JAK1/2 and pan-deacetylase inhibitor combination therapy yields improved efficacy in preclinical mouse models of JAK2V617F-driven disease

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Translational Relevance

Although JAK inhibitors have demonstrated rapid and durable reductions in splenomegaly, as well as improvement in symptoms and quality of life in patients with the myeloproliferative neoplasm myelofibrosis, the impact on the mutant allele burden and bone marrow fibrosis has been modest, indicating that combinations with other agents may yield additional benefits. Recently, the pan-deacetylase inhibitor panobinostat has shown encouraging single-agent activity in phase I/II myelofibrosis trials. Using mouse models of JAK2^{V617F}-driven diseases, we demonstrate that the combination of the JAK1/2 inhibitor ruxolitinib with the pan-deacetylase inhibitor panobinostat results in markedly improved efficacy compared to the single agents. Our preclinical findings suggest that combining JAK1/2 with pan-deacetylase inhibitors warrants clinical evaluation for myelofibrosis therapy.

Abstract

Purpose: The myeloproliferative neoplasm myelofibrosis is characterized by frequent deregulation of JAK/STAT signaling, and JAK inhibitors were shown to reduce splenomegaly and ameliorate disease-related symptoms. However, the mutant clone and bone marrow fibrosis persist in the majority of patients. Using preclinical models we explored if JAK and pan-deacetylase inhibitor combination yielded additional benefits.

Experimental design: The combination of the JAK1/2 inhibitor ruxolitinib and panobinostat was investigated using two different mouse models of JAK2^{V617F}-driven disease. A Ba/F3 JAK2^{V617F} cells-driven leukemic disease model was used to identify tolerated and efficacious doses. The drugs were then evaluated alone and in combination in a mouse model of myeloproliferative neoplasm-like disease based on
transplantation of bone marrow transduced with a retrovirus expressing JAK2$^{V617F}$.

Exposures were determined in blood and tissues, and phosphorylated STAT5 and acetylated histone H3 pharmacodynamic readouts were assessed in spleen and bone marrow. Histological analysis was carried out on spleen and bone marrow, including staining of reticulin fibers in the latter organ.

**Results:** The combination of ruxolitinib and panobinostat was found to have a more profound effect on splenomegaly, as well as on bone marrow and spleen histology, compared to either agent alone, and the analysis of pharmacodynamic readouts demonstrated that ruxolitinib and panobinostat have non-overlapping and complementary effects.

**Conclusion:** Combining JAK1/2 and pan-deacetylase inhibitors was fairly well tolerated and resulted in improved efficacy in mouse models of JAK2$^{V617F}$-driven disease compared to the single agents. Thus, the combination of ruxolitinib and panobinostat may represent a promising novel therapeutic modality for myeloproliferative neoplasms.

**Introduction**

The discovery in 2005 of the somatic activating JAK2$^{V617F}$ mutation in chronic myeloproliferative neoplasms (MPN) polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) provided a rationale for the development of JAK2 inhibitors (1), which rapidly entered clinical trials for patients with myelofibrosis (MF) (2). Consistent with data obtained in preclinical animal models (3-5) JAK inhibitors have demonstrated a rapid and durable reduction of splenomegaly in patients with myelofibrosis, but also a substantial improvement of constitutional
symptoms (6-8). However, the impact of JAK inhibitors on the JAK2\textsuperscript{V617F} mutant allele burden and bone marrow fibrosis has been modest (9). In order to yield additional benefits for patients with myelofibrosis, combinations of JAK inhibitors with other agents seem warranted. Several other drugs are being explored clinically, including deacetylase inhibitors, immunomodulatory agents, smoothened antagonists, recombinant interferon-alpha, and the rapalog everolimus (10). Encouragingly, some of these drugs have shown early signs of clinical activity, offering opportunities for eventual combinations with JAK inhibitors. However, given the number of different agents being investigated, the choice and prioritization of the combination partner represents a considerable challenge.

On one hand, combination strategies with JAK inhibitors could be devised to target parallel pathways activated by aberrant JAK signaling, such as the PI3K/mTOR and MAPK pathways, and/or downstream STAT effector molecules and their targets (e.g. anti-apoptotic Bcl-2 family members, Pim kinases, c-Myc etc.) (2, 11). These approaches may result in more profound and/or more sustained inhibition of JAK2\textsuperscript{V617F} signaling. Alternatively, combination strategies with JAK inhibitors could also be aimed at addressing biological pathways and processes that may cooperate with aberrant JAK signaling. Along these lines, the immunomodulatory drug lenalidomide plus prednisone (12) and the deacetylase inhibitor panobinostat (13-15) have shown promising activity in terms of disease-modifying potential, evidenced by anemia responses, reduction of mutant allele burden and reversal of bone marrow fibrosis in some patients with MF, thus, representing potentially appealing combination partners for JAK inhibitors.
To gain confidence in a particular combination modality, preclinical models may provide useful guidance. For instance, in-vitro studies using JAK2V617F mutant cells have shown that pan-deacetylase inhibition resulted in enhanced JAK2 inhibitor-mediated suppression of aberrant JAK/STAT signaling and synergistic apoptosis induction (16). Furthermore, the combination also displayed greater cytotoxicity than either agent alone on primary CD34+ cells from MF patients. Interestingly, combined pan-deacetylase and JAK2 inhibition triggered less apoptosis in CD34+ cells from healthy subjects as compared with CD34+ cells from patients with MF (16). These in vitro results suggest that combined JAK and deacetylase inhibition could be promising for the treatment of MPNs, however, the combination has not yet been explored in respective in vivo disease models.

