Inhibition of juvenile myelomonocytic leukemia cell growth in vitro by farnesyltransferase inhibitors

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Juvenile myelomonocytic leukemia (JMML) is an early childhood disease for which there is no effective therapy. Therapy with 13-cis retinoic acid or low-dose chemotherapy can induce some responses, but neither mode is curative. Stem cell transplantation can produce lasting remissions but is hampered by high rates of relapse. The pathogenesis of JMML involves deregulated cytokine signal transduction through the Ras signaling pathway, with resultant selective hypersensitivity of JMML cells to granulocyte-macrophage colony-stimulating factor (GM-CSF). A JMML mouse model, achieved through homozygous deletion of the neurofibromatosis gene, confirmed the involvement of deregulated Ras in JMML pathogenesis. With this genetic knowledge, mechanism-based treatments are now being developed and tested. Ras is critically dependent on a GTPase-activating protein, and it serves to hydrolyze Ras from its active GTP-bound state to its inactive GDP-bound state. The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 U.S.C. section 1734.

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selective hypersensitivity to GM-CSF as do JMML cells, and transplantation of these cells into irradiated recipient mice results in the development of a myeloproliferative disorder similar to the human JMML syndrome and characterized by activated Ras-MAP kinase signaling in hematopoietic cells. Furthermore, recent studies show that murine cells lacking Nf1 are critically dependent on GM-CSF for growth. Given these compelling data linking JMML pathogenesis to deregulated GM-CSF signal transduction through the Ras intracellular pathway, it seems reasonable to begin to explore mechanism-based therapy for JMML. Because JMML hematopoietic progenitor cells do not produce sufficient GM-CSF themselves to sustain in vitro colony growth, JMML is not an autocrine-driven disease. Rather, because of their inherent hypersensitivity to GM-CSF, JMML progenitors are dependent on the basal production of GM-CSF from monocytes. IL-10 has been shown to inhibit the monocytic production of GM-CSF and specifically to inhibit JMML cell growth. The GM-CSF antagonist and apoptotic agent, E21R, has also been shown to inhibit JMML in vitro cell growth and JMML cell engraftment in immunodeficient mice. Finally, 1 of 2 recently developed GM-CSF/diphtheria toxin fusion proteins has been shown to inhibit JMML cell growth in vitro. It is hypothesized that most of these potential therapies interfere with GM-CSF-cell interactions at the JMML cell surface. Whether any of these potential therapies can actually abolish the entire malignant clone is a matter of ongoing investigation.

Another feasible way to block the GM-CSF hypersensitive growth of JMML cells is to block the intracellular signaling pathway. For Ras to be active as a master switch for signal transduction, it must localize to the inner surface of the plasma membrane; this occurs after a series of posttranslational modifications. The first obligatory step in this series, which is essential for Ras cell-transforming activity, is the addition of a 15-carbon isoprenyl (farnesyl) group to Ras through a covalent link. The addition of the farnesyl moiety to the cysteine residue of the COOH-terminal CAAX motif (C, cysteine; A, usually an aliphatic residue; X, any other amino acid) is catalyzed by the enzyme farnesyl-protein transferase (FPTase). Several inhibitors of FPTase, representing broad structural diversity, have been synthesized. Some of these compounds, now termed farnesyltransferase inhibitors (FTIs), have been evaluated in several different in vitro and in vivo preclinical systems and have demonstrated significant antitumor effects. They have demonstrated an ability to inhibit the Ras-induced transformation of tissue culture cells and several cancer cell lines (primarily solid tumor types) and to block the proliferation of Ras-activated xenografts in nude mice. Further, FTIs have shown efficacy in transfusional mouse models of mammary and salivary carcinomas in which the Ras expression was forced by a mammary tumor virus. Finally, FTIs have demonstrated efficacy in blocking some of the phenotypic changes in NF1-deficient cells. Given this background of the developmental design of the FTIs and the pathogenic mechanisms involving Ras and NF1 in JMML, the goal of this study was to determine the effectiveness of the CAAX peptidomimetic FPTase inhibitors L-739,749 and L-744,832 to abrogate JMML cell growth in vitro.

Materials and methods

Acquisition of donor samples

With the approval of the respective institutional review boards and after obtaining parental consent, peripheral blood samples, bone marrow samples, or both were obtained from children with JMML and from a child with another disorder to serve as an age-matched control. The diagnosis of JMML was based on uniform criteria as agreed on by the International JMML Working Group and confirmed by the demonstration of selective hypersensitivity to GM-CSF in all children.

Normal controls were volunteer adults who donated bone marrow samples after informed consent and with the approval of the Institutional Review Board of the University of Alabama at Birmingham.

