

MOLECULAR TARGETS FOR THERAPY (MTT)



***In vitro* antiproliferative activity of the farnesyltransferase inhibitor R115777 in hematopoietic progenitors from patients with myelofibrosis with myeloid metaplasia**

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R115777 is an orally bioavailable farnesyltransferase inhibitor (FTI) that has displayed encouraging activity in patients with acute myeloid leukemia. To determine whether R115777 might exert similar activity in myelofibrosis with myeloid metaplasia (MMM), we evaluated its effects on circulating myeloid progenitor cells from patients with MMM ($n=25$) using *in vitro* colony-forming assays. The median R115777 concentrations that inhibited colony formation by 50% were 34 and 2.7 nM for myeloid and megakaryocytic colonies from MMM patients, respectively. Progenitors from normal controls and patients with other myeloproliferative disorders demonstrated similar sensitivity. Since the ras polypeptides are one putative target of FTIs, the potential role of ras effectors was examined by incubating parallel progenitor assays with the phosphatidylinositol-3 (PI-3) kinase inhibitor LY294002 and the mitogen-activated protein kinase 1 inhibitor PD98059. MMM progenitor colonies ($n=7$) were highly sensitive to LY294002 but not to PD98059, implying that the PI-3 kinase pathway may be critical for survival and proliferation of these cells. In addition to indicating that MMM progenitors are sensitive to clinically achievable R115777 concentrations *in vitro*, these results provide a potential explanation for the thrombocytopenia observed with R115777 during the treatment of other hematologic malignancies.

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Introduction

Myelofibrosis with myeloid metaplasia (MMM) is a clonal hematopoietic stem cell disorder characterized by expansion of the circulating myeloid progenitor pool, splenomegaly, and intramedullary fibrosis.¹ Clinically, these patients experience progressive cytopenias, expansive hepatosplenomegaly, debilitating constitutional symptoms, blast transformation, and death.¹ Current therapy for MMM is very limited and largely palliative. Allogeneic² and autologous stem cell transplantation³ are potentially curative options, but are of limited applicability because of the age and comorbidities in MMM patients. Splenectomy⁴ or the use of androgens,^{5,6} steroids, supplemental erythropoietin,⁷ interferon-alpha,⁸ or hydroxyurea⁹ offers lim-

ited palliative benefit. However, no pharmacologic agent has been shown to have any impact on the natural history of the disease.

Current understanding of the pathogenesis of MMM is incomplete. No causative genetic mutation or mechanism for the origin of the disease has been identified. It has, however, been established that a clonal population of myeloid cells (potentially megakaryocytes or monocytes) is markedly expanded.^{10,11} In addition, profibrotic or fibrogenic cytokines, including transforming growth factor- β ,¹² platelet-derived growth factor (PDGF),¹³ vascular endothelial growth factor,¹⁴ and tumor necrosis factor- α (TNF- α), are increased. These cytokines are believed to be responsible for polyclonal fibroblast proliferation,¹⁵ intramedullary deposition of excess connective tissue, and ineffective hematopoiesis that are characteristic of MMM. Recent pilot clinical trials have tested agents that inhibit the actions of these cytokines, including pirfenidone (inhibits various cytokines),¹⁶ thalidomide (inhibits various cytokines),¹⁷ STI571 (inhibits PDGF),¹⁸ and etanercept (inhibits TNF- α),¹⁹ with limited success. The relatively disappointing results of these trials highlight the need to focus investigative efforts on therapeutic agents that inhibit the aberrant clone rather than the secondary cytokine cascade.

R115777 is a nonpeptidomimetic inhibitor of farnesyl protein transferase,²⁰ the enzyme that transfers the 15-carbon farnesyl group to the carboxyl terminal end of selected polypeptides. Among the polypeptides that are normally farnesylated in cells are the ras proteins (N-ras, H-ras, K-ras), which require prenylation for their attachment to the plasma membrane.^{21–24} Farnesyltransferase inhibitors (FTIs) were originally synthesized and tested on the premise that farnesyl protein transferase inhibition would inhibit the membrane targeting and function of oncogenic ras mutants.^{22,23,25,26} Subsequent studies, however, have demonstrated that FTIs inhibit the proliferation of transformed cells *in vitro* and *in vivo* even if ras mutations are absent.^{27,28} Along with H-ras, other farnesylated proteins, including rhoB, the centromere proteins CENP-E and CENP-F, and a currently unidentified molecule in the phosphatidylinositol-3 (PI-3) kinase/Akt pathway, have been implicated as potential FTI targets in neoplastic cells that lack ras mutations.^{27–30}