Here, we evaluated the activity and tolerability of the JAK1/2 inhibitor ruxolitinib (INCB018424/INC424 (17)) in combination with the pan-deacetylase inhibitor panobinostat (LBH589 (18)) in animal models of JAK2V617F-dependent disease. The combination was first explored in a mouse mechanistic model of Ba/F3 JAK2V617F cells-driven leukemic disease, followed by assessment in a bone marrow transplant model of JAK2V617F-mediated MPN-like disease. The combination of ruxolitinib with panobinostat showed superior suppression of splenomegaly, as well as improved bone marrow and spleen histology, as compared with the single agents. Furthermore, ruxolitinib and panobinostat demonstrated non-overlapping and complementary effects on the proximal pharmacodynamic readouts phosphorylated-STAT5 (p-STAT5) and actetylated histone H3.
Materials and Methods

Compounds, formulations and treatment schedules

Ruxolitinib mono-phosphate was formulated in 0.5% HPMC (Pharmacoat 603, Dow Chemical, Midland, MI, USA) at a concentration of 7.9 mg/mL and was administered orally (p.o.) twice daily (q12h/bid) in an application volume of 10 mL/kg at a free-base equivalent dose of 60 mg/kg. The lactate salt of panobinostat was formulated in isotonic D5W (5% Dextrose; B. Braun Medical Inc., Bethlehem, PA, USA) at a concentration of 1.5 mg/mL, 1 mg/mL and 0.5 mg/mL, and administered 3 times a week (Monday/Wednesday/Friday (M/W/F)) in a volume of 10 mL/kg by intraperitoneal (i.p.) injection. The final free-base equivalent doses were 11.90, 7.94 and 3.97 mg/kg, respectively (rounded up values reported in text, figures and tables). For combination treatments, ruxolitinib and panobinostat were administered simultaneously and with the same dosing regimen used for the single agents.

Mouse models of JAK2V617F-driven disease

The mouse model of Ba/F3 JAK2V617F-luc cells-driven leukemic disease (19) and the mouse bone marrow transplant model of JAK2V617F-driven MPN-like disease were used as previously described (20-22). All animal experiments were performed in strict adherence to Swiss laws for animal welfare and approved by the Swiss Cantonal Veterinary Office of Basel-Stadt.

Detection of green fluorescent protein (GFP)-positive cells in blood samples by flow cytometry

10 μL whole blood was used to detect circulating GFP-positive cells. Blood was distributed into a 96-well round bottom plate (#3795, Corning Life Sciences, New York,
NY, USA) and red blood cells (RBC) were lysed with 200 µL of RBC lysis buffer (R-7757, Sigma, St. Louis, MO, USA). After 7 minutes incubation in the dark on a plate agitator, cells were centrifuged (5 minutes, 300 g) and the supernatant discarded by inversion. After 3 washes in FACS buffer (phosphate buffered saline (D-PBS), 3% FBS and 0.02% sodium azide), nucleated cells were resuspended in 200 µL of cold FACS buffer and processed for GFP detection using a LSRII flow cytometer (BD Biosciences, Heidelberg, Germany).

**Western blotting**

Western blotting was carried out as previously described (19). Typically, 40-100 µg of protein lysates were resolved by NuPAGE Novex 4-12% Bis-Tris Midi Gels (Life Technologies, Carlsbad, CA, USA) and transferred to PVDF membranes by semi-dry blotting. The following antibodies were used: JAK2 (#3230), Phosphorylated-STAT5 (Tyr694) (#9359), acetylated lysine (#9441), PARP (#9542) and GAPDH (#2118) were from Cell Signaling Technology (Beverly, MA, USA). STAT5 (#sc-835) and β-tubulin (#T4026) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA), respectively.

**Statistical analysis**

Results in figures and tables represent mean±SEM. Absolute values or transformed values (log₁₀ or as specified) for all parameters were analyzed by one-way ANOVA analysis followed by Dunnett’s test to compare all treatment groups to vehicle group. Multiple comparisons were performed using the Tukey’s test to compare each single agent to combination groups (all treatment groups were included in the test). The
significance level was set to p<0.05. Calculations were performed using Graph Pad Prism 5.00.

