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AKT activation is a feature of *CALR* mutant Myeloproliferative Neoplasms

Chunling Fu^{1,2}, Qiang Jeremy Wen², Christian Marinaccio², Te Ling², Wei Chen^{1,2}, Marinka Bulic², Terra Lasho³, Ayalew Tefferi³, John D. Crispino^{2,*}, and Kailin Xu^{1,*}

¹Blood Disease Institute, Xuzhou Medical University, Xuzhou, China

²Division of Hematology/Oncology, Northwestern University, Chicago, IL, USA

³Division of Hematology/Oncology, Mayo Clinic, Rochester, MN, USA

Among the driver mutations in the MPNs, the mechanism by which *CALR* mutations activate JAK/STAT signaling is unique in that the novel C-terminus of *CALR* mutant proteins binds directly to MPL leading to constitutive activation (1, 2). *CALR* mutants have also been shown to activate the MAPK pathway (3). Whether *CALR* mutants further activate PI3K/AKT signaling is controversial, with several studies reporting a lack of (or modest) activation (2, 4), but another demonstrating potent activation (5). The former studies relied upon transduction of Ba/F3 TpoR cells with *CALR* mutant alleles whereas the latter probed the pathway in cells lines with megakaryocytic potential. Moreover, the extent to which PI3K/AKT signaling is a target in *CALR* mutant MPNs has been debated, with at least one study reporting that inhibitors of this pathway do not synergize with ruxolitinib (2).

To address the discrepancy, we assayed for activation of JAK/STAT and AKT in multiple relevant settings. First, we overexpressed the *CALR* type 1 (del52) and type 2 (ins5) mutants in primary murine c-kit⁺ bone marrow progenitors and cultured the cells for 48 hours. We observed enhanced activation of both STAT5 and AKT (Fig 1a). Next we transplanted bone marrow transduced with *CALRdel52* mutant or empty vector to irradiated recipient mice and assessed AKT activation. Both intracellular flow and western blots of spleen cells revealed increase in p-AKT (Fig 1b,c). Finally, we assayed for total and phospho-AKT in primary *CALR* mutant MPN samples. We observed robust increase in AKT phosphorylation in patient derived CD34⁺ cells (Fig 1d and Supplementary Table 1). This was accompanied by an increase in cyclinD3 consistent with the increased proliferation of the *CALR* mutant cells. These cells also expressed MPL, consistent with the manner in which *CALR* mutants activate signaling (Fig 2d).

We previously reported that inhibition of AKT activity with the selective AKT inhibitor MK-2206 resulted in reduced growth of MPLW515L expressing cells both in vitro and in vivo, suggesting that this pathway is a therapeutic target in ET and PMF (6). To determine

*Co-corresponding authors: Kailin Xu, MD, Blood Disease Institute, Department of Hematology, The Affiliated Hospital of Xuzhou Medical University, 84 West Huahai Road, Xuzhou, Jiangsu, 221002, China, 0516-8580-2382, lihmd@163.com, John Crispino, PhD, Northwestern University, 303 East Superior Street, 5-123, Chicago, IL 60611, 312-503-1504, j-crispino@northwestern.edu.

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whether *CALR* mutant expressing cells are similarly dependent on PI3K/AKT signaling, we treated CD34⁺ cells from PMF patients or healthy individuals with MK-2206. We found that *CALR* mutant cells were susceptible to AKT inhibition and more sensitive than healthy progenitor cells in colony assays (Fig 1e,f). Of note, the differential effect was much more significant for megakaryocyte colonies (Fig 1f) than for myeloid colonies (Fig 1e), consistent with a reliance of megakaryocytes on MPL signaling. Finally, while the level of p-AKT was higher in bone marrow cells from mice transplanted with *CALRdel52* expressing cells compared to those with empty vector, the degree of inhibition was similar (Fig 1g).

MK-2206 was well tolerated in healthy C57Bl/6 mice with no evidence of myelosuppression or impaired hematopoiesis (6). To determine the extent to which the drug suppressed thrombocytosis and enhanced megakaryopoiesis induced by mutant *CALR*, we transplanted *CALRdel52* mutant expressing hematopoietic progenitor cells into irradiated recipients. Recipients developed splenomegaly, hepatomegaly and thrombocytosis by 14 weeks (Fig 2a, f), similar to previous reports (1, 4, 7). Treatment of mice with MK-2206 20 weeks post-transplant led to significant reductions in spleen and liver weights (Fig 2b), as well as the total cell count in the spleen, but not the bone marrow (Fig 2c). The absence of a striking bone marrow effect could be the result of a greater dependence of hematopoiesis in the spleen in this animal model. We also observed decreases in the percentages of c-kit⁺ and CD41⁺ cells and the number of megakaryocytes in the spleen, but not the bone marrow (Fig 2d-g). Analysis of p-AKT levels revealed decreases in both total spleen and bone marrow cells (Fig 2h). This inhibition of AKT signaling failed to reduce the degree of thrombocytosis (Fig 2i), but it did prevent a further increase in the platelet count (Fig 2j). MK-2206 also had no significant effect on the hematocrit or other peripheral blood parameters (Fig 2i and data not shown). Given the modest effect of AKT inhibition as a single agent, we next combined MK-2206 with ruxolitinib. We observed synergy in inhibition of colony formation from murine hematopoietic progenitor cells expressing

