinical trial of patients with refractory AML. In those patients, assessment of toxicity toward normal hematopoiesis is difficult because of the advanced stage of disease. In this study, we show that MLN518 has only moderate effects on normal hematopoiesis, alone and in combination with chemotherapy, opening the possibility of integrating the drug into conventional AML induction or consolidation protocols. Given that higher doses of MLN518 and more intensive chemotherapy may be used in such studies, careful monitoring of toxicity will nonetheless be required.

References

22.Appending BNF1852
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Effects of MLN518, a dual FLT3 and KIT inhibitor, on normal and malignant hematopoiesis

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