Fasudil, a clinically safe ROCK inhibitor, decreases disease burden in a Cbl/Cbl-b deficiency-driven murine model of myeloproliferative disorders

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Objectives: Mutations in Cbl or Cbl-b gene occur in 10% of myeloproliferative disorder (MPD) patients and are associated with poor prognosis. Hematopoietic Cbl/Cbl-b double knockout (DKO) leads to a disease in mice phenotypically similar to human MPDs. The aim of this study was to evaluate the anti-MPD activity of a clinically safe drug, Fasudil, identified in an *in vitro* kinase inhibitor as an inhibitor of proliferation of DKO mouse hematopoietic stem/progenitor cells (HSPCs).

Methods: Fasudil exhibited relatively selective anti-proliferative activity against Cbl/Cbl-b DKO vs. control murine bone marrow HSPCs. We established a mouse model with uniform time of MPD onset by transplanting Cbl/Cbl-b DKO HSPCs into busulfan-conditioned NOD/SCID/gamma chain-deficient mice. Four weeks post-transplant, mice were treated with 100 mg/kg fasudil (13 mice) or water (control, 8 mice) daily by oral gavage, followed by blood cell count every 2 weeks.

Results: By 2 weeks of treatment, total white cell and monocyte counts were significantly lower in mice treated with fasudil. We observed a trend towards improved survival in fasudil-treated mice that did not reach statistical significance. Notably, prolonged survival beyond 27 weeks was observed in two fasudil-treated mice, nearly twice the 16-week average life-span in the Cbl/Cbl-b DKO MPD model.

Conclusions: Our results suggest a therapeutic potential for fasudil, a clinically safe drug with promising results in vascular diseases, in the treatment of MPDs or other mutant Cbl-driven myeloid disorders.

Keywords: Myeloproliferative diseases, Fasudil, Rho kinase, Cbl, Myosin light chain, Mouse model

Introduction

Myeloproliferative neoplasms/disorders (MPNs/MPDs) are clonal hematopoietic stem cell disorders characterized by over-proliferation of one or more myeloid cell lineages in the bone marrow (BM) and increased numbers of mature and immature myeloid cells in the peripheral blood. Excess proliferation is frequently associated with splenomegaly and cardiovascular complications as well as increased risk of transformation to acute leukemia. MPNs/MPDs are heterogeneous disorders with variable clinical courses.[1] Patients with long-standing disease frequently develop myelofibrosis with extra-medullary hematopoiesis, and therapeutic options are extremely limited in that situation. Allogeneic hematopoietic stem cell transplantation is currently the only curative option for advanced MPDs, however many patients are ineligible for transplantation because of advanced age, medical co-morbidities, and multiple organ dysfunctions secondary to systemic fibrosis.[2]

Recently, mutations in the Cbl gene family member Cbl, and less commonly Cbl-b, were reported by multiple independent investigators to be present in about

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10% of patients with MPNs/MPDs, and these patients tend to have poorer prognosis.[3,4] Cbl and Cbl-b are highly related E3 ubiquitin ligases that function in hematopoietic cells to negatively regulate signaling of tyrosine kinase-coupled cell-surface receptors activated by growth factors, antigenic signals, cytokines, and other stimuli.[5] Mutations in the Cbl gene lead to loss of negative regulatory control mediated by Cbl proteins, uncontrolled signaling from surface growth factor/cytokine receptors, and uncontrolled cellular proliferation that may evolve into malignancy.[6]

We have previously demonstrated that the Cbl/Cbl-b double knockout (DKO) mice, in which a floxed Cbl gene is deleted by MMTV-Cre and Cbl-b gene is constitutively deleted, develop a hematological disease that is phenotypically similar to human MPNs/MPDs.[7] We undertook an *in vitro* tyrosine kinase screen that demonstrated the differential anti-proliferative activity of fasudil on BM cells isolated from Cbl/Cbl-b DKO mice compared to control BM cells from mice from same genetic background (data not shown). Fasudil is already clinically approved for use in Japan for the prevention of vasospasm that follows subarachnoid hemorrhage.[8,9] It has shown clinically beneficial activity in patients with pulmonary hypertension1[10] and clinical trials are ongoing to confirm such observations.[11] If fasudil activity can be shown in a disease model for MPNs/MPDs, it would be a highly attractive agent to be taken to early phase clinical trials in patients with MPNs/MPDs given the fact that fasudil is a clinically approved drug and has demonstrated safety profile in hundreds of patients.[12]