On the basis of the presence of ras mutations in a subset of acute myeloid leukemias,³¹ as well as patient tolerance of nonmyeloid drug side effects in solid tumor patients,^{32,33} R115777 was examined in a phase I trial in acute leukemia.³⁴

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Responses were observed in 29% of patients with relapsed or primary refractory acute myeloid leukemia, none of whom had ras mutations. Pharmacokinetic data showed peak and trough serum concentrations of 3.4 and 0.9 μM , respectively, at the maximum tolerated dose (MTD) of 900 mg twice a day. Interestingly, R115777 concentrations in leukemic marrow were three- to four-fold higher than corresponding serum concentrations. Ancillary studies demonstrated inhibition of farnesylation in blast cells at this dose. In addition, there are recent reports^{35–37} of preliminary clinical activity of R115777 in other myeloid malignancies, including myelodysplasia and chronic myeloid leukemia. Interestingly, these latter trials of R115777 have revealed dose-limiting thrombocytopenia in patients with pre-existing marrow dysfunction.³⁷

Aside from a single study showing that hematopoietic progenitors from patients with juvenile myelomonocytic leukemia are exquisitely sensitive to FTIs *in vitro*,³⁸ there is little information about the effects of this class of agent on progenitors from patients with myeloproliferative syndromes. The current lack of effective therapy for MMM and the intriguing clinical activity reported for R115777 in other myeloid disorders led us to evaluate this agent for activity against MMM progenitors *in vitro*.

Methods

Patients

After documentation of informed consent, samples of peripheral blood were obtained according to a protocol approved by the Mayo Clinic Institutional Review Board. Enrollment included patients who met standard diagnostic criteria³⁹ for MMM ($n=25$) and a comparison population of normal volunteers ($n=20$) and patients with other chronic myeloproliferative disorders – polycythemia vera (PV $n=6$) and essential thrombocythemia (ET $n=7$).

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from 10 ml EDTA-anticoagulated peripheral blood by double-layer Ficoll–Hypaque density centrifugation according to the method described by English and Andersen.⁴⁰ The top layer representing PBMC was removed, washed in Dulbecco's phosphate-buffered saline, and counted on a hemocytometer. PBMC were then resuspended in Iscove's modified Dulbecco's medium (Life Technology, Rockville, MD, USA) with 20% heat-inactivated fetal bovine serum (Biosource, Walkersville, MD, USA).

Nonmegakaryocytic myeloid colony assays

Aliquots containing 600 000 PBMC ($2 \times 10^6/\text{ml}$) were placed in Methocult methylcellulose medium (StemCell Technologies, Vancouver, Canada) containing diluent or graded drug concentrations, plated into culture dishes with 35-mm grids and incubated for 14 days at 37°C with 5% CO₂. The Methocult medium contains all the cytokines necessary for the growth of granulocyte/erythrocyte/macrophage/monocyte (GEMM) colonies, granulocyte/macrophage (GM) colonies, erythroid colonies, and erythroid bursts, including stem cell factor, GM-colony-stimulating factor, granulocyte-colony-stimulating factor, erythropoietin, and interleukins 3 and 6. After a 14-day incubation, progenitor colony numbers and subtypes were

counted by inverted light microscopy at $\times 25$ magnification according to morphologic criteria established by the manufacturer.

Megakaryocytic colony assays

Samples containing 500 000 PBMC ($1 \times 10^7/\text{ml}$) were placed in Iscove's modified Dulbecco's medium (without fetal calf serum) in Megacult collagen-based medium (StemCell Technologies), plated onto sterile dual-chambered culture dishes containing diluent or graded drug concentrations and incubated for 12 days at 37°C with 5% CO₂. The assay contains all the requisite cytokines necessary for megakaryocyte development, including erythropoietin, interleukins 3 and 6, and thrombopoietin. At the completion of the incubation, the collagen gels were fixed in 1:3 methanol:acetone and stained for platelet glycoprotein IIb/IIIa as instructed by the supplier to distinguish megakaryocyte colonies from other colonies. Megakaryocyte colony-forming units (CFU-MK) were then counted as colonies of more than three clustered megakaryocytes.

