Uniform sensitivity of FLT3 activation loop mutants to the tyrosine kinase inhibitor midostaurin

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Small molecule inhibitors that target fms-like tyrosine kinase 3 (FLT3)–activating mutations have potential in the treatment of leukemias. However, certain mutations can simultaneously activate the tyrosine kinase, and confer resistance to small molecule inhibitors. We therefore tested the sensitivity of 8 FLT3 activation loop mutants to midostaurin. Each mutant conferred IL-3 factor–independent proliferation to Ba/F3 cells, and each resulted in the constitutive activation of FLT3 and its targets, signal transducer and activator of transcription 5 (STAT5) and extracellular stimuli-responsive kinase (ERK). For each mutant tested, midostaurin inhibited cell growth and phosphorylation of FLT3, STAT5, and ERK. In contrast, midostaurin did not inhibit Ba/F3 cells stably transduced with FLT3-internal tandem duplications containing a G697R mutation that confers resistance to midostaurin, demonstrating that midostaurin inhibition of FLT3 activation loop mutants was not due to off-target effects. We conclude that midostaurin is a potent inhibitor of a spectrum of FLT3 activation loop mutations, and that acute myeloid leukemia patients with such mutations are potential candidates for clinical trials involving midostaurin. (Blood. 2007;110:4476-4479)

Introduction

Fms-like tyrosine kinase 3 (FLT3), a cell surface receptor tyrosine kinase, is among the most commonly mutated genes in acute myeloid leukemia (AML).1 Stimulation of FLT3 activates signal transduction pathways such as signal transducer and activator of transcription 5 (STAT5), RAS/mitogen-activated protein kinase (RAS/MAPK), phosphoinositide 3-kinase (PI3K), src homologous and collagen gene (SHC), SH2-containing inositol-5-phosphatase (SHIP), and cytoplasmic tyrosine phosphatase with 2 Src-homology 2 (SH2) domains (SHP2), which play important roles in cellular proliferation, differentiation, and survival.2,3

There are 2 types of activating mutations in FLT3 described in patients with leukemia. These include a spectrum of internal tandem duplications (ITD) occurring within the auto-inhibitory juxtamembrane domain,4,4 and activation loop mutations that include Asp835Tyr (D835Y), Asp835Val (D835V), Asp835His (D835H), Asp835Glu (D835E), Asp835Ala (D835A), Asp835Asn (D835N), Asp835 deletion and Ile836 deletion.5-10 These activating mutations result in constitutive phosphorylation and activation of FLT3, and subsequent activation of downstream targets.10,11

The importance of FLT3 mutations in the pathogenesis of leukemias has been well established and, in most studies, these have been shown to confer a poor prognosis, with decreased survival.12-14 Therefore, attention has been focused on developing small molecule inhibitors that target FLT3. Midostaurin (formerly known as PKC412) is a selective inhibitor of FLT3, as well as vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), c-kit receptor tyrosine kinase (KIT), and fibroblast growth receptor 1 (FGFR-1).15-17

In vitro, midostaurin induces apoptosis in Ba/F3 cells that have been transformed to IL-3-independent growth by FLT3-ITD.18 Furthermore, midostaurin is effective in FLT3-ITD-induced disease in a murine bone marrow transplantation model.18

Midostaurin has been investigated in phase 1 and phase 2 studies, and is generally well tolerated. In a phase 2 study in patients with relapsed or refractory AML, single-agent midostaurin resulted in significant reductions in peripheral blood and bone marrow blasts, with a 35% response rate.19 Midostaurin also showed activity in a phase 1 trial in combination with daunorubicin and cytarabine in newly diagnosed AML, in particular those patients with FLT3-ITD mutations.20 Based on these promising findings, a randomized phase 3 Cancer and Leukemia Group B (CALGB)/Southwest Oncology Group (SWOG)/Eastern Cooperative Oncology Group (ECOG) Intergroup trial is planned to assess efficacy of induction chemotherapy with midostaurin in patients with mutant FLT3.

Studies have analyzed the sensitivity of FLT3-activating mutations to small molecule inhibitors, and have demonstrated that certain activation loop mutations simultaneously activate the kinase and confer resistance to small molecule inhibitors.21 Therefore, defining the precise spectrum of sensitivity of the range of FLT3 activation loop mutations to specific kinase inhibitors may be critical to determine patient eligibility for treatment, and evaluation of response to therapy. Here we report that midostaurin is a potent inhibitor of 8 activation loop mutations tested.
Figure 1. FLT3 activation loop mutants and the effects of midostaurin. (A) FLT3 activation loop mutations result in constitutive activation of FLT3, STAT5, and extracellular stimuli-responsive kinase (ERK). Ba/F3 cells expressing each of the FLT3 mutations were grown in the absence of IL-3. Cell lysates were prepared and the proteins resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was then performed using the indicated antibodies. (B) Midostaurin inhibits proliferation of Ba/F3 cells expressing FLT3-ITD and activation loop mutations. Ba/F3 cells expressing FLT3-ITD or FLT3 activation loop mutations were incubated with increasing concentrations of midostaurin for 48 hours. The number of viable cells was then determined using a colorimetric assay. Results are expressed as a percentage of viable cells after 48 hours of growth (± SD) in the presence of dimethyl sulfoxide (DMSO) without the drug. (C) Restoration of cellular proliferation by IL-3. The experiment was repeated in the presence of IL-3. Results are again expressed as a percentage of viable cells after 48 hours of growth (± SD) in the presence of DMSO without the drug. (D) Inhibition of FLT3 autophosphorylation by midostaurin. Ba/F3 cells expressing FLT3-ITD or FLT3 activation loop mutations were incubated with increasing concentrations of midostaurin for 20 minutes. Cell lysates were prepared, resolved by SDS-PAGE, then immunoblotted with either anti-phospho-FLT3 antibody or anti-FLT3 antibody, as indicated. (E) Inhibition of STAT5 phosphorylation by midostaurin. Whole cell lysates were prepared and resolved by SDS-PAGE as before, and immunoblotted with anti-phospho-STAT5 or anti-STAT5 antibodies, as indicated. (F) Inhibition of ERK phosphorylation by midostaurin. As before, cell lysates were prepared, resolved by SDS-PAGE, and then probed with anti-phospho–ERK or anti-ERK antibodies, as indicated.
Materials and methods