Materials and methods

Reagents and antibodies

Fasudil was purchased from LC Laboratories (Woburn, MA). Busulfan and Cremophor were purchased from Sigma-Aldrich (St Louis, MO). Fluorochrome-labeled monoclonal antibodies to CD45.1 and CD45.2 were purchased from BD Biosciences (San Jose, CA). The murine recombinant cytokines stem cell factor (SCF), thrombopoietin (TPO), and FMS-like tyrosine kinase 3 ligand (FLT3L) were obtained from PeproTech (Rocky Hill, NJ). X-VIVO medium was obtained from Lonza (Basel, Swiss). Antibodies to myosin light chain (MLC) 2 were purchased from Cell Signaling Technology (Danvers, MA).

In vitro BM cell proliferation assay

Cell proliferation was assayed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol. In brief, lineage-negative cells were purified from the whole BM cells using lineage-depletion kit (Miltenyi Biotec, San Diego, CA) to remove mature cell populations, thus yielding enriched hematopoietic stem and progenitor cell fraction. Purified cells (3×10^3) were plated per well in 96-well plates and cultured in the presence of 50 ng/ml SCF, 10 ng/ml TPO, and 10 ng/ml FLT3L for 3 days. Luminescent signal after adding the CellTiter-Glo reagent was read using LUMIstar OPTIMA (BMG LABTECH, Cary, NC).

Animals

$Cbl^{flox/flox}$; $Cblb^{-/-}$ and MMTV-Cre transgenic mice were backcrossed to C57BL/6 for at least five generations before being intercrossed to generate the MMTV-*Cre*; $Cbl^{flox/flox}$; $Cblb^{-/-}$ mice as described previously.[7] For ease, these mice will be referred to as the Cbl/Cbl-b DKO mice in the remainder of the text. NOD/SCID/gamma chain-deficient (NSG) mice (6–8 weeks old) used for BM transplantation-based MPD model were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were housed under specific pathogen-free conditions in the animal care facility at the Center for Comparative Medicine of the University of Nebraska Medical Center (UNMC). All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the UNMC.

BM transplantation

The BM cell donor Cbl/Cbl-b DKO mice were sacrificed at 2 months after birth, when they had started to show signs of MPD. BM was collected from the femurs and leg bones, and marrow cells were suspended in phosphate-buffered saline (PBS). Busulfan was dissolved in DMSO at 100 mg/ml and mixed with nine volumes of 50% Cremophor formulation (10% Cremophor EL, 15% propylene glycol, 25% ethanol, and 50% PBS). Immediately before injection, the above solution was diluted with 5% glucose to adjust the final concentration of busulfan to 4 mg/ml. Recipient NSG mice were conditioned by intraperitoneal injection of two doses of busulfan at 20 mg/kg body weight 24 hours apart, as described in Hayakawa *et al.*[13] Twenty-four hours after the second busulfan injection, 4×10^6 total BM cells isolated from Cbl/Cbl-b DKO donors were injected intravenously in recipient mice. Peripheral blood was obtained by submandibular vein bleeding[14] every 2 weeks, starting at 4 weeks after transplant, and total white blood cell (WBC), granulocyte (GRA), and monocyte (MON) counts were performed on a Scil Vet abc Animal Blood Counter (Scil Animal Care, Gurnee, IL). Mice were euthanized when they exhibited severe distress from disease.