Materials

R115777 was kindly provided by Janssen Research Institute (Beerse, Belgium). LY294002 and PD98059 were purchased from Calbiochem (San Diego, CA, USA) and Alexis (San Diego, CA, USA), respectively. All drugs were dissolved in dimethyl sulfoxide at 1000 times the indicated final concentrations.

Statistics

The median, minimum, and maximum nonmegakaryocytic progenitor colony values were obtained on samples from three groups: patients with MMM ($n=13$), patients with ET/PV ($n=10$), and normal controls ($n=10$). Nonmegakaryocytic progenitor colony values were compared for the three groups using the Kruskal–Wallis test. All other analyses were descriptive.

Results

On the basis of the promising activity of R115777 in other myeloid disorders, we evaluated the effect of this agent on circulating hematopoietic progenitors from patients with MMM. Concentrations used in these studies were well below the marrow concentrations (reported as 3 μg R115777/g (range 2–3.6) of cell pellet, roughly equivalent to 6 μM) observed in leukemic blasts from patients treated at the MTD of 900 mg twice a day.³⁴ Initial experiments ($n=4$) using R115777 concentrations in excess of 100 nM resulted in complete inhibition of MMM colony growth. Accordingly, subsequent experiments were performed with R115777 concentrations of 0–100 nM ($n=13$).

Dose–response curves were constructed by treating circulating progenitors from the patients with MMM with increasing concentrations of R115777 for 14 days *in vitro*. This assay utilized a continuous-exposure paradigm in an attempt to mimic the exposure obtained during the 21-day dosing period used clinically.³⁴ The patients providing cells for these assays encompassed a spectrum of MMM disease subtypes (including post-polycythemic myeloid metaplasia and post-thrombocytopenic myeloid metaplasia), disease manifestations, and concur-

Table 1 Characteristics of 17 patients with MMM demonstrating *in vitro* sensitivity to R115777

Type of MMM, no. of patients (%)	
AMM	8 (47)
PPMM	5 (29)
PTMM	4 (24)
Sex, no. of patients (%)	
Male	11 (65)
Female	6 (35)
Age year, mean (range)	
At diagnosis	57 (31–72)
At time of trial	63 (39–78)
Time elapsed since diagnosis, months	54 (0–178)
Concurrent therapy, no. of patients (%)	
None	6 (35)
Hydroxyurea	5 (29)
Anagrelide	1 (6)
Thalidomide	3 (18)
Interferon-alpha	1 (6)
Disease status, no. of patients (%)	
Transfusion-dependent anemia	5 (29) (median 2.5 U/month)
Weight loss (>20% baseline)	5 (29)
Fevers	3 (18)
Night sweats	6 (35)
Bone pain	3 (18)
Laboratory studies/physical exam at enrollment, mean (range)	
Hemoglobin (g/dl)	10.5 (6.6–16.2)
Leukocyte count ($\times 10^9/l$)	24.2 (1.9–112)
Neutrophils	14.6 (0.9–66.0)
Lymphocytes	2.1 (0.6–8.8)
Monocytes	1.1 (0–5.4)
Eosinophils	0.5 (0–4.2)
Basophils	0.6 (0–2.3)
Metamyelocytes	0.8 (0–8.9)
Myelocytes	2.0 (0–22.4)
Myeloblasts	1.2 (0–8)
Nucleated erythrocytes (per 100 leukocytes)	1.8 (0–9.8)
Peripheral blood CD34+ count ($n=6$) ($/\mu l$)	427 (0–2229)
Platelet count ($\times 10^9$)	236 (16–604)
Spleen size (cm below LCM)	8.8 (0–20)
Liver size (cm below RCM)	0.5 (0–4)
Concurrent bone marrow ($n=8$)	
Cellularity (%)	75 (10–100)
Reticulin fibrosis grade, no. of patients	1=1, 2=5, 3=2
Osteosclerosis grade, no. of patients	0=5, 1=1, 2=1, 3=1
Karyotype	Abnormal in five (55%)

AMM, agnogenic myeloid metaplasia; LCM, left costal margin; PPMM, post-polycythemic myeloid metaplasia; PTMM, post-thrombocytopenic myeloid metaplasia; RCM, right costal margin.