Mononuclear cell isolation and colony assays

Peripheral blood or marrow mononuclear cells for patient and control samples were isolated by density gradient centrifugation as described previously. Soft agar assays for granulocyte-macrophage colonies (CFU-GM) were established in 1-mL cultures of 0.3% agarose with McCoy’s 5A medium plus nutrients and 15% fetal bovine serum as described previously. Cultures were incubated for 14 days at 37°C in a 5% CO2 atmosphere. CFU-GM colonies (>40 cells/colony) were scored at day 14 using a dissecting microscope. GM-CSF (R & D, Minneapolis, MN) was added to the control cultures from the normal adult volunteer samples to stimulate growth at final concentrations of either 0.32 ng/mL or 2 ng/mL.

Addition of farnesyltransferase inhibitor to CFU-GM assays

The FTIs, L-739,749 and L-744,832, were supplied by Drs Allen Oliff and Jackson Gibbs of Merck Research Laboratories (West Point, PA) and were dissolved in a stock solution of 50% methanol at a concentration of 100 mmol/L and stored at −20°C. Three methods of adding the FTI to the CFU-GM assays were evaluated: (1) L-739,749 or L-744,832 was added only once, 24 hours after the cultures were established, duplicating the type of in vitro assay that established the effectiveness of 13-cis retinoic acid; (2) the one-time dosing of FTI was delayed and was added at either day 3, day 5, or day 7 after the cultures were established; or (3) the cells were exposed to FTI before the establishment of the semisolid agar cultures. In the latter experiment, the mononuclear cells were placed in liquid suspension in McCoy’s 5A medium with nutrients and 15% fetal bovine serum and then the FTI inhibitor was mixed in. Cells were exposed to the FTI inhibitor for 1, 3, or 5 days, then washed twice and placed in agar assays without any further addition of FTI inhibitor. In all types of cultures, the appropriate methanol dilutions for the respective FTI concentrations were simultaneously established to control for any effects on CFU-GM growth imposed by the methanol itself. Appropriate dilutions of the FTI or methanol control were made such that, for each dose, 100 µL of volume was spread uniformly over the agarose surface.

Results and discussion

Samples from 12 patients with JMML were evaluated. All patients fulfilled the diagnostic criteria for JMML and demonstrated selective GM-CSF hypersensitivity of hematopoietic progenitor cells in vitro. Only 5 of 12 patient samples have been fully evaluated for NF1 or RAS abnormalities. Of the 5 fully studied, 2 had RAS mutations (both KRAS point mutations) and the other 3 had NF1 abnormalities (unpublished observations, Snyder RC, Emanuel PD and ref. 39).

In the first series of experiments, the FTI was added once 24 hours after the establishment of the cultures. Numbers of CFU-GM (>40 cells/colony) were counted, and the amount of inhibition by the FTI was calculated as a percentage of the maximal colony growth. As depicted in Figures 1 and 2, there was inhibition of JMML spontaneous CFU-GM colony growth at all concentrations of either FTI, L-739,749, or L-744,832. At concentrations ≥10 µmol/L FTI, there was complete abrogation of growth, and virtually no colonies were present in any patient sample. At a concentration of 1 µmol/L FTI, significant inhibition of CFU-GM colony growth was noted, and the colonies were smaller (fewer
cells) than in the no addition controls. Because the FTI was in a methanol stock solution, appropriate methanol controls were also established, and these showed no significant effect on CFU-GM growth (data not shown).