Results

Activity and tolerability of ruxolitinib and panobinostat, alone and in combination, in a mouse model of Ba/F3 JAK2^{V617F} cells-driven leukemic disease

A mechanistic mouse model of JAK2^{V617F}-driven leukemic disease (19) was used to assess the activity and tolerability of ruxolitinib and panobinostat alone or in combination. Ruxolitinib was given at 60 mg/kg orally twice daily, while panobinostat was given at i.p. doses of 4, 8, 12 mg/kg. For ruxolitinib a somewhat lower dose than that previously reported in a similar model (17) was chosen in order to be able to observe modulation of efficacy readouts upon titration of panobinostat in the combination setting. Animals were treated for one week and bioluminescence signals were measured during the course of the treatment and at study end. Ruxolitinib and panobinostat single agents produced significant antitumor effects, reflected by the reduction in light emission (Fig. 1A). Enhanced efficacy was observed upon combination of panobinostat with ruxolitinib, particularly with the two higher panobinostat dose-levels. The combination of ruxolitinib with the 12 mg/kg panobinostat regimen was most efficacious with a treatment over control ratio (T/C) of 3% (near stasis), which was significantly different from the efficacy seen with the single agents (Fig. 1A and Supplementary Table S1). Spleen weight, used as another index of tumor load, was recorded at sacrifice. Treatment with ruxolitinib alone normalized spleen weights, while panobinostat given alone reduced spleen weights, but not to normal values (Fig. 1B). In all combination groups spleen weights were below normal
values, although not significantly different from ruxolitinib mono-therapy values. There was no major impact on tolerability, as assessed by body weight changes, between panobinostat given alone or in combination with ruxolitinib (Supplementary Fig. S1).

**Effects of ruxolitinib and panobinostat, alone and in combination, on pharmacodynamic and histological readouts in a mouse model of Ba/F3 JAK2V617F cells-driven leukemic disease**

Pharmacodynamic readouts for target modulation in spleen by ruxolitinib and panobinostat (phosphorylated-STAT5 and protein acetylation) were assessed post-therapy. Ruxolitinib reduced phosphorylation of the JAK2 downstream target STAT5, whereas panobinostat (at the 12 mg/kg dose level) reduced total STAT5 levels and markedly increased overall lysine acetylation (Fig. 2A). Levels of phosphorylated and total STAT5 were further reduced in mice treated with the combination of ruxolitinib and panobinostat, while the overall lysine acetylation pattern was comparable to panobinostat mono-therapy (Fig. 2A). In the bone marrow, ruxolitinib, panobinostat (at 12 mg/kg), or both combined, reduced levels of STAT5 phosphorylation (Fig. 2B), with a trend for strongest suppression when ruxolitinib was combined with the highest panobinostat dose (Supplementary Fig. S2A). These pharmacodynamic changes were recapitulated with Ba/F3 JAK2V617F and human JAK2V617F-mutant MB-02 cells in culture (Supplementary Fig. S2B and S2C). Consistent with histone deacetylase inhibition, mice treated with panobinostat displayed strongly increased levels of acetylated histone H3 in bone marrow sections (Fig. 2B), reaching a peak with the 8 mg/kg dose, and without further modulation in the combination arms (Fig. 2B). Histological assessment of bone marrow sections revealed that the 12 mg/kg panobinostat dose level, alone or in combination with ruxolitinib, restored normal bone
marrow proliferation due to suppression of Ba/F3 JAK2V617F cell proliferation.

Panobinostat, alone or in combination with ruxolitinib, induced elevated levels of cleaved-caspase-3 as compared to vehicle and ruxolitinib treated groups (Supplementary Fig. S2D), indicating that apoptosis induction is involved in the suppression of leukemic cell spreading. Activated caspase-3 has also been reported to play a direct role in the degradation of STAT5 (23). Pharmacokinetic analyses did not indicate drug-drug interaction between ruxolitinib and panobinostat (Supplementary Tables S2 and S3).

**Efficacy and tolerability of ruxolitinib in a mouse model of JAK2V617F-driven MPN-like disease**

Based on the encouraging results obtained in the mouse mechanistic model, we next set out to establish the efficacious and tolerated regimens of ruxolitinib and panobinostat, first given as mono-therapies, in a mouse bone marrow transplant model of JAK2V617F-driven MPN-like disease (20, 21). These studies also served to establish the doses of the agents to be assessed in combination. Transplanted mice were randomized on day 27 based on hematocrit (67-68% on average per treatment group) and treated with ruxolitinib at 30, 60 or 90 mg/kg twice daily for 21 consecutive days. The spleen weight was significantly reduced after ruxolitinib therapy, with a trend for dose-dependency (Fig. 3A). Body weight changes were modest (Supplementary Fig. S3A), indicating that ruxolitinib was well tolerated. Elevated hematocrit (Supplementary Fig. S3B), as well as increased reticulocyte and white blood cell (WBC) counts (Fig. 3B and C), were reduced after ruxolitinib therapy, and the latter two parameters were within the normal ranges at study end. Platelet count was not affected by ruxolitinib treatment and slightly below normal range in all groups (Supplementary Fig. S3C). The percentage of
circulating GFP-positive cells was determined by FACS analysis after 2 weeks of treatment as a surrogate readout for JAK2$^{V617F}$ mutant allele burden changes. Treatment with ruxolitinib was found to reduce the fraction of GFP-positive cells (Fig. 3D), although dose-dependency was not observed.