CALRdel52 (Supplementary Table 2). Of note, CFU-Mk assays revealed a much stronger synergy of MK-2206 with ruxolitinib than myeloid colonies. These results suggest that although MK-2206 showed modest activity as a single agent, combining it with ruxolitinib may enhance the anti-tumor effect of both drugs, especially against cells that are dependent upon MPL.

Although ruxolitinib shows similar efficacy patients with any of the three driver mutations (8), it has limited use in that most patients progress or become intolerant within 2–3 years (9). Therefore, novel agents to enhance or follow ruxolitinib are needed. Here we demonstrate that AKT signaling is indeed increased in *CALR* mutant MPN patients and further reveal that AKT is a therapeutic target in this group of patients. With development of new PI3K/AKT inhibitors, inhibition of this pathway should be pursued in combination with ruxolitinib for the group of patients with *CALR* mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: PI3K/AKT signaling is active in *CALR* mutant MPNs.

a) Western blot and quantitation of STAT5 and PI3K/AKT activation (pS473) in murine bone marrow cells expressing wild-type *CALR*, *CALRdel52* or *CALRins5* mutants versus those with *Migr1* empty vector. b) AKT phosphorylation as measured by intracellular flow cytometry of spleen cells from recipients of bone marrow cells transduced with *CALRdel52* or empty vector, 20 weeks post transplantation. Right) Mean fluorescence intensity (MFI) of the flow cytometry data. Mean $\pm$ SD is shown for n=2 *Migr1* and 3 *CALRdel52* samples. * p<0.05. c) Western blot and quantitation of pS473 and total AKT levels in spleen cells from recipients of bone marrow cells transduced with *CALRdel52* or empty vector. d) Western blots and quantitation of AKT phosphorylation in human MPN patient samples. e,f) CFU-Myeloid (e) and CFU-MK (f) assays for *CALR* mutant MPN versus healthy CD34⁺ cells treated with MK-2206. n=3; the samples correspond to MPN-1, MPN-2, and MPN-3. The responses are significantly different as measured by 2-way ANOVA, p=0.031 (e) and p<0.001 (f). g) Western blot and quantitation of AKT phosphorylation in bone marrow cells harvested from mice transplanted with *CALRdel52* expressing cells cultured in the presence

of increasing doses of MK-2206 *in vitro* for 24 hours. Bar graphs depict mean $\pm$ SD. n=3 biological replicates. Grb2 is included as a loading control for the western blots.

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Figure 2: AKT is a therapeutic target in *CALR* mutant MPNs.

Captisol (vehicle) or 120 mg/kg MK-2206 were administered to *CALRdel52* mice by oral gavage for four weeks, five days a week, beginning 20 weeks post-transplant. Hematopoiesis was analyzed at 24 weeks post-transplant. **a**) Images of spleens and livers from captisol versus MK-2206 treated animals. **b**) Spleen and liver weights from mice treated with captisol or MK-2206. Typical spleen and liver weights of healthy mice are depicted by a dotted line. **c**) Total spleen and bone marrow cell counts with captisol or MK-2206 treatment. **d,e**) Percentages of GFP⁺ CD41⁺ (**d**) or GFP⁺ c-kit⁺ (**e**) cells in the spleen or bone marrow of mice following captisol or MK-2206 treatment. **f**) Hematoxylin/eosin stained spleen and sternum bone marrow sections from mice treated with captisol or MK-2206. Scale bars=100 μm (20x) and 50 μm (40X). Original magnification 200X and 400X. **g**) Quantitation of the numbers of megakaryocytes in the bone marrow and spleen of treated mice. **h**) Western blot evaluating the levels of AKT, p-AKT and cyclinD3 in treated mice. Grb2 is included as a loading control. **i**) Hematocrit and platelet counts of mice at different

time points post-transplant. Mice were treated with MK-2206 beginning at 20 weeks. Left and right arrows indicate the initiation and cessation of treatment, respectively. j) Fold change in platelet counts during MK-2206 therapy. * $p < 0.05$, ** $p < 0.01$.

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