Because of the nature of JMML, finding suitable controls for these experiments was problematic. We have demonstrated that JMML peripheral blood-derived or marrow-derived progenitor cells are essentially equivalent with regard to spontaneous growth patterns and to GM-CSF hypersensitivity patterns. Normal peripheral blood mononuclear cells did not show spontaneous colony growth, and normal marrow mononuclear cells did so only sporadically. Numerous normal age-matched marrow samples were not obtainable because of ethical concerns. Therefore, as controls for these experiments, we used normal adult marrow mononuclear cells stimulated with GM-CSF to simulate colony growth similar to that observed in JMML. We examined the inhibitory effects of the FTI on normal CFU-GM colony growth under several conditions: freshly obtained specimens stimulated with maximal concentrations of GM-CSF (2 ng/mL), freshly obtained specimens stimulated with threshold concentrations of GM-CSF (0.32 ng/mL), and specimens that had been stored in a liquid nitrogen environment for a period of time. The latter 2 conditions were attempted to simulate JMML conditions as much as possible. Because JMML cells are hypersensitive to GM-CSF, we stimulated normal cells with a GM-CSF concentration obtained from our dose-response curve data that represents a threshold concentration (0.32 ng/mL GM-CSF) at which JMML cells show an initial growth response. This was compared with effects at 2 ng/mL, which represents a maximal stimulation situation. Therefore, both physiologic and pharmacologic situations were simulated. No significant difference was noted in normal adult marrow cultures between the 2 GM-CSF concentrations (Figure 1). In each situation the amount of colony growth inhibition by the FTI was always less than that seen in the JMML samples. The third situation simulated the JMML conditions in that several of our JMML samples had been stored in liquid nitrogen for various periods of time before evaluation. The storage conditions did have some increased effects on the amount of colony growth inhibition in the normal adult control samples (Figure 1), but these effects were still less than those seen in JMML samples. Taking into account the hypersensitivity to GM-CSF in JMML cells, these results are predictable. Finally, we were able to evaluate an age-matched marrow sample from a patient whose hematopoietic progenitor cells did in fact show spontaneous CFU-GM colony growth. This patient was ultimately diagnosed with thrombocytopenia with absent radii (TAR). The child did not meet the diagnostic criteria for JMML. CFU, colony-forming unit; CSF, colony-stimulating factor; GM, granulocyte macrophage; JMML, juvenile myelomonocytic leukemia.

Figure 1. Inhibition of CFU-GM colony growth by the addition of L-739,749. L-739,749 was a one-time addition 24 hours after establishment of the cultures, with respective concentrations of L-739,749 as indicated. Results in all cases are the mean of experiments performed in triplicate and are expressed as the percentage inhibition of maximal spontaneous CFU-GM colony growth. Error bars indicate the standard error of the mean. Solid bars: Inhibition of JMML spontaneous CFU-GM colony growth. Results represent the mean of experiments performed in triplicate from 10 different patients with JMML. Hatched bars: Inhibition of GM-CSF–stimulated normal donor CFU-GM colony growth from normal mononuclear cells obtained fresh from 5 different normal adult marrow donors. Results are expressed as the percentage inhibition of maximal CFU-GM colony growth stimulated by GM-CSF (either 2 ng/mL or 0.32 ng/mL). Diagonal bars: Inhibition of GM-CSF–stimulated normal donor CFU-GM colony growth from normal mononuclear cells that had been previously obtained from 3 different normal adult marrow donors and had been put in liquid nitrogen for long-term storage. Results are expressed as the percentage inhibition of maximal CFU-GM colony growth stimulated by GM-CSF (2 ng/mL). Open bars: Inhibition of spontaneous CFU-GM colony growth from the bone marrow mononuclear cells of an age-matched patient diagnosed with thrombocytopenia with absent radii (TAR syndrome). The child did not meet the diagnostic criteria for JMML. CFU, colony-forming unit; CSF, colony-stimulating factor; GM, granulocyte macrophage; JMML, juvenile myelomonocytic leukemia.

In a final series of experiments to determine the effectiveness of L-739,749 on spontaneous JMML CFU-GM colony growth.

Figure 3 demonstrates a clear-cut, time-dependent loss of inhibitory effect of L-739,749 on spontaneous JMML CFU-GM colony growth. Inhibition of spontaneous CFU-GM colony growth from the bone marrow mononuclear cells of an age-matched patient diagnosed with thrombocytopenia with absent radii (TAR syndrome). The child did not meet the diagnostic criteria for JMML. CFU, colony-forming unit; CSF, colony-stimulating factor; GM, granulocyte macrophage; JMML, juvenile myelomonocytic leukemia.

Given that either FTI was able to inhibit 100% of spontaneous CFU-GM growth in virtually all patients with JMML at concentrations of 10 μmol/L, in the next series of experiments we sought to determine whether variations in time of exposure to the FTI would affect the degree of growth inhibition. Therefore, L-739,749 was added at either days 1, 3, 5, or 7 after the establishment of cultures. Because of limited supplies of L-739,749, this type of multiple-dosing experiment could be performed on only 1 patient sample. Figure 3 demonstrates a clear-cut, time-dependent loss of inhibitory effect of L-739,749 on spontaneous JMML CFU-GM colony growth.
ase inhibitors, initially developed for their potential to block Ras signal transduction, profoundly inhibited JMML cell growth in vitro. The value of this study stems from the fact that these cell culture studies were performed using primary cells from patient samples obtained from 12 different patients with confirmed diagnoses of JMML. These patients accurately represented a spectrum of JMML: some had NF1 abnormalities, some had RAS abnormalities, and some probably had neither. The pathogenesis of JMML is intricately linked with the deregulation of signal transduction through the Ras signaling pathway. This deregulation, regardless of where in the Ras pathway the causative mutation(s) may occur, ultimately results in JMML cells demonstrating in vitro selective hypersensitivity to GM-CSF in granulocyte-macrophage colony-forming proliferation assays (CFU-GM). Activating RAS mutations are found in 15% to 30% of patients with JMML, and NF1 gene abnormalities are found in as many as 30% more. NF1 is a tumor-suppressor gene that encodes for the protein neurofibromin, which serves to inactivate Ras by hydrolyzing it from its active guanosine triphosphate (GTP)-bound state to an inactive guanosine diphosphate (GDP)-bound state. Loss of heterozygosity or other loss of function mutations of NF1, therefore, are essentially equivalent to activating Ras point mutations. All JMML samples examined in this study demonstrated similar growth inhibition by FTIs regardless of the identification within individual samples of RAS mutations, NF1 abnormalities, or other as yet undefined mutations.