Spleen and bone marrow samples were collected post-therapy and sections were assessed for p-STAT5 by IHC. Ruxolitinib was found to suppress levels of p-STAT5 after 2 hours, with the higher doses eliciting stronger p-STAT5 suppression, and levels recovered by 12 hours (Supplementary Fig. S4). Histological analysis revealed marked proliferation of erythroid and myeloid cells in spleen, leading to a disruption of splenic architecture (Supplementary Fig. S5A). A decrease in severity of extramedullary hemopoiesis was observed in the spleen of ruxolitinib treated animals when compared with the vehicle treated group (Supplementary Fig. S5A, upper panel). In parallel, a decrease in incidence and severity of bone marrow hypercellularity was observed in the sternum of ruxolitinib treated animals in a dose-dependent manner as compared to the vehicle treated group (Supplementary Fig. S5A, lower panel). Fine reticulin fibers in the bone marrow were reduced to some extent in the ruxolitinib treated arms, with the best effect seen at the 60 mg/kg dose (Supplementary Fig. S5B).

Taken together, in this model of JAK2$^{V617F}$ driven MPN-like disease ruxolitinib therapy was found to suppress splenomegaly, elevated hematocrit, reticulocyte and WBC counts, the mutant allele-burden surrogate, hypercellularity and reticulin fibers in bone marrow, as well as extramedullary erythropoiesis in the spleen. Differences between the ruxolitinib dose levels on these efficacy parameters were either relatively modest or not evident, and all doses were well tolerated.
Efficacy and tolerability of panobinostat in a mouse model of JAK2V617F-driven MPN-like disease

Next, panobinostat was evaluated in the mouse MPN model. Mice were randomized on day 27 post-transplantation based on hematocrit (70% on average per treatment group) and panobinostat was given at doses of 4, 8, or 12 mg/kg i.p. on a thrice-weekly schedule for 19 consecutive days. A somewhat stronger MPN disease phenotype was observed compared with the ruxolitinib monotherapy experiment, with average spleen weights exceeding 1 g, hematocrits of 76% and a WBC count of 300·10^9/L in the control group at the time of sacrifice. Spleen weight was significantly reduced, but not normalized, in a dose-dependent manner by panobinostat treatment as compared with the vehicle group (Fig. 4A). Panobinostat treatment led to some decrease of body weight (-8% on average) in the 12 mg/kg dose group (Supplementary Fig. S6A).

Panobinostat had modest, if any, effects on hematocrit (Supplementary Fig. S6B) and reticulocyte count (Fig. 4B), but significantly reduced WBC count (Fig. 4C). Platelet count was at the lower end of the normal range in the vehicle group and significantly reduced below normal range with all panobinostat doses (Supplementary Fig. S6C). Dose-dependency of panobinostat-mediated effects on blood parameters was either minimal or not discernible. Finally, the 8 and 12 mg/kg panobinostat dose levels also reduced the percentage of circulating GFP-positive (i.e. JAK2V617F-mutant) cells (Fig. 4D).

Levels of acetylated lysine were markedly increased in spleen samples of panobinostat treated animals (Supplementary Fig. S7A). Acetylation of histone H3 was assessed in the bone marrow by IHC, revealing a dose-dependent increase of the
pharmacodynamic marker in panobinostat treated animals (Supplementary Fig. S7B). Histological analysis showed that panobinostat treatment with the 12 mg/kg dose level led to a slight decrease of bone marrow hypercellularity as compared to vehicle-treated animals (Supplementary Fig. S8A). A trend for a decrease of fine reticulin fibers in the bone marrow was noted with all panobinostat doses (Supplementary Fig. S8B).

In summary, panobinostat reduced splenomegaly and aberrant WBC count in the mouse MPN disease model. These changes in efficacy parameters were consistent with deacetylase inhibition, as judged by assessment of pharmacodynamic readouts in target organs. However, treatment of mice with panobinostat also led to some degree of body weight loss and reduced platelet count.

**Efficacy and tolerability of ruxolitinib and panobinostat, alone and in combination, in a mouse model of JAK2^{V617F}-driven MPN-like disease**

Having established the single-agent efficacious doses, we next evaluated the combination of ruxolitinib and panobinostat in the MPN disease model. Based on the monotherapy efficacy studies in this model, we tested ruxolitinib at a dose of 60 mg/kg bid, as this dose yielded efficacy, but reasoning that it would still enable detecting modulation of efficacy in the combination setting with another drug. Panobinostat was tested at the 8 mg/kg dose level for the same reasons, and to have some margin in terms of tolerability. Mice were randomized on day 27 post-transplantation based on hematocrit (67% on average per treatment group) and treated either with vehicle, panobinostat, ruxolitinib or the combination of ruxolitinib with panobinostat for 21 consecutive days. Panobinostat administered alone reduced spleen weight but not to normal, while ruxolitinib showed a trend to reduce spleen weight, but with high
variability (range 67-1153 mg) (Fig. 5A). The combination significantly improved efficacy in terms of spleen weight, which normalized or was even somewhat below normal range (in 6/9 animals) (Fig. 5A). Ruxolitinib was well tolerated as judged by monitoring body weight, while panobinostat showed moderate body weight loss (-5% on average), and body weight decreased further in the combination arm (Supplementary Fig. S9A), but still less than the maximal tolerated body weight loss (-15%).