rent therapies, as outlined in Table 1. Results of all of the nonmegakaryocytic myeloid progenitor assays are summarized in Figure 1. All of the precursors from MMM patients assayed (CFU-GEMM, CFU-GM, BFU-E (erythroid burst-forming units), CFU-E (erythroid colony-forming units), and CFU-MK) were very sensitive to R115777 *in vitro*, as illustrated in Figure 2. Nonmegakaryocytic colony formation was inhibited by 50% at R115777 concentrations of 7.5–74.5 nM (median 34 nM) in the MMM samples (Figure 1). Megakaryocytic colony formation was even more sensitive, with R115777 concentrations as low as 6.25 nM resulting in almost complete CFU-MK inhibition (Figure 3). In fact, 6.25 nM resulted in complete inhibition of CFU-MK in all but one patient with MMM. Statistical analyses failed to show any correlation between *in vitro* sensitivity to

R115777 and various parameters shown in Table 1, including type of MMM, age, concurrent therapy, or disease status.

To determine whether the sensitivity of circulating progenitors to R115777 was unique to MMM samples, we performed additional assays with the myeloid progenitors from patients with other myeloproliferative diseases (ET and PV, $n=11$, for a dose range of 0–100 nM R115777) and normal controls ($n=10$, for a dose range of 0–100 nM R115777). Nonmegakaryocytic progenitors from ET and PV patients were just as sensitive as the MMM progenitors (median IC_{50} 20 nM, range 5–47 nM, $P=0.17$) (Figure 1). In contrast, higher median R115777 IC_{50} concentrations were required for inhibition of nonmegakaryocytic progenitors from normal controls (median 46 nM, range 6–118 nM) (Figure 1), although this difference was not statistically

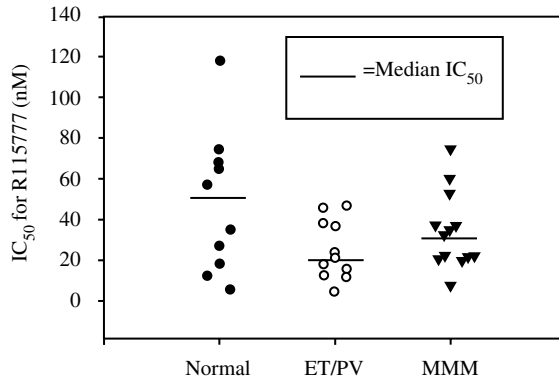


Figure 1 Summary results of R115777 dose-response curves in nonmegakaryocytic progenitors from patients with MMM, patients with ET/PV, and normal controls. From dose-response curves (see Figure 2), concentrations that inhibited nonmegakaryocytic (CFU-GEMM, CFU-GM, and erythroid (BFU-E and CFU-E)) colony formation by 50% (IC₅₀s) were calculated. Median IC₅₀s were 34 nM R115777 in nonmegakaryocytic progenitors from MMM patients, 20 nM in progenitors from ET/PV patients, and 46 nM in progenitors from normal controls.

significant among any of the three groups because of the wide range in sensitivity within each group. Megakaryocytic progenitors from normal controls, MMM patients, and patients with other chronic myeloproliferative syndromes exhibited similar, and profound, R115777 sensitivity, with virtually complete growth suppression at 6.25 nM. To better define the sensitivity of the CFU-MK, we performed experiments with circulating progenitors from additional MMM patients ($n=8$) and normal controls ($n=7$), using R115777 at 1, 2.5, 5, 10, 12.5, and 25 nM. These assays yielded mean IC₅₀ values for the CFU-MK of 2.7 and 9.1 nM for the MMM patients and normal controls, respectively (Figure 4). Although there did appear to be greater

sensitivity to R115777 among the CFU-MK of normal controls, there was no statistical difference in this small population.

Even with the identification of additional farnesylated polypeptides, ras remains a potentially important target for the antiproliferative effects of FTIs.^{26,41} To delineate the potential dependence of MMM myeloid progenitors on pathways downstream of ras, we examined the growth-inhibitory effects of agents known to inhibit pathways at least partially dependent upon ras activation. In particular, myeloid progenitor assays were performed in the absence or presence of LY294002 (MMM $n=8$, ET/PV $n=7$, normal $n=4$), an inhibitor of PI-3 kinase,⁴² and PD98059 (MMM $n=7$, ET/PV $n=9$, normal $n=8$), an inhibitor of the mitogen-activated protein kinase kinase (Mek) pathway.⁴³ As shown in Figure 5, nonmegakaryocytic myeloid progenitors from all three groups of subjects showed marked sensitivity to LY294002 but no appreciable sensitivity to PD98059 under the conditions of these *in vitro* assays.

