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Combined MEK and JAK inhibition reduces osteopontin plasma level and bone marrow fibrosis in a myelofibrosis mouse model

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Dear Editor,

Myelofibrosis (MF), either primary or secondary, is the most aggressive among Philadelphia-negative myeloproliferative neoplasms (MPNs), since it is characterized by an increased risk of leukemic progression and an inferior life expectancy [1]. Key features of MF include clonal myeloproliferation, extramedullary hematopoiesis, extensive bone marrow (BM) fibrosis deposition, and chronic inflammation [2]. MF progression is primarily driven by neoplastic clone hyperproliferation and the excessive production of proinflammatory and profibrotic mediators [3]. While considerable progress has been made in identifying the genomic alterations responsible for clonal myeloproliferation, particularly driver mutations in *JAK2*, *MPL*, and *CALR* that activate the JAK/STAT pathway [4], the molecular mechanisms underlying the abnormal mediator production and the disrupted interaction between malignant hematopoietic cells and the BM microenvironment are still not fully understood. Currently, with the exception of hematopoietic stem cell transplantation, no medical or pharmacologic treatment cures MF. Targeted JAK1/2 inhibitors, while effective in reducing splenomegaly and inflammation-related symptoms, fail to address BM fibrosis and disease burden in most patients [5, 6]. Therefore, the development of novel therapeutic strategies aimed at restoring BM architecture and targeting both clonal myeloproliferation and fibrosis remains a critical unmet need in MF management.

We previously showed that the transcription factor MAF, whose levels have been found to be elevated in MF patients, upregulates the production of proinflammatory and profibrotic mediators from differentiated myeloid cells (mainly monocytes and megakaryocytes) derived from malignant CD34+ hematopoietic stem and progenitor cells. *SPP1* (encoding osteopontin, OPN), a direct target of MAF, was identified as a key player in this process, with OPN overproduction contributing to fibroblast proliferation and collagen deposition, thereby driving myelofibrosis. Moreover, OPN plasma levels were significantly elevated in

MF patients compared to healthy donors, with even higher levels observed in overtly fibrotic versus pre-fibrotic patients. These elevated OPN levels also correlated with poorer overall survival, highlighting the role of OPN in MF pathogenesis [5]. Building on these results, we further identified that ERK1/2 signaling supports OPN production, and pharmacological inhibition with Ulixertinib reduced BM and spleen fibrosis in a thrombopoietin receptor agonist (TPO-RA- treated mouse model of MF. These effects were primarily due to the reduction of OPN expression and function, as similar effects were observed with an anti-OPN neutralizing antibody [6].

It is well established that constitutive activation of the JAK/STAT pathway via driver mutations leads to downstream activation of the mitogen-activated protein kinase (MAPK) pathway, thereby contributing to MF pathogenesis [7], with OPN representing one of the mediators involved in the process.

Several studies have emphasized the value of co-targeting the MAPK pathway alongside JAK inhibition, as resistance to JAK inhibitors often arises through compensatory activation of MAPK signaling [7–9]. Notably, it has been observed that inhibition of JAK2 triggers Ras/MAPK signaling in JAK2-mutant cell lines, and that simultaneous targeting of both JAK2 and Ras/MAPK pathways in primary PMF cells in vitro leads to a significant reduction in colony formation [10, 11]. Consistently, combined inhibition strategies, targeting MEK1/2, ERK1/2, or AXL together with JAK inhibitors, have shown improved therapeutic efficacy in MPN mouse models [7–9]. Despite growing preclinical evidence, clinical translation remains limited. Several clinical trials have explored the use of MAPK pathway inhibitors in myeloid malignancies, including a phase I trial of the MEK inhibitor Selumetinib (NCT03326310) in combination with azacitidine for high-risk chronic myeloid neoplasms; a phase I/II trial of the ERK1/2 inhibitor Rineterkib (NCT04097821) in combination with Ruxolitinib in MF patients; and a phase II trial evaluating the MEK inhibitor Trametinib (NCT04487106) in combination with azacitidine and venetoclax in refractory acute myeloid leukemia. However, to date, none of these studies has led to the establishment of a novel therapeutic intervention.