Flow cytometry

All transplant recipient NSG mice were monitored by peripheral blood analysis every 2 weeks after

transplantation. Peripheral blood was obtained by submandibular vein bleeding.[14] Red blood cells were lysed by ACK lysis buffer (Quality Biological, Gaithersburg, MD) and mononuclear cells were labeled with antibodies against CD45.1 (recipient cells) and CD45.2 (donor cells). Flow cytometry was performed on a BD LSRII or Aria II at the UNMC Flow Cytometry Core Facility. Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

Fasudil treatment

Starting 4 weeks after transplantation, 13 mice received 100 mg/kg fasudil dissolved in water by gavage once daily (treatment group) and eight mice received the same volume of water (control group). Treatments were continued until the end of the experiment. Total WBC, GRA, and MON counts were performed as above, every 2 weeks, until the mice were euthanized due to severe distress from disease. The onset and persistence of disease over time, and survival times were recorded.

Western blotting

Peripheral blood samples were obtained prior to euthanasia of mice. Protein lysates were prepared by lysing cells in a RIPA buffer and Western blot was carried out as described previously.[15]

Statistical analysis

The statistical significance of differences in total WBC, GRA, and MON counts between control and fasudil-treated groups were determined using Student's *t*-test. Log-rank (Mantel–Cox) test was used to compare survival between treated and control groups. GraphPad Prism was used for statistical analysis. P values <0.05 were considered statistically significant.

Results

Fasudil selectively inhibits Cbl/Cbl-b DKO cell expansion in vitro

To validate the potential anti-proliferative activity of fasudil observed in a larger screen using a kinase inhibitor library (data not shown), we first performed a proliferation assay, in which lineage-negative (Lin−) BM cells representing hematopoietic stem and progenitor cell populations were isolated from the wild-type (WT) control or the Cbl/Cbl-b DKO mice and cultured *in vitro* in the presence of cytokines for 3 days with water alone or with various concentrations of fasudil (Fig. 1). Fasudil, at 0.01–1 μM concentrations, induced a dose-dependent inhibition of proliferation of both the control and the DKO BM cells. However, the inhibitory effect was more pronounced on the Cbl/Cbl-b DKO cells, especially at 0.1 and 1 μM concentrations, with the differences at 1 μM being statistically significant. These results not only confirmed the anti-proliferative activity of fasudil against hematopoietic cells but also suggested the relative selectivity of fasudil against Cbl/Cbl-b DKO cells compared to control BM cells.

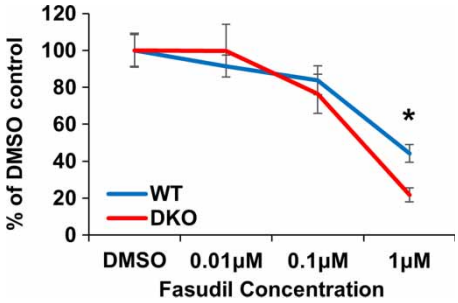

Figure 1 Fasudil inhibited Cbl/Cbl-b DKO Lin− BM cell proliferation *in vitro*. BM cells were collected from WT control or Cbl/Cbl-b DKO mice and Lin− cells were purified using magnetic beads and 3 × 103 Lin− cells were plated in each well in 96-well plate and cultured in X-VIVO medium with SCF 50 ng/ml, TPO 10 ng/ml, and FLT3L 10 ng/ml. Cell proliferation was assayed using CellTiter-Glo® Luminescent Cell Viability Assay. Data shown are one representative experiment of three independent repeats with similar results. Data are shown as mean ± SD (*$P < 0.05$).

All recipient NSG mice developed MPD

Because leukocytes derived from the recipient NSG and Cbl/Cbl-b DKO donor mice express CD45.1 and CD45.2 allotypes, respectively,[16] cell origins could be readily distinguished by cell-surface staining for CD45 allotypes followed by flow cytometry. Analysis of the transplanted NSG recipient mice demonstrated that only those receiving the Cbl/Cbl-b DKO BM donor cells, but not those receiving the control BM donor cells, exhibited severe MPD, as demonstrated by the leukocytosis, hepatosplenomegaly, and rapid lethality (Fig. 2A–D). Using this transplantation system, we next established a cohort of recipient NSG mice that were transplanted with the DKO BM cells. All recipient NSG mice survived transplantation and demonstrated engraftment with donor cells, with a mean donor chimerism of 88% (Fig. 3A). All mice developed signs of MPD, based on elevated total WBC, GRA, and MON counts as early as 4 weeks after transplantation (Fig. 3B–D).