Discussion

In this study, we compared the effects of the FTI R115777 on the proliferation of hematopoietic progenitors from patients with MMM, those with other chronic myeloproliferative disorders, and normal controls. The results of this analysis demonstrated that normal progenitors were slightly more resistant to the effects of R115777 *in vitro*. In addition, these experiments showed that megakaryocytic precursors are more sensitive to the inhibitory effects of R115777 than other hematopoietic progenitors. This finding might be of particular significance because MMM is characterized by expansion of the megakaryocytic pool in the marrow,⁴⁴ and this lineage is believed to be central to the pathogenesis of the disease.

Evaluation of potential therapeutic agents in MMM has been hampered by the lack of either an established cell line or a definitive animal model.¹ MMM is characterized by a marked

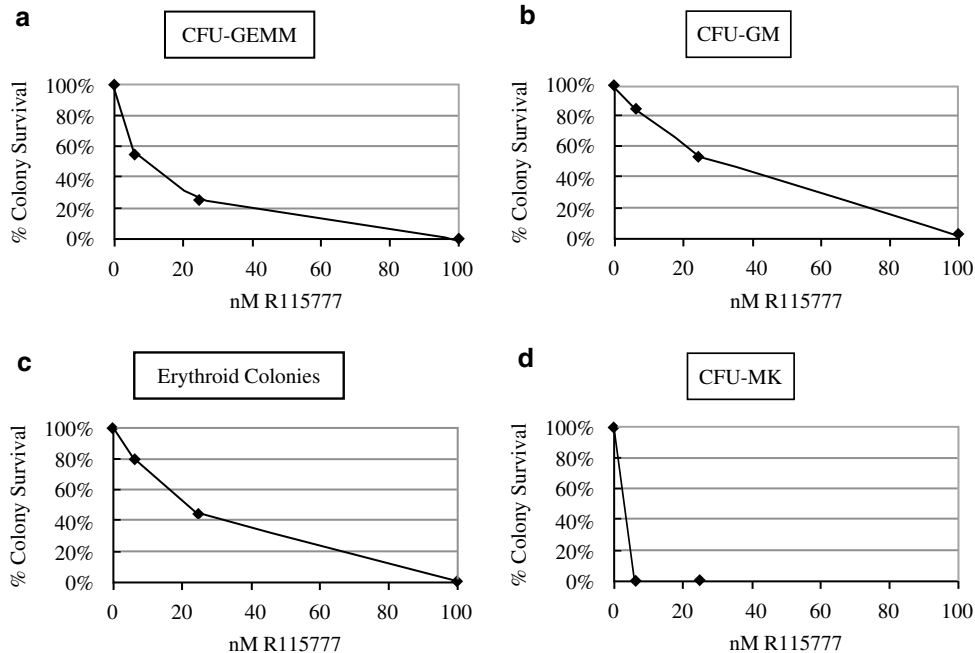


Figure 2 Dose-response curves from a representative patient were constructed by treating circulating progenitors from patients with MMM with increasing concentrations of R115777 for 14 days. Megakaryocytic and nonmegakaryocytic (CFU-GEMM, CFU-GM, and erythroid (BFU-E and CFU-E)) progenitor colonies were quantitated separately.