The combination showed a trend for a stronger impact on elevated red blood cell parameters, particularly on reticulocyte count, although this effect was not significantly different from single agent arms (Fig. 5B and Supplementary Fig. S9B). WBC count was reduced by panobinostat alone and in combination with ruxolitinib, despite absence of leukocytosis in this experiment, except for single animals in the vehicle and ruxolitinib groups, respectively (Fig. 5C). Platelet count was below normal range in the vehicle arm and reduced further by panobinostat treatment. However, platelet count reduction by panobinostat was not significantly exacerbated upon combination with ruxolitinib (Supplementary Fig. S5C). In this experiment, ruxolitinib had little impact on the mutant allele burden surrogate readout, while a trend for a reduction was observed in the panobinostat and combination groups, respectively (Fig. 5D). The relative levels of \( JAK2^{V617F} \) in bone marrow and spleen samples were measured post-therapy using quantitative PCR (Supplementary Fig. S10) and showed a similar trend as the levels of circulating GFP-positive cells (Fig. 5D).

Pharmacodynamic marker assessments by IHC showed a clear reduction in phosphorylated-STAT5 upon treatment with ruxolitinib alone and in combination with panobinostat (Fig. 6A and B), while panobinostat markedly increased levels of
acetylated histone H3, alone and in combination with ruxolitinib (Fig. 6A and C).

However, the 8 mg/kg panobinostat dose level did not appreciably reduce levels of phosphorylated-STAT5 in the spleen (Fig. 6A), suggesting that the combination of ruxolitinib and panobinostat, at tolerated doses, has non-overlapping and complementary effects on these proximal pharmacodynamic marker readouts in MPN target organs.

Treatment with either ruxolitinib or panobinostat improved splenic architecture, with the strongest effect being observed with the combination regimen (Supplementary Fig. S11A). A decrease in bone marrow hypercellularity was observed for all drug treatment groups, with combination treatment resulting in the greatest effect, but with mild bone-marrow depletion. Both mononuclear megakaryocytes and megakaryocytes with multi-lobulated nuclei were observed in all treatment groups, without specific differences in their proportion. Despite high variability, the ruxolitinib and the combination groups showed a tendency for reduced fibrosis score, as assessed by staining reticulin fibers on sternum sections (Supplementary Fig. S11B). Cleaved caspase-3 levels in bone marrow sections were highest in the panobinostat-treated group, while a trend for increased levels was also observed in the ruxolitinib and the combination groups, although the differences did not reach statistical significance.

Importantly, the combination did not impact tissue exposure to each compound (Supplementary Tables S4 and S5).

In summary, the combination of ruxolitinib with panobinostat showed a significant improvement on splenomegaly reduction, as well as on bone marrow and spleen histology, compared to the drugs given alone. Pharmacodynamic marker
analyses demonstrated that ruxolitinib and panobinostat were given at active doses and have non-overlapping and complementary effects on the proximal readouts phosphorylated-STAT5 and acetylated histone H3, respectively. Panobinostat therapy led to some degree of body weight loss in the animals, which decreased further upon combination with ruxolitinib. However, overall, the combination of ruxolitinib with panobinostat was fairly well tolerated in this model of MPN-like disease.

**Discussion**

The cMPNs PV, ET and PMF are characterized by a high incidence of the JAK2<sup>V617F</sup> mutation (2). JAK inhibitors rapidly entered clinical trials for patients with MF following the discovery of aberrant JAK/STAT pathway activation in the disorder, and demonstrated encouraging reductions in splenomegaly, improvement in symptoms and quality of life for patients (24). However, the impact of JAK inhibitors on the mutant allele burden and bone marrow fibrosis has been modest so far (25), suggesting that combinations with other drugs may yield additional benefits. More recently additional mutations, albeit at lower frequencies and in varying combinations, have been identified in cMPNs, affecting e.g. TET2, ASXL1, EZH2, CBL, IDH1, IDH2, LNK and IKZF1 (26). The loss of function mutations in CBL (27) and LNK (28), which are negative regulators of JAK/STAT signaling, would be expected to raise JAK activity. Interestingly, the remainder of mutations identified to date are thought to affect chromatin states, the epigenome and/or gene expression, suggesting that alterations at these levels contribute to MPNs and may cooperate with aberrant JAK/STAT activation in MPN pathogenesis (26). Thus, interfering with altered chromatin and gene expression in MPNs may represent yet another potential avenue for MPN therapy. In support of
this notion, Akada et al. have recently shown that treatment of JAK2V617F knock-in mice with the pan-deacetylase inhibitor vorinostat led to a reduction in splenomegaly, aberrant blood counts and mutant allele burden (29). Encouragingly, anemia responses, as well as reduction of mutant allele burden and bone marrow fibrosis, have been observed in some MF patients treated with the pan-deacetylase inhibitor panobinostat (13-15). In terms of promising combination strategies, preclinical studies have shown that sequential treatment of CD34+ cells from patients with MF with the hypomethylating agent 5-aza-2'-deoxycytidine followed by the deacetylase inhibitor trichostatin A was capable of correcting aberrant CXCR4 expression and trafficking behavior of the mutant clone (30). Furthermore, studies with JAK2V617F mutant cells demonstrated that pan-deacetylase inhibition enhanced suppression of JAK/STAT signaling by a JAK2 inhibitor, and the combination exerted synergistic apoptosis induction (16). Finally, in JAK2 or FLT3 mutant AML xenograft models the HDAC inhibitor pracinostat displayed synergistic tumor growth inhibition in combination with the dual JAK2/FLT3 inhibitor pacritinib (31).