Fasudil decreases MPD disease burden in mice

After 2 weeks of treatment with fasudil, total WBC, GRA, and MON counts were significantly lower (Fig. 3) in mice treated with fasudil ($P = 0.004$, 0.012, and 0.009, respectively). For the entire fasudil-treated mouse group (Fig. 4), we observed a trend towards improved survival compared to that in the control group, although this did not reach statistical significance ($P = 0.07$). However, an analysis of the male recipients only ($n = 6$ for control and $n = 7$ for fasudil treatment) revealed a significant survival

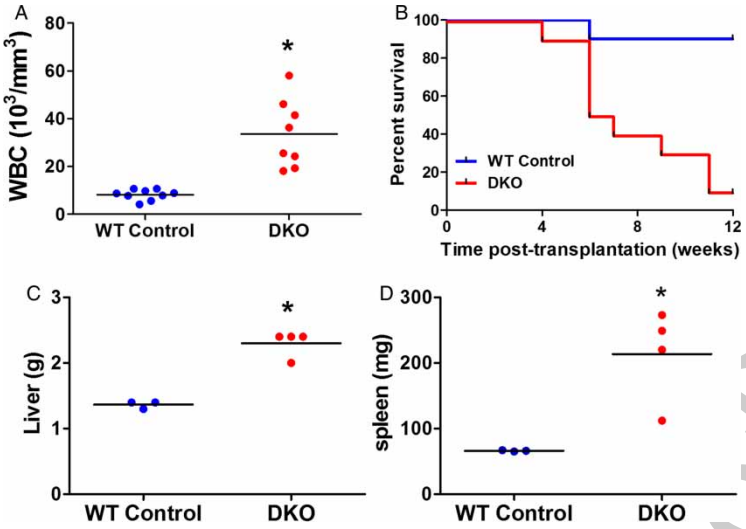

Figure 2 DKO donor cell induced MPD in NSG recipient mice. (A, B) Whole BM cells (4×10^6) from control or DKO mice were transplanted into pre-conditioned NSG recipients. Six weeks after transplantation, whole blood cell count was performed (A) and overall survival was recorded (B). (C, D) Whole BM cells (2×10^6) from control or DKO mice were transplanted into pre-conditioned NSG recipients. Recipients were sacrificed at 4 weeks after transplantation and liver (C) and spleen (D) were weighted to evaluate the hepatosplenomegaly. Each dot represents one individual recipient (*$P < 0.05$).

advantage in the fasudil-treated group compared to the control group ($P = 0.04$).

Fasudil treatment imparted prolonged survival in a subset of mice with Cbl/Cbl-b DKO BM transplant-induced MPD

While all untreated mice succumbed to MPD between 12 and 16 weeks after transplant, survival beyond 27 weeks was observed in two fasudil-treated mice, which is nearly twice the mean life-span of the untreated mice with the Cbl/Cbl-b DKO BM transplant-induced MPD (16 weeks). The two long-term survivors had undetectable levels of MLC, a downstream target of ROCK phosphorylation (Fig. 5).

Discussion

In this study, we assessed the potential therapeutic activity of fasudil, a Rho kinase inhibitor, in a model of MPD arising from the transplant of Cbl/Cbl-b DKO BM hematopoietic stem and progenitor cells into NSG recipient mice. This effort was based on an *in vitro* screen that identified fasudil with preferential a relatively selective anti-proliferative activity against the highly proliferative Cbl/Cbl-b DKO mouse BM cells compared to the control cells. Given its safety profile in clinical use in hundreds of patients with vascular conditions, our results support a potential therapeutic role of fasudil in MPDs.