In the search for novel compounds targeting the MAPK pathway, with the aim of identifying a rapidly translatable and effective therapeutic strategy to counteract OPN production, we selected Cobimetinib for further investigation. Cobimetinib is a clinically approved and well-tolerated MEK1 inhibitor for the treatment of melanoma and histiocytic neoplasms [12, 13], thereby representing a compound with high translational relevance and potential for clinical repurposing in MF. We tested Cobimetinib in vitro on human primary CD14+ monocytes, that represent one of the main sources of OPN among neoplastic clone–derived

hematopoietic cells in MF patients [5]. Increasing concentrations of Cobimetinib (1 to 10 µM) did not affect cell viability (Fig. 1A), but significantly reduced *SPP1* expression levels, as measured by real-time qRT-PCR (Fig. 1B), and OPN secretion, as assessed by ELISA in culture supernatants after 72 and 96 h of treatment (Fig. 1C, D), in human primary monocytes.

We previously demonstrated that MF mouse models, including those treated with a Thrombopoietin receptor agonist (TPO-RA) and those harboring the *JAK2V617F* mutation, exhibited elevated plasma levels of OPN, which were associated with extensive BM and spleen fibrosis deposition [6]. Given that JAK2-mutant mice display a Polycythemia Vera-like phenotype and develop fibrosis at a later stage (7–8 months of age) [14], and that Cobimetinib requires twice-daily oral administration over an extended period, we opted to evaluate in vivo the efficacy of the compound in the TPO-RA-induced mouse model of MF, which rapidly develops extensive BM and spleen fibrosis after 15 days of treatment [15].

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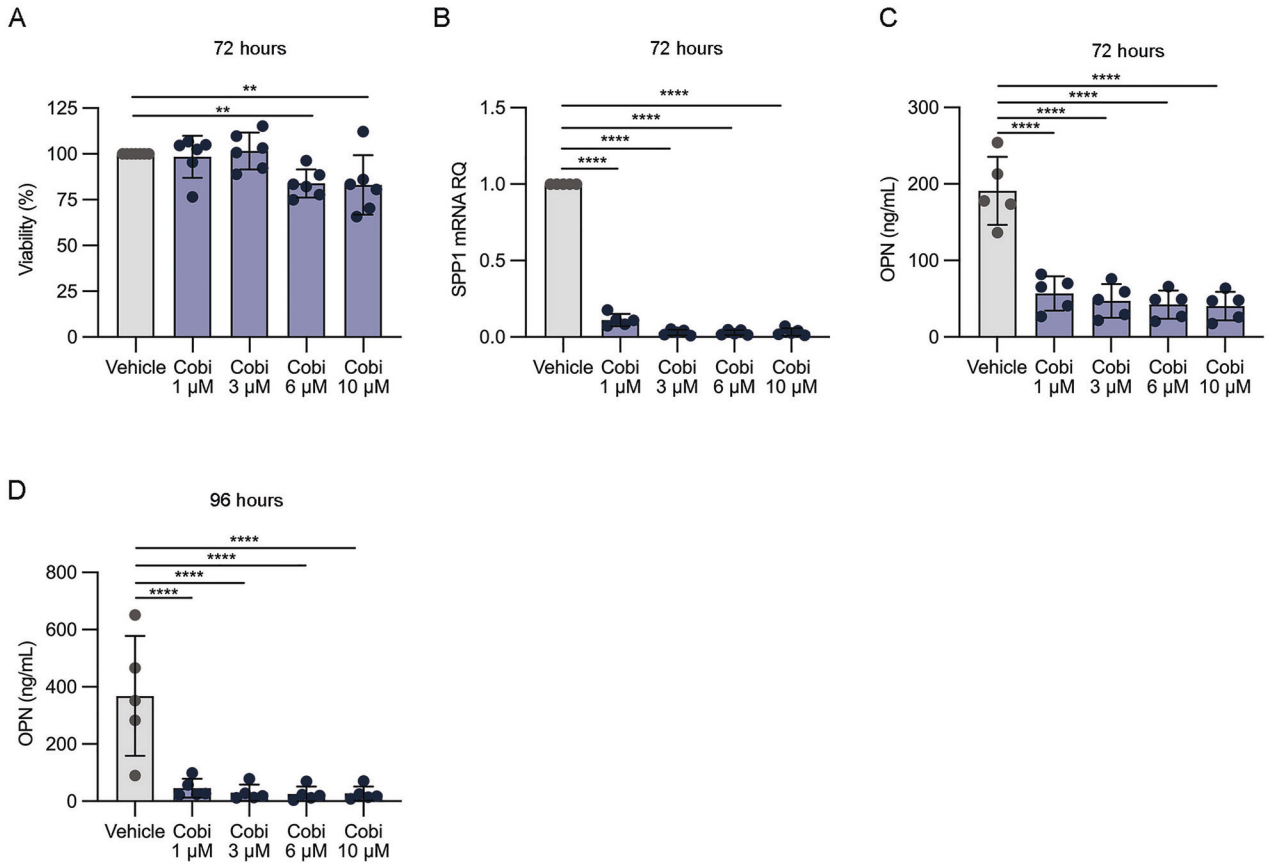


