Farnesyltransferase inhibitor tipifarnib (Zarnestra™, R115777) preferentially inhibits
in vitro autonomous erythropoiesis of polycythemia vera patient cells.

Short tile for running head: tipifarnib in polycythemia vera

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ABSTRACT

Polycythemia vera (PV) is an acquired myeloproliferative disorder with primary expansion of the red cell mass leading to an increased risk of thrombosis and less frequently to myelofibrosis and secondary acute leukemia. Standard therapies include cytoreduction with either phlebotomy and chemotherapeutic agents and antithrombotic drugs. Because long term exposure to cytotoxic chemotherapy may increase the risk of acute transformation, new therapeutic options are needed. Tipifarnib is a non-peptidomimetic inhibitor of farnesyl transferase that was developed as a potential inhibitor of RAS signalling. In the present study we report that tipifarnib used at pharmacologically achievable concentrations strongly inhibits the BFU-E autonomous growth which characterizes PV patients. Moreover at low tipifarnib concentrations (0.15 µM), the inhibitory effect was preferentially observed in PV BFU-E progenitors and not in normal BFU-E progenitors and was not rescued by EPO. Thus tipifarnib may specifically target PV stem cells and may be of clinical interest in the treatment of PV patients.
INTRODUCTION

Polycythemia vera (PV) is a chronic myeloproliferative disorder characterized by an increased production of mature red cells. Despite the fact that the molecular mechanism in PV is still unknown, hypersensitivity of PV hematopoietic progenitors to cytokines and growth factors including erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor, interleukin-3, insulin-like growth factor or stem cell factor has been demonstrated. Thus, spontaneous erythroid burst-forming units (BFU-E) growth represents one of the major features of in vitro PV studies. PV as well as ET (essential thrombocythemia), may progress, with time, to blastic transformation but genetic alterations associated with disease progression have not been extensively studied. Though P53 and N- and K-Ras mutations have been described in a small subset of PV patients in blastic transformation, disruptions of these genes appear to be extremely rare in non-transformed PV patients.

Farnesyl transferase inhibitors (FTIs) have emerged as promising new targeted therapies for malignant diseases. FTIs inhibit farnesylation of low molecular weight GTPases. Among these, RAS proteins require farnesylation to induce activation and oncogenic transformation. Farnesylated proteins other than RAS are also targeted by FTIs. It has been demonstrated that FTI-mediated tumor growth inhibition does not always correlate with Ras mutation status and other pathways such as RhoB, PI3K/Akt/BAD or centromere-associated proteins CENP-E and –F are potential targets for FTIs. Interestingly, FTIs abrogate p38 MAPK activation, shown to be upregulated in the majority of PV patients. Tipifarnib is a nonpeptidomimetic inhibitor of farnesyl transferase with demonstrated activity in human tumor cell lines with either wild-type or mutant Ras. In humans, tipifarnib has so far demonstrated clinical activity in advanced cancer, myelodysplasia, acute myelogenous leukemia and multiple myeloma.
In this study, we show that tipifarnib inhibits spontaneous erythroid progenitor growth from PV patients and may be a novel therapeutic approach in polycythemia vera.
STUDY DESIGN

Patients

Thirteen consecutive PV patients were included in the study after informed consent. The diagnosis of PV was established according to the Polycythemia Vera Study Group diagnostic criteria. None of the patients had entered blastic phase of the disease. Peripheral blood was collected during routine biological control at diagnosis.

Reagents

Farnesyl transferase inhibitor tipifarnib was kindly provided by Janssen and preserved protected from air. Recombinant human EPO was purchased from Roche (Basel, Switzerland) and used at a final concentration of 2 U/mL.

Cell preparation and semisolid clonogenic assay

Fresh peripheral blood mononuclear cells (PBMC) from 13 patients with PV and bone marrow mononuclear cells (BMMC) from 5 healthy donors were isolated by Ficoll-Hypaque gradient centrifugation. Positive selection of CD34+ cells was performed in 3 of the normal BMMC by means of magnetically activated cell sorting (MACS) CD34 isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany). For clonogenic assay, PV PBMC were counted and seeded in semisolid medium (StemCell Technologies) at a concentration of 5 X 10^5 mononuclear cells/mL in 35-mm Petri dishes, in the presence or absence of tipifarnib at a concentration of 10^{-9} to 10^{-6} M. Percentages of inhibition were expressed as the ratio between the number of colonies observed in control and in tipifarnib treated samples. As a control for the non specific activity of tipifarnib, BMMC and CD34 positive cells from normal individuals (n = 5) were studied in the same conditions (BMMC: 10^5 cells/mL, CD34+ cells: 10^3 cells/mL). Spontaneous colony growth was assessed in the absence of exogenous cytokine
in the culture medium. Ability of EPO to rescue PV PBMC colony growth inhibition by tipifarnib was performed by adding EPO (2 U/mL) to the culture medium in the presence or absence of tipifarnib. Results were expressed as the ratio between the number of colonies observed in EPO treated control and in tipifarnib + EPO treated samples. Cultures were incubated at 37°C and 5% CO₂ in air for 14 days, at which time colonies were visualized and scored with an inverted microscope which allows the distinction between CFU-GM, CFU-G, CFU-M and BFU-E/CFU-E. Analyses were performed in triplicate.