Here, we expanded on these findings and describe the combination of ruxolitinib with panobinostat in two preclinical mouse models of JAK2V617F-driven disease. We first took advantage of a mechanistic model of Ba/F3 JAK2V617F-cells driven leukemic disease, as it readily enables to determine tolerated and efficacious dose ranges of drug combinations. Panobinostat was found to inhibit the growth of JAK2V617F-driven leukemic cells and the combination of panobinostat with ruxolitinib further improved activity, while being well tolerated. Next, we established the efficacious doses for ruxolitinib and panobinostat in a mouse JAK2V617F bone marrow transplant model of MPN-like disease, and then tested the drugs in combination. The combination of
ruxolitinib with panobinostat markedly reduced splenomegaly in the mouse JAK2$^{V617F}$-dependent MPN disease model, returning spleen weights to normal. The combination also improved bone marrow hypercellularity and spleen histology. These findings were consistent with modulation of phosphorylated-STAT5 and acetylated histone H3 by ruxolitinib and panobinostat, respectively, showing that, when given at active and tolerated doses, the two drugs have non-overlapping and complementary effects on these proximal pharmacodynamic marker readouts, which may underlie some of the observed beneficial effects of the combination. Depending on the dose of panobinostat combined with ruxolitinib, greater suppression of aberrant JAK2/STAT5 signaling can be achieved compared to ruxolitinib, as exemplified by decreased levels of both phospho- and total STAT5 in the mechanistic model. Recently, it was reported that panobinostat can impact STAT5 levels in MF patients receiving the drug, albeit at a high starting dose that was not well tolerated (32). Thus, MPN patients may tolerate and benefit from deacetylase inhibitors alone or combined with other agents if they receive the deacetylase inhibitor at lower doses, and over prolonged periods of time (15, 32, 33). In the mouse disease model the combination of panobinostat with ruxolitinib further decreased body weight seen with panobinostat alone, although this was not seen in the mechanistic mouse model, and unlikely to be attributed to enhanced exposure due to drug-drug interaction based on pharmacokinetic and pharmacodynamic analyses.

Panobinostat therapy in the MPN disease model decreased the platelet count. Although ruxolitinib alone did not decrease platelet count, and did not exacerbate the panobinostat-mediated drop in platelet count when combined with the deacetylase inhibitor, it should be pointed out that both drugs exhibited thrombocytopenia as the dose-limiting toxicity in clinical trials (7, 8, 15). Thus, given this overlapping toxicity, platelet count will have to be carefully monitored in the clinic. Although human
exposure is not straightforward to replicate in mouse models, due to species differences in clearance or plasma protein binding for example, the ruxolitinib and panobinostat doses used in our mouse models yielded exposures consistent with those achieved clinically. For instance, in healthy volunteers the $C_{\text{max}}$ and exposure (AUC) determined in plasma at the maximum tolerated ruxolitinib doses of 25 mg bid or 100 mg qd were 1.16 μM and 4.32 μM·h, or 4.78 μM and 16.60 μM·h, respectively (34). The recommended phase II dose of oral panobinostat in a study in patients with MF was determined as 25 mg thrice weekly (15), and at an oral dose of 20 mg the drug yielded plasma $C_{\text{max}}$ and exposure (AUC) values of 0.04 μM and 0.44 μM·h, respectively (35).

Taken together, our preclinical in vivo studies show that the combination of ruxolitinib with panobinostat is fairly well tolerated, affords a significant reduction in splenomegaly and ameliorates histology of the bone marrow and spleen, as compared to either drug given alone. The added benefit is seemingly consistent with pharmacodynamic marker analyses, which revealed complementary effects of the ruxolitinib and panobinostat combination. The combination of ruxolitinib and panobinostat is currently being investigated in a phase Ib clinical trial in patients with myelofibrosis (36).

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Author Contributions

E.E., N.E., and C.R. performed the in vivo studies, participated in the design of experiments, analyzed data and helped draft parts of the manuscript. V.R., R.A. Z.Q., A.D. and E.D. performed ex vivo pharmacodynamic marker analysis. D.S. performed pharmacokinetic analyses. R.C. and F.H. provided conceptual input. F.B., M.M. and T.R. conceived the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

Disclosure of Potential Conflicts of Interest

All authors are full-time employees of Novartis Pharma AG. A patent application on combination of ruxolitinib with panobinostat has been filed.