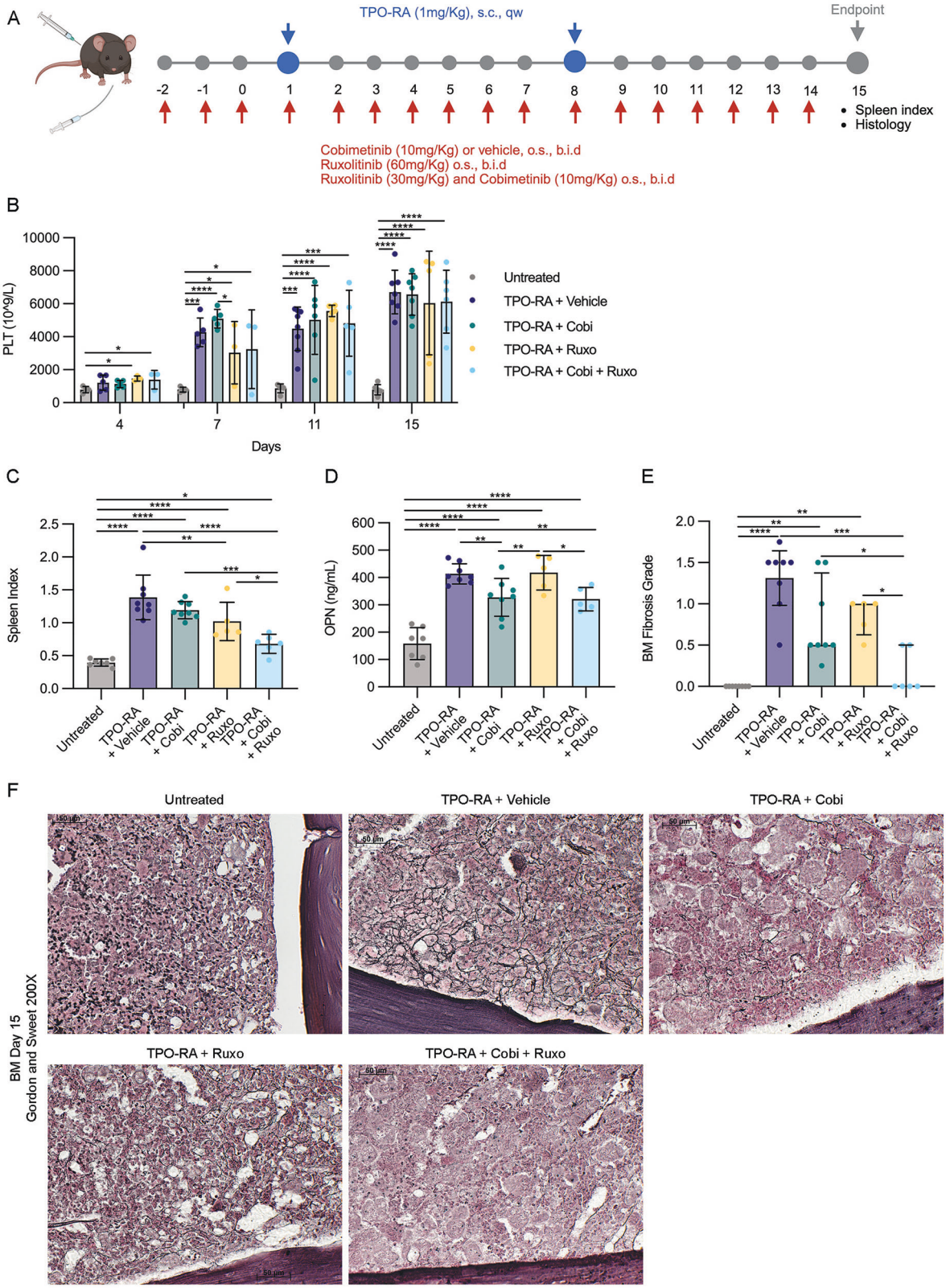
Fig. 1 MEK1 inhibition using Cobimetinib reduces OPN production and secretion by monocytes in vitro. Increasing concentrations of the MEK inhibitor Cobimetinib were tested in vitro on human monocytes from healthy donors. **A** The viability of monocytes was assessed using XTT assay after 72 h of treatment. **B** SPP1 mRNA expression was assessed by real-time qRT-PCR after 72 h of treatment. Monocyte-derived OPN levels were evaluated in culture supernatants by ELISA (Enzyme-linked immunosorbent assay) at 72 (**C**) and 96 h (**D**) of treatment. Histograms represent the mean values while bars indicate the standard deviation. Comparisons were performed by means of one-way. ANOVA. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. Cobi Cobimetinib, SPP1 osteopontin, RQ relative quantity, OPN osteopontin.