**Statistical analysis**

A Student t test was used for BFU-E number comparisons. A value of $P < 0.05$ was used to define statistical significance.
RESULTS AND DISCUSSION

For all PV patients tested, spontaneous growth of erythroid colonies was observed in the absence of EPO as expected. The median BFU-E number was 36 (range 15-130) per 5 X 10^5 mononuclear cells. No spontaneous BFU-E growth could be detected in PBMC or CD34+ cells from healthy donors (n=5) tested in the same period. EPO induced a 4-fold increase of the median BFU-E number of PV PBMC (median 120 per 5 X 10^5 mononuclear cells, range 55-230, P = .0003). Tipifarnib inhibition of spontaneous BFU-E growth was assessed at three different concentrations, 15 nM, 150 nM and 1.5 µM. As shown in Figure 1, tipifarnib-mediated inhibition was dose-dependent with a mean percentage of inhibition of 41% ± 24% (n = 13 at 15 nM), 65% ± 19% (n = 12 at 150 nM) and 82% ± 9% (n = 8 at 1.5 µM). Of note, these concentrations can be easily achieved in patients with a daily dose ranging from 25 mg to 300 mg.16

Tipifarnib-erythroid progenitor growth inhibition in PV patients was next assessed in the presence of EPO. Mean number of BFU-E was of 128.5 ± 53 (n = 13) in the presence of EPO alone. In EPO treated samples, addition of tipifarnib 1.5 µM, drastically reduced BFU-E number in all patients to a mean value of 4.5 ± 2 (Figure 2A). Interestingly, presence of EPO did not rescue BFU-E growth inhibition. Inhibition persisted up to a 15 nM concentration of tipifarnib. Partial rescue was only observed at 1.5 nM of tipifarnib (Figure 2B). Thus, an inverse dose response relationship was observed between inhibition of BFU-E colony formation and efficacy of rescue by EPO, suggesting that tipifarnib is able to interfere with EPO and may be other growth factor signaling pathways in PV precursor cells.

In order to assess the specificity of tipifarnib-induced-growth arrest in BFU-E from PV patients, normal bone marrow purified mononuclear cells from healthy donors were incubated with identical concentrations of the drug in the presence of EPO 2 U/ml (n = 3). While tipifarnib equally inhibits BFU-E growth of normal progenitors, a 10 fold higher
concentration is required to obtain the same inhibitory effect observed in PV erythroid progenitors (Figure 1). Furthermore, tipifarnib concentrations below 150 nM did not reduce normal GM-CFU or G-CFU colony numbers (n = 3, data not shown) indicating a specific effect of tipifarnib on PV BFU-E.

Our results indicate that tipifarnib might be useful in the treatment of patients with PV. We found a differential effect of the drug on malignant and non-malignant cells in the presence of EPO, at tipifarnib doses below 150 nM. These observations suggest that PV erythroid precursor cells might be more sensitive to tipifarnib than normal progenitor cells. The target of tipifarnib in PV remains unknown. As p38 MAPK upregulation has been shown in PV patients,9 tipifarnib may either target upstream or downstream p38 MAPK signaling pathways and result in PV cell growth arrest. Imatinib mesylate, which targets tyrosine kinases encoded by oncogenes such as Bcr-Abl, c-Kit and PDGF-R, reduces PV progenitor spontaneous growth but unlike tipifarnib fails to inhibit the BFU-E proliferation in the presence of hematopoietic growth factors.17

Conventional treatment of PV relies on myelosuppressive drugs such as hydroxyurea or pipobroman which unfortunately may increase the risk of leukemia. Clinical trials with other drugs such as anagrelide or interferon alpha are currently being evaluated with the aim to propose alternative therapies. Nevertheless, as these compounds are not well tolerated and do not appear as effective as conventional treatments to achieve rapid and sustained long term remission, other approaches should be tested.

Clinical trials with tipifarnib have shown that the drug was well tolerated. In a phase I study in refractory and relapsed leukemic patients, drug-induced myelosuppression (absolute neutrophil count < 100/ml) occurred only at the 600 mg level and above.18 Our results suggest that tipifarnib used at low doses (around 100 mg daily) could be safely investigated in PV patients.
REFERENCES


**LEGEND**

**Figure 1: Dose-dependent effect of tipifarnib on spontaneous BFU-E growth.** 5 X 10⁵/ml peripheral blood mononuclear cells from PV patients were plated for 14 days in the presence of various concentrations of tipifarnib (15 nM to 1.5 µM) without addition of EPO in the medium and BFU-E numbered. Results are given as mean ± SD of triplicates BFU-E. Controls are BFU-E obtained from bone marrow mononuclear cells of healthy donors cultured in the presence of EPO 2 UI/mL and tipifarnib (15 nM to 1.5 µM).

**Figure 2: EPO does not relieve tipifarnib BFU-E growth inhibition.** (A). BFU-E colony number in PV patients (n = 8) at 1.5 µM tipifarnib concentration in the absence or presence of EPO. (B). ♦ **dose-dependent BFU-E growth inhibition:** ratio of BFU-E colony growth assessed in PV samples between medium alone and tipifarnib treated samples (n = 8); ■, **EPO rescue of tipifarnib BFU-E growth inhibition:** ratio of BFU-E colony growth between EPO and tipifarnib + EPO treated samples (n = 8). BFU-E colonies were counted at day 14 with each experiment assessed in triplicate.
Figure 1.
Figure 2.

A.

B.

% of BFU-E inhibition (%) vs. % of BFU-E rescue (%) for different concentrations of Tipifarnib.
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