References


Figure legends

Figure 1. Activity of ruxolitinib (RUX) and panobinostat (PAN), alone and in combination, in a mouse model of Ba/F3 JAK2V617F cells-driven leukemic disease. SCID beige mice were intravenously inoculated with Ba/F3 JAK2V617F-luc cells. On day 4 post-cell injection detectable luciferase signals could be measured and mice were randomized and treated with vehicle, panobinostat at 4, 8 and 12 mg/kg i.p. on a M/W/F dosing schedule, alone or in combination, with ruxolitinib at a dose of 60 mg/kg p.o. bid
for 7 days. (A) Representative bioluminescence images on day 11 post-cell injection (7
days of treatment). The difference from control was calculated by the ratio of
bioluminescence fold-increase (treated/control)·100. *p<0.05 versus vehicle treated
animals (one-way ANOVA followed by post-hoc Dunnett’s test on log_{10} transformed
values), †p<0.05 versus ruxolitinib treated animals, and ‡p<0.05 versus panobinostat
treated animals at the same dose (one-way ANOVA followed by post-hoc Tukey’s test
on log_{10} transformed values). (B) Spleen weights (mean±SEM, n=7/group) were
recorded at sacrifice, which was 2 hours after receiving the final dose after 7 days of
treatment. Average spleen weight of age-matched SCID beige mice is 75 mg. *p<0.05
versus vehicle treated animals (one-way ANOVA followed by post-hoc Dunnett’s test
on log_{10} transformed values), †p<0.05 versus ruxolitinib treated animals, ‡p<0.05 versus
panobinostat treated animals at the same dose (one-way ANOVA followed by post-hoc
Tukey’s test on log_{10} transformed values). Similar results were obtained in an
independent experiment.

**Figure 2. Modulation of pharmacodynamic markers following treatment with
ruxolitinib (RUX) and panobinostat (PAN), alone and in combination, in a mouse
model of Ba/F3 JAK2^{V617F} cells-driven leukemic disease.** SCID beige mice were
intravenously inoculated with Ba/F3 JAK2^{V617F}-luc cells. On day 4 post-cell injection
mice were randomized and treated with vehicle, panobinostat at 4, 8 and 12 mg/kg i.p.
on a M/W/F dosing schedule, alone or in combination, with ruxolitinib at a dose of 60
mg/kg p.o. bid for 7 days. (A) Mice were given a last dose and sacrificed 2 hours later.
Spleen lysates from 4 mice/group (focusing on the highest dose of panobinostat alone
and in combination with ruxolitinib) were analyzed by Western blotting for
phosphorylated STAT5 (p-STAT5), total STAT5, acetylated lysine (Acetyl-Lys), β-
Figure 3. Efficacy of ruxolitinib (RUX) in a mouse model of JAK2V617F-driven MPN-like disease. Balb/c mice transplanted with mouse JAK2V617F expressing bone marrow and displaying a PV-like phenotype received either vehicle or ruxolitinib given at 30, 60, and 90 mg/kg p.o. bid for 21 consecutive days. Animals were sacrificed on day 48 (half of the groups) and on day 49 (second half of the groups) post-bone marrow transplant. (A) Change in spleen weight at sacrifice depicted as means±SEM (n=8/group). Stippled line depicts normal spleen weight for age-matched non-transplanted Balb/c female mice (98 mg). *p<0.05 versus vehicle treated group (one-way ANOVA followed by Dunnett’s test or Tukey’s test on log10 transformed values for spleen weight). (B) Reticulocyte count and WBC count (C) post-therapy. Shaded areas depict historic ranges of non-transplanted Balb/c female mice. (D) Percentage of GFP-positive cells (of total nucleated cells) in circulation was assessed by FACS analysis after 2 weeks of treatment for each individual animal. *p<0.05 versus vehicle-treated group (one-way ANOVA followed by Dunnett’s test). Results are depicted as means±SEM (n=6-8/group). Similar results were obtained in an independent experiment.

Figure 4. Efficacy of panobinostat (PAN) in a mouse model of JAK2V617F-driven MPN-like disease. Balb/c mice transplanted with mouse JAK2V617F expressing bone marrow and displaying a PV-like phenotype received either vehicle or panobinostat given at 4, 8, and 12 mg/kg i.p. M/W/F for 19 consecutive days. (A) Change in spleen weight at sacrifice depicted as means±SEM (n=8/group). Stippled line depicts normal spleen weight for age-matched non-transplanted Balb/c female mice (98 mg). *p<0.05 versus vehicle treated group (one-way ANOVA followed by Dunnett’s test or Tukey’s test on log10 transformed values for spleen weight). (B) Reticulocyte count and WBC count (C) post-therapy. Shaded areas depict historic ranges of non-transplanted Balb/c female mice. (D) Percentage of GFP-positive cells (of total nucleated cells) in circulation was assessed by FACS analysis after 2 weeks of treatment for each individual animal. *p<0.05 versus vehicle-treated group (one-way ANOVA followed by Dunnett’s test). Results are depicted as means±SEM (n=6-8/group). Similar results were obtained in an independent experiment.
weight at time of sacrifice depicted as means±SEM (n=6-8/group; 2 animals (on day 9 and 10) in the 4 mg/kg arm, and 1 in the 12 mg/kg arm (on day 14) had to be sacrificed due to poor animal condition). Stippled line in (A) depicts normal spleen weight for age-matched non-transplanted Balb/c female mice (98 mg). *p<0.05 (one-way ANOVA followed by Dunnett’s test (versus vehicle-treated group) or Tukey’s test for multiple comparisons). (B) Reticulocyte count and WBC count (C) post-therapy. Shaded areas depict historic ranges of non-transplanted Balb/c female mice. (D) Percentage of GFP-positive cells (of total nucleated cells) in circulation was assessed by FACS analysis.