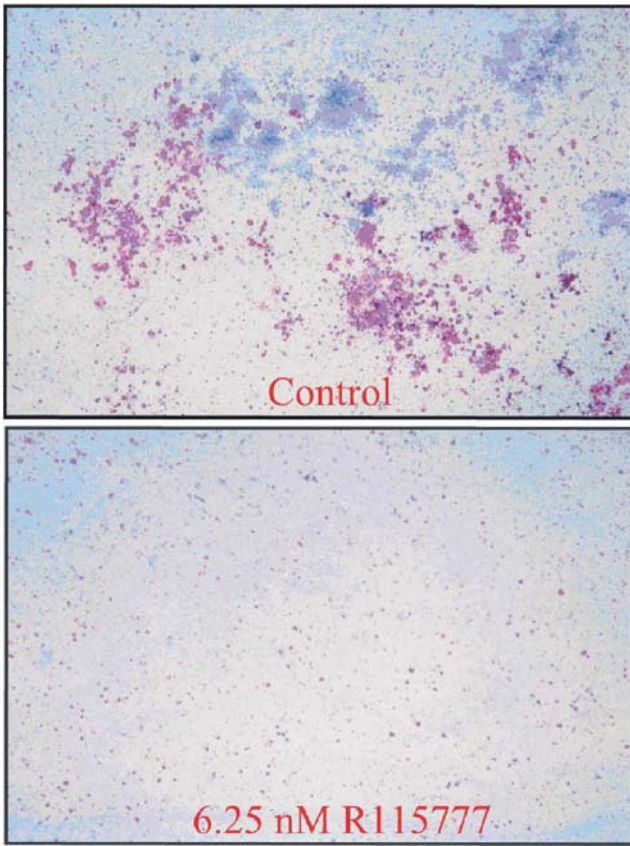


Figure 3 Megakaryocytic colony formation was inhibited by concentrations of R115777 as low as 6.25 nM. Complete inhibition of CFU-MK growth after continuous 14-day exposure to 6.25 nM R115777 (upper panel, 0.1% dimethyl sulfoxide; lower panel, 6.25 nM R115777).

increase in the circulating pool of myeloid progenitors.^{45,46} We have previously reported evidence suggesting that these circulating myeloid progenitors have the same karyotypic abnormalities as are seen in marrow progenitors, indicating that circulating myeloid progenitors in MMM are progeny of the aberrant clone.⁴⁷ This increase in circulating myeloid progenitors has been associated with an adverse prognosis⁴⁵ and contributes to extramedullary hematopoiesis that occurs in the spleen, liver, and other sites in patients with MMM. In the present study, we examined the ability of R115777 to suppress the growth of these circulating myeloid progenitors. The advantage of such an approach is ready access to the clonal population of disease-associated cells for assessment of therapeutic activity. The disadvantage is that this model does not adequately reflect the potential effects of any drug on the interaction between the myeloid progenitors and the bone marrow microenvironment.

The median concentrations of R115777 found to inhibit production of nonmegakaryocytic and megakaryocytic progenitor colonies by 50% were 34 and 2.7 nM, respectively. Since the progenitor cells represent only a small fraction of the circulating PBMC population, it is difficult to perform biochemical studies to assess the mechanism of action of R115777 on these cells. The low concentration of the drug required for inhibition of colony growth suggests, however, that R115777 is acting through inhibition of myeloid cell proliferation as opposed to induction of apoptosis. The concentrations of R115777 that suppress progenitor colony formation compare favorably with

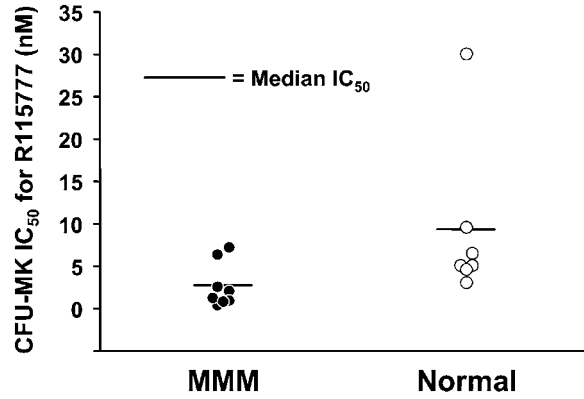


Figure 4 Summary results of R115777 dose–response curves in megakaryocytic progenitors from patients with MMM and normal controls. From experiments employing 0, 1, 2.5, 5, 10, 12.5, and 25 nM R115777, concentrations that inhibited CFU-MK colony formation by 50% (IC_{50}) were calculated. Median IC_{50} was 2.7 nM R115777 in progenitors from MMM patients and 9.1 nM in progenitors from normals, but the difference was not statistically significant because of overlap of the two populations.