Cobimetinib was administered twice daily, starting 3 days prior to the first dose of TPO-RA (designated as day 1) and continued until sacrifice (day 15, Fig. 2A). Blood count analysis revealed the anticipated severe thrombocytosis induced by TPO-RA, which was not reversed by any of the treatments (Fig. 2B). Similarly, additional hematological parameters, including white blood cell, mean platelet volume, red blood cell, hemoglobin (HGB), and hematocrit (HCT), were unaffected by Cobimetinib administration (Supplementary Fig. 1). As expected, treatment with Ruxolitinib resulted in a significant reduction of the spleen index (Fig. 2C). Interestingly, Cobimetinib alone did not reduce spleen size, whereas the combination of the MEK1 inhibitor with Ruxolitinib induced a synergistic effect, leading to a substantial reduction in splenomegaly (Fig. 2C), although Ruxolitinib was administered at half the dose (30 mg/Kg) compared to the monotherapy regimen (60 mg/Kg).

Additionally, the inhibition of MEK1 exerted a profound impact on OPN production, BM and spleen fibrosis. Cobimetinib, either alone or in combination with Ruxolitinib, significantly attenuated the production of OPN observed in the TPO-RA mouse model, as evidenced by the quantification of plasma OPN levels at day 11 (Fig. 2D). Furthermore, the reduction in

OPN levels in Cobimetinib-treated mice was accompanied by a marked reduction in BM (Fig. 2E, F) and spleen fibrosis (Supplementary Fig. 2). Additionally, it is well established that Ruxolitinib, while alleviating disease symptoms, has a limited effect on reducing BM fibrosis [16]. Treatment with Ruxolitinib in TPO-RA mice induced a modest and nonsignificant reduction in fibrosis in some animals. In contrast, and strikingly, the combination of the JAK1/2 inhibitor with Cobimetinib demonstrated a synergistic effect, resulting in a profound reduction in BM fibrosis, with the complete disappearance of reticulin fibers in most treated animals (Fig. 2E). Histological analysis of BM sections (Fig. 2F) revealed extensive coarse reticulin fiber deposition in the femurs of vehicle-treated TPO-RA mice. In contrast, Cobimetinib-treated mice showed a significant reduction in BM fibrosis, with most areas devoid of detectable reticulin fibers, though a few thin, loosely arranged fibers remained. Notably, mice treated with both Cobimetinib and Ruxolitinib demonstrated an even more pronounced reduction in reticulin fibers, with virtually no reticulin deposition observed, suggesting that simultaneous inhibition of MEK1, and thereby OPN, via Cobimetinib, alongside JAK1/2, can lead to a potent synergistic effect capable of mitigating bone marrow fibrosis in an MF mouse model. Similarly, spleen fibrosis was strongly reduced in TPO-RA mice receiving MEK1 and JAK1/2 inhibitors (Supplementary Fig. 2).