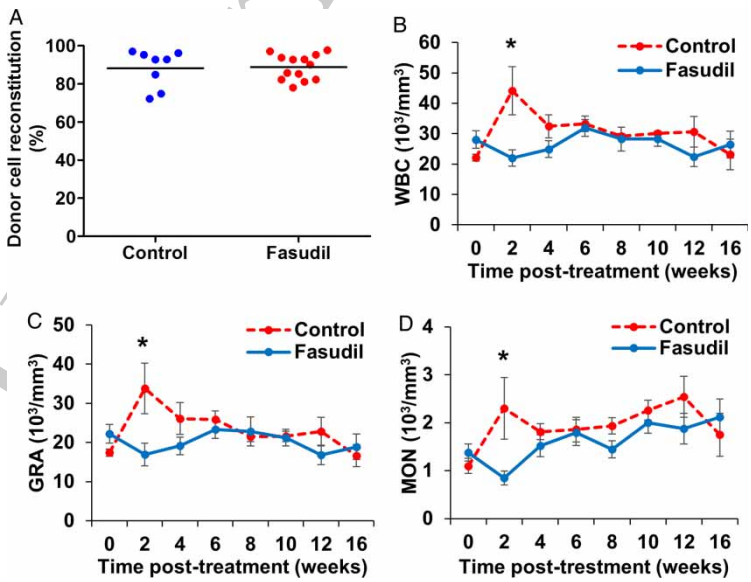

Figure 3 Fasudil decreased MPD disease burden. Four weeks after BM transplantation (week 0), engraftment of donor cells was analyzed by flow cytometry (A). Recipient mice were then treated with water (control) or fasudil. Peripheral blood was collected and analyzed every 2 weeks for total WBC (B), GRA (C), and MON (D) counts. Data are shown as mean ± SD (*$P < 0.05$).

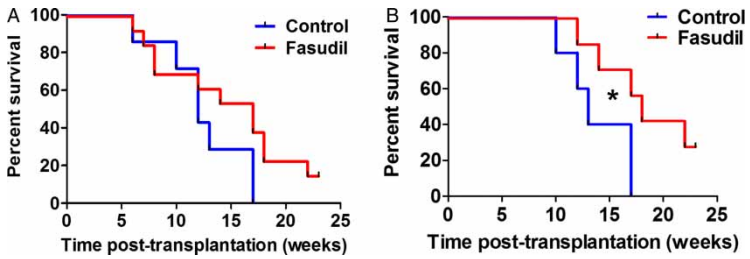

Figure 4 Survival of Cbl/Cbl-b DKO mice treated with fasudil ($n = 13$) compared to controls ($n = 8$). (A) All mice (no significant difference seen; $P = 0.07$) and (B) male mice ($n = 12$) showing a statistically significant difference in survival with fasudil treatment (*$P = 0.04$).

Rho kinases (ROCKs), which are targeted by fasudil, are involved in regulating a myriad of physiologic functions including cytoskeletal reorganization, cell migration, adhesion, survival, and proliferation. They do so via activating several different downstream pathways such as MLC phosphatase. To date, most of the conclusions with regard to the function of ROCKs have involved the use of cell lines, pharmacologic inhibitors, and dominant negative approaches. Importantly, the role of ROCK in hematopoiesis, myeloid biology, or leukemogenesis *in vivo* remains poorly understood. Mali *et al.*[17] demonstrated constitutive activation of ROCK in leukemic cells expressing activating forms of KIT, Flt-3, and breakpoint cluster region–Abelson kinase, which are oncogenes commonly found in patients with systemic mastocytosis, acute myeloid leukemia, and chronic myeloid leukemia, respectively. Mali *et al.*[17] showed that pharmacologic inhibition of ROCK in leukemic cells inhibited the growth and survival of leukemic cells *in vitro* and, importantly, prolonged survival of leukemic mice.