FTIs were developed to exploit Ras signal transduction physiology. For Ras proteins to serve as molecular master switches in mitogenic signal transduction, they must be in a membrane-bound, GTP-bound state. Obligatory for Ras cell-transforming activity is the prenylation reaction that attaches a farnesyl group (a 15-carbon isoprenyl group) to Ras to allow its association to the outer membrane of the cell, thus becoming a fully mature protein. Prenylated proteins share characteristic C-terminal sequences such as the CAAX motif. Farnesyl-protein transferase is 1 of 3 enzymes that catalyze protein prenylation. The others are geranylgeranyl protein transferase (GGPTase) types I and II. Selective inhibition of FPTase was explored and developed because geranylgeranylation of normal cellular proteins is 5 to 10 times more prevalent than farnesylation. Several farnesylated proteins play important roles in normal cells, including nuclear lamins essential for nuclear structural integrity, proteins of the retinal visual signal transduction system, the human homologue of the yeast molecular chaperone YDJ1, the skeletal muscle phosphorylase kinase, and others. The question that arises is how can the FTIs exert selective effects on tumor cells when so many normal cells also depend on farnesylation. The answer is likely not simple, but it may in part relate to the sensitivity of particular proteins to the FTIs, the ability of some proteins also to use GGPTase-I, and the dependency of the tumor cell on the Ras signal pathway rather than on the other redundant pathways in normal cells. Reports published recently indicate...
that KRAS-transformed cells may not be nearly as sensitive to FTIs as HRAS-transformed cells in which much of the preliminary testing of the FTIs was performed. These reports demonstrate that KRAS proteins can be prenylated by geranylgeranyl protein transferase if the farnesylation pathway is blocked by an FTI. KRAS is the gene form most often mutated in human tumors. However, the specific RAS mutational status of human tumor cells may not necessarily correlate with their sensitivity to FTIs. In these cases the sensitivity to FTIs may lie in the dependency of the tumor cells on the Ras pathway, or it may raise the possibility that other farnesylated proteins besides Ras contribute to their biologic phenotypes. In this regard, recent studies imply RhoB as a potential alternative or an additional target for the block of farnesylation. Therefore, though FTIs were developed to be specific compounds to block Ras signal transduction, it is becoming evident that inhibiting the farnesylation of proteins other than Ras may play a major role in their mechanism of action. In addition, since the initial development of the FTIs, more knowledge is emerging about the trafficking of Ras. A recent report shows that prenylated CAAAX proteins do not, in fact, associate directly with the plasma membrane; rather, they associate with the endomembrane and are subsequently transported to the plasma membrane. Therefore, it is clear that the full function and mechanism of action of the FTIs remain to be elucidated.

The first FTI used in this study, L-739,749, is the methyl ester of the produg L-739,750. In the initial report on the development of these compounds, L-739,750 was shown to have profound effects on blocking prenylation in vitro, and this was highly selective for farnesylation over geranylgeranylation, as studied by prenylation assays. However, though L-739,750 could block prenylation on a subcellular level, it demonstrated minimal effectiveness in inhibiting whole-cell growth. Conversely, the methyl ester L-739,749 demonstrated less effectiveness in prenylation experiments (though selectively was maintained), but it did have profound effects specifically blocking Ras-transformed growth in whole-cell experiments. The inhibition of Ras-transformed Rat1 cell growth displayed a clear dose dependency of L-739,749 in the range from 1 µmol/L to 20 µmol/L. Similarly, in the current study, we observed a dose-dependent inhibition of JMML spontaneous CFU-GM growth over the same drug concentration range of 1 to 20 µmol/L. The second FTI used in this study was L-744,832, the isopropyl ester of the produg L-739,750. It was employed to demonstrate that the effect of L-739,749 on JMML hematopoietic progenitor cells was not simply the result of a nonspecific toxicity event.

References

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