These results provide a compelling rationale for exploring combined Cobimetinib and Ruxolitinib inhibition as a novel therapeutic strategy in MF. Importantly, the dual inhibition not only addresses the proinflammatory and profibrotic mediators but also holds potential for overcoming some of the current limitations of existing therapies, such as the inability to effectively reverse BM fibrosis [16]. Given the translational relevance of Cobimetinib as a clinically approved drug for



melanoma and histiocytic neoplasms, this combination therapy could be rapidly evaluated in clinical trials for MF, offering a promising avenue for patients with advanced disease who currently have limited treatment options. In conclusion, while further studies are necessary to refine the optimal dosing and combination regimens, our findings provide a strong foundation for the development of combination therapies using Cobimetinib to target both the neoplastic clone and the fibrotic microenvironment in MF. The potential to reverse BM fibrosis and improve long-term outcomes in MF patients remains a critical area of ongoing research, and our study adds valuable insights into this endeavor.

Fig. 2 Combined MEK1 and JAK1/2 inhibition improves splenomegaly, reduces OPN plasma levels, and reverses BM fibrosis induced by TPO-RA. A Overview of the experimental setup. Myelofibrosis was induced by sub-cutaneous injection of TPO-RA (Romiplostim, 1 mg/kg, once weekly). Mice received vehicle (10% DMSO in 20% SBE-β-CD in saline) (TPO-RA + Vehicle), Cobimetinib 10 mg/kg (TPO-RA + Cobi), Ruxolitinib 60 mg/kg (TPO-RA + Ruxo) or a combination of Cobimetinib 10 mg/kg and Ruxolitinib 30 mg/kg (TPORA + Cobi + Ruxo) through oral gavage twice daily starting 3 days before the first TPO-RA administration. Mice were sacrificed after 15 days of TPO-RA treatment. **B** Platelet count was assessed at days 4, 7, 11 and 15 ($n = 3$–8/group). **C** Spleen index was calculated at sacrifice (day 15) ($n = 5$–8/group). **D** At day 11 after the first TPO-RA administration, plasma OPN concentration was measured using ELISA (enzyme-linked immunosorbent assay) in control mice (Untreated) and TPO-RA-treated mice receiving vehicle (TPO-RA + vehicle), Cobimetinib (TPO-RA + Cobi), Ruxolitinib (TPO-RA + Ruxo) or a combination of Cobimetinib and Ruxolitinib (TPO-RA + Cobi + Ruxo) ($n = 5$–8/group). **B**–**D**, histograms represent mean values while bars indicate the standard deviation. Comparisons were performed by means of one-way ANOVA. **E** Fibrosis grading was assessed blindly by an expert pathologist ($n = 5$–8/group). Histograms represent median values while bars indicate the interquartile range. Comparisons were performed by means of Kruskal–Wallis' test. **F** Gordon and Sweet's reticulin-stained bone marrow sections from representative mice of each experimental group. Magnification 200X. $^{*}P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$; $^{****}P \le 0.0001$. *n* number of samples, s.c. sub-cutaneous, qw once weekly, o.s. oral gavage, b.i.d. twice daily, PLT platelets, TPO-RA Thrombopoietin receptor agonist, Cobi Cobimetinib, Ruxo Ruxolitinib, OPN osteopontin, BM bone marrow.

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DATA AVAILABILITY

Data are contained within the letter or in Supplementary Materials.

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AUTHOR CONTRIBUTIONS

RM designed the study and supervised the manuscript; LT wrote the manuscript, performed in vivo experiments, and analyzed data; MB, AN, EP, SR performed in vivo experiments and analyzed data; EB, MMirabile, RN performed in vitro experiments and analyzed data; SP and CC performed emocytometric analysis, CT performed mouse organ isolation; MMalerba performed reticulin stainings; LF performed paraffin-embedding of mouse organs; LL performed quantification of bone marrow fibrosis; PG, ET and AMV provided support in animal experiments and supervised the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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