Our results suggest a promising role of fasudil in decreasing the morbidity of MPD through reduction in WBC, GRA, and MON counts. There was a trend towards survival advantage as well, which did not reach statistical significance in the overall cohort analyzed. Given the fact that fasudil significantly impaired the Cbl/Cbl-b DKO BM cell expansion *in vitro* (Fig. 1), the lack of a statistically significant difference between the groups could be explained by: (1) the small sample size, or (2) superior metabolism of fasudil in rodents with reduced systemic exposure with single daily dosing, as has been shown.[8,18–20] The fasudil effect however was statistically significant when the males alone were compared. The differential effect of sex on the survival of transplanted NSG mice is of interest. We observed a more aggressive disease phenotype in female mice in our Cbl/Cbl-b DKO murine model, which likely explains the poorer survival and lesser benefit of fasudil treatment in this subset (Fig. 5). Sanchez-Aguilera *et al.*[21] have recently shown that estrogen receptor (ER) differentially regulates the survival and proliferation of hematopoietic stem/progenitor cells (HSPCs) and that tamoxifen treatment blocked the development of $JAK2^{V617F}$-induced MPN *in vivo*, induced the apoptosis of human $JAK2^{V}617^{F+}$ HSPCs in a xenograft model, and sensitized MLL-AF9+ leukemias to chemotherapy. Apoptosis was selectively observed in mutant cells, and tamoxifen treatment only had a minor impact on steady-state hematopoiesis in disease-free animals.[21] $JAK2^{V}617^{F}$ is the most common genetic abnormality reported in MPDs[22,23] and is associated with constitutive activation of downstream STAT5 signaling and heightened sensitivity to erythropoietin and other hematopoietic growth factors leading to myeloid lineage expansion and subsequently MPD.[23] A differential effect of ER signaling akin to that demonstrated on $JAK2^{V617F}$-driven clonal hematopoiesis may explain the more aggressive disease phenotype we observed in female recipient mice in our Cbl/Cbl-b DKO murine model.

Lastly, the association between the prolonged survival observed in two mice treated with fasudil (>27 weeks), which is almost double the mean survival time in our Cbl/Cbl-b DKO model, and the total depletion of MLC in the peripheral blood of these survivors (Fig. 5) is intriguing. Mali *et al.*[17] found MLC to be constitutively hyperphosphorylated on Ser19 in leukemic cells, whose activation could be rapidly inhibited upon treating the leukemic cells with ROCK inhibitors, suggesting that the antileukemic

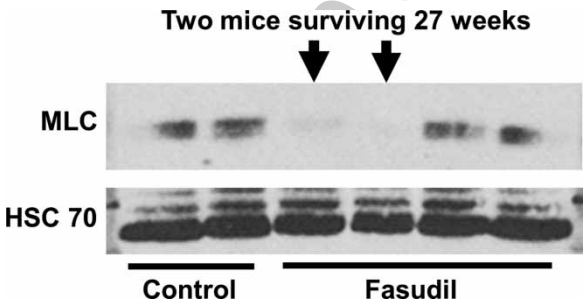

Figure 5 Fasudil reduced MLC level in recipient mice. Whole BM cells were collected from recipient mice and analyzed for the expression MLC; a downstream target of fasudil. Undetectable MLC, by Western blot, in the two mice that had extended survival (beyond 27 weeks).

activity of ROCK inhibition is mediated through inhibition of the downstream MLC signaling. Inhibition of MLC leads to the destabilization of actin filaments and subsequently cell death.[24] It remains unclear why such a dramatic benefit was seen in these two mice compared to the others, despite an identical genetic background.

Our data suggest that fasudil has the potential to ameliorate the disease burden in MPD and, given its demonstrated safety in large cohorts of patients with subarachnoid hemorrhage, testing its safety and efficacy in early phase clinical trials in patients with MPD is warranted. There is an oral formulation of fasudil that is currently in clinical trials in patients with primary pulmonary hypertension (PH). PH is an under-recognized complication of long-standing MPD (in the 'spent phase') and leads to significant morbidity. Ruxolitinib, a JAK2 inhibitor currently in clinical use to ameliorate systemic symptoms of MPD, was shown to improve echocardiographic findings in 66% of patients with MPD-associated PH in a small trial of 12 patients. That improvement was associated with reduction in nitric oxide plasma levels.[25] Given the lack of overlapping toxicities between fasudil and JAK2 inhibitors, a clinical trial combining both of these agents in patients with MPDs would be of considerable interest given the potential for their additive, and possibly synergistic, effects.

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Disclaimer statements

Contributors H.B. and V.B. conceived the study. H.B., B.W., W.A., and D.F. designed experiments. B.W., W.A., D.F., S.N., B.M., and M.S. performed experiments. B.W., W.A., and H.B. analyzed data. B.W., W.A., and H.B. wrote and S.N., B.M., M.S., and V.B. edited the manuscript.

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Ethics approval None.

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