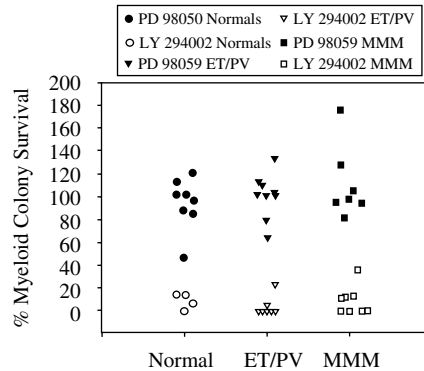


Figure 5 Nonmegakaryocyte myeloid progenitors from MMM patients, ET/PV patients, and normal controls showed marked sensitivity to LY294002 (40 μ M) but no appreciable sensitivity to PD 98059 (30 μ M). Colony formation was assessed after progenitors were exposed to LY294002 or PD98059 for 14 days.

levels achieved in patients receiving R115777. In the phase I trial of R115777 in acute leukemia,³⁴ serum peak and trough concentrations of 1660 and 180 nM, respectively, were observed in patients treated with 300 mg R115777 twice a day, a dose that was half the dose recommended for evaluation in phase II trials in patients with acute leukemia. At a dose of 300 mg twice a day, R115777 was well tolerated. These observations suggest that R115777 might be active against MMM at therapeutically achievable concentrations. In this context, the marked sensitivity of megakaryocytic progenitors from MMM patients to the effects of R115777 is of interest in two respects. First, megakaryocytes show marked intramedullary proliferation in MMM and are believed to be integral to the pathogenesis of MMM. Second, in the reported clinical trials of R115777 in patients with nonhematologic malignancies, thrombocytopenia was uncommon and occurred only at very high dose levels,³³ raising the possibility that R115777 might have somewhat selective effects on the megakaryocytic lineages in afflicted MMM patients.

On the other hand, the effects of R115777 on normal marrow progenitors raise the concern that it may be excessively

myelosuppressive in MMM and other chronic myeloproliferative disorders. In both the phase I acute leukemia trial and recent pilot studies of R115777 in other myeloid diseases (myelodysplasia³⁵ and chronic myeloid leukemia³⁷), R115777 doses of 600 mg twice a day and larger were associated with reversible myelosuppression, which required dose reduction in up to 48% of patients. Indeed, thrombocytopenia (\geq grade 3) was seen in 48% of patients with chronic myelogenous leukemia³⁷ treated with 600 mg twice a day. Our observations indicate that megakaryocytic progenitors are particularly sensitive to the antiproliferative effects of R115777, providing a potential explanation for these recent findings.

At present, the critical target(s) of FTIs downstream of farnesyltransferase remains unclear (see Introduction). One of the postulated targets is H-ras. Constitutive activation of H-ras as a consequence of upstream signaling has been implicated in the pathogenesis of a variety of proliferative disorders,²³ including chronic myelogenous leukemia.^{27,48,49} According to current understanding, H-ras signals through at least two pathways: one involving raf, Mek1 and the erks, and another involving PI-3 kinase and Akt.⁴¹ It has been shown that both the PI-3 kinase pathway and the Mek pathway are involved with downstream signaling from a variety of critical hematopoietic growth factors, including erythropoietin and stem cell factor.^{50,51} In addition, inhibition of the PI-3 kinase and Mek1 pathways has been shown to inhibit the growth of erythroid progenitors through potentially different mechanisms.^{50,51} Since circulating progenitor cells in MMM constitute only a small fraction of the circulating mononuclear cells, it was not feasible to assess the effects of R115777 on these pathways using a biochemical approach. Examination of other inhibitors, however, indicated that MMM progenitors (and normal hematopoietic progenitors) were much more sensitive to the antiproliferative effects of the PI-3 kinase inhibitor LY294004 than the Mek1 inhibitor PD98059. The ability of a PI-3 kinase inhibitor to mimic the effects of R115777 is consistent with recent data suggesting that FTIs might slow proliferation and induce apoptosis by inhibiting the farnesylation of H-ras or a protein in the PI-3 kinase/Akt pathway.⁴¹ In addition, these results suggest that Mek1 inhibitors such as PD98059 or CI1040, which are undergoing preclinical testing in acute myelogenous leukemia,⁵² are unlikely to be active in MMM, whereas PI-3 kinase and Akt inhibitors might warrant further investigation.

Conclusion

R115777 appears to inhibit circulating MMM hematopoietic progenitors *in vitro* at clinically achievable concentrations. The specificity of this agent in MMM is not clear and deserves further scrutiny. Farnesyltransferase deserves further attention as a possible therapeutic target for MMM.

Acknowledgements

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