Similar results were obtained in an independent experiment.

**Figure 5. Efficacy of ruxolitinib (RUX) and panobinostat (PAN), alone and in combination, in a mouse model of JAK2 V617F-driven MPN-like disease.** Balb/c mice transplanted with JAK2 V617F expressing bone marrow and displaying a PV-like phenotype received vehicle (D5W + HPMC 0.5%), panobinostat at 8 mg/kg i.p. M/W/F, ruxolitinib at 60 mg/kg p.o. bid, or the combination of panobinostat with ruxolitinib for 21 consecutive days. (A) Change in spleen weight at time of sacrifice depicted as means±SEM (n=9/group). Stippled line in (A) depicts normal spleen weight for age-matched non-transplanted Balb/c female mice (98 mg). (B) Reticulocyte count and WBC count (C), post-therapy. Shaded areas depict historic ranges, and means of non-transplanted Balb/c female mice. (D) Percentage of GFP-positive cells (of total nucleated cells) in circulation was assessed by FACS analysis. *p<0.05 versus vehicle-treated group or single agent (one-way ANOVA followed by Dunnett’s test or Tukey’s test on the reciprocal values for spleen weight and on log-transformed values for WBC counts, n=7-9/group). Similar results were obtained in an independent experiment.
Figure 6. Modulation of pharmacodynamic markers following treatment with ruxolitinib (RUX) and panobinostat (PAN), alone and in combination, in a mouse model of JAK2V617F-driven MPN-like disease. Balb/c mice transplanted with mouse JAK2V617F expressing bone marrow and displaying a PV-like phenotype received either vehicle, panobinostat at 8 mg/kg i.p. M/W/F, ruxolitinib at 60 mg/kg p.o. bid, or the combination of panobinostat with ruxolitinib for 21 consecutive days. Mice were sacrificed 2 hours after the last dose and tissue samples were processed for pharmacodynamic marker analysis. (A) Detection of acetylated histone H3 and p-STAT5 in spleen and bone marrow sections (scale bar: 50 µm). (B) p-STAT5 staining was quantified in bone marrow using the Definiens image analysis software. (C) Acetylated histone H3 signals in spleen and bone marrow sections (manual quantification). *p<0.05 versus vehicle or single agent treated group. Similar results were obtained in an independent experiment.
Figure 1

A

% of control

Vehicle

PAN 4 mg/kg

PAN 8 mg/kg

PAN 12 mg/kg

RUX 60 mg/kg

RUX 60 mg/kg + PAN 4 mg/kg

RUX 60 mg/kg + PAN 8 mg/kg

RUX 60 mg/kg + PAN 12 mg/kg

% of control

40% *

27% *

22% *

20% *

11% *

15% †

3% ††

B

Spleen weight (mg) (mean ± SEM)

Vehicle

PAN 4 mg/kg, 3qw

PAN 8 mg/kg, 3qw

PAN 12 mg/kg, 3qw

RUX 60 mg/kg, q12h

Combo RUX + PAN 4 mg/kg

Combo RUX + PAN 8 mg/kg

Combo RUX + PAN 12 mg/kg
Figure 2
Figure 3

A

Change in body weight (%)
(mean ± SEM)

Vehicle, q12h
RUX 30 mg/kg, q12h
RUX 60 mg/kg, q12h
RUX 90 mg/kg, q12h

B

Reticulocyte count (x 10^12/L)
(mean ± SEM)

Vehicle, q12h
RUX 30 mg/kg, q12h
RUX 60 mg/kg, q12h
RUX 90 mg/kg, q12h

C

WBC count (10^9/L)
(mean ± SEM)

D

Circulating GFP cells (%)
(mean ± SEM)

Vehicle, q12h
RUX 30 mg/kg, q12h
RUX 60 mg/kg, q12h
RUX 90 mg/kg, q12h
Figure 4

A

B

C

D

vehicle
PAN 4 mg/kg, 3qw
PAN 8 mg/kg, 3qw
PAN 12 mg/kg, 3qw

vehicle
PAN 4 mg/kg, 3qw
PAN 8 mg/kg, 3qw
PAN 12 mg/kg, 3qw

Spleen weight (mg) (mean ± SEM)

Reticulocyte count (x 10^12/L) (mean ± SEM)

WBC count (10^9/L) (mean ± SEM)

Circulating GFP cells (%) (mean ± SEM)
Figure 6

A

Vehicles

<table>
<thead>
<tr>
<th>Spleen p-STAT5</th>
<th>PAN</th>
<th>RUX</th>
<th>PAN + RUX</th>
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B

p-STAT5 positive nuclei (% (mean ± SEM)

Vehicle | PAN | RUX | PAN + RUX

C

Spleen Acetyl-histone H3 histology score (mean ± SEM)

Vehicle | PAN | RUX | PAN + RUX

BM Acetyl-histone H3 histology score (mean ± SEM)

Vehicle | PAN | RUX | PAN + RUX
JAK1/2 and pan-deacetylase inhibitor combination therapy yields improved efficacy in preclinical mouse models of JAK2V617F-driven disease

Emeline Evrot, Nicolas Ebel, Vincent Romanet, et al.

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