DNA constructs and retroviral transduction

DNA constructs of FLT3-ITD, and FLT3 with D835A, D835E, D835H, D835N, D835V, D835 deletion, I836 deletion, and D835Y point mutations were created and cloned into the murine stem cell virus (MSCV)-neo vector as previously described.14,21 IL-3-dependent murine hematopoietic Ba/F3 cells were transduced as previously described.21,22 In addition, a DNA construct consisting of a FLT3 G697R kinase domain point mutation was cloned into the MSCV-FLT3-ITD vector and transduced into Ba/F3 cells.23 Transduced cells were grown in absence of IL-3 to confirm factor independence.

Midostaurin dosing

Midostaurin (Novartis Pharma, Basel, Switzerland) was prepared in a 10mM stock solution in DMSO and stored at −10°C. Serial dilutions were made in DMSO to obtain the final concentrations used for immunoblotting and cell growth assays.

Ba/F3 cell growth assays and dose-response curves

Each cell line (1.5 × 10⁵ cells/mL) was grown with varying concentrations of midostaurin in DMSO for 48 hours. The number of viable cells was then determined by a colorimetric assay. Results are expressed as a percentage of viable cells after 48 hours of growth (± SD) in the presence of DMSO without the drug. The experiment was repeated in the presence of IL-3. (B) Midostaurin does not inhibit phosphorylation of FLT3, STAT5, or ERK in Ba/F3 ITD cells with the G697R mutation. Whole cell lysates were prepared from cells incubated with various concentrations of midostaurin for 20 minutes, resolved by SDS-PAGE as before, and immunoblotted with the antibodies indicated.

Results and discussion

We confirmed that each of the activation loop mutations resulted in constitutive activation of FLT3 and its downstream effectors STAT5 and ERK. Figure 1A demonstrates that FLT3 was constitutively phosphorylated in all mutants. In addition, blotting with phospho-specific antibodies for STAT5 and ERK also demonstrated constitutive activation of these targets of activated FLT3.

Each of the transduced Ba/F3 cell lines was then tested for sensitivity to midostaurin. Ba/F3 cells expressing each of the activation loop mutations as well as FLT3-ITD were incubated with varying concentrations of midostaurin. At 48 hours, viable cells were quantitated using a colorimetric assay. Inhibition of growth was abrogated by the addition of IL-3, supporting that inhibition by midostaurin is specific to the FLT3 pathway (Figure 1C). The cellular concentration that inhibited response by 50% (IC50) was less than 10 nM for all of the mutants tested, with a range of 1 nM to 10 nM (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article), demonstrating that midostaurin resulted in a decrease in cellular proliferation of all of the Ba/F3 constructs at relatively low concentrations. Pharmacokinetic studies in phase 1 and 2 trials of midostaurin have shown that these are values that should be readily achieved in patients.25

To assess the effect of midostaurin on FLT3 phosphorylation, lysates were prepared from cells incubated with various concentrations of midostaurin, followed by immunoblotting with...
anti-phospho-FLT3 antibody. There was inhibition of FLT3 phosphorylation in all of the activation loop mutants at relatively low concentrations of midostaurin (Figure 1D), with an IC₅₀ for inhibition of phosphorylation comparable to that observed for inhibition of cell growth. We also observed inhibition of phosphorylation of STAT5 and ERK, at comparably low concentrations of midostaurin (Figures 1E,F).

Thus, all of the activation loop mutants tested were sensitive to inhibition with midostaurin. However, midostaurin is known to inhibit kinases other than FLT3, and thus it was possible that the observed results, although consistent with FLT3 as a target of midostaurin, could be explained by simultaneous inhibition of other kinases essential for cell growth. To demonstrate that the effect of midostaurin was wholly attributable to inhibition of FLT3, and not due to off-target effects, we repeated the experiments using Ba/F3 cells stably transduced with a FLT3-ITD G697R allele (Ba/F3 FLT3-ITD-G697R cells), that we have previously reported to confer resistance to midostaurin. In the cell growth assay (Figure 2A), there was no inhibition of cellular proliferation at doses of midostaurin up to 300 nM. In addition, immunoblot assays (Figure 2B) demonstrated that midostaurin did not inhibit phosphorylation of FLT3, STAT5, or ERK in Ba/F3 FLT3-ITD-G697R cells. These findings indicate that inhibition of the respective mutant FLT3 activating alleles in this cellular context is due to specific inhibition of FLT3 by midostaurin.

These findings have a direct bearing on phase 3 trials that are currently being planned in the Intergroup setting for treatment of newly diagnosed AML patients with FLT3-ITD mutations with midostaurin and induction chemotherapy. In contrast to other small molecule FLT3 inhibitors, midostaurin inhibits the full spectrum of FLT3 activation loop mutations. Thus, these data indicate that AML patients harboring such activation loop mutations in FLT3 should be considered as candidates for enrollment, in addition to those with FLT3-ITD alleles.

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