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Original Article

Fedratinib and gandotinib induce apoptosis and enhance the efficacy of tyrosine kinase inhibitors in human mast cells

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Abstract: Mastocytosis is characterized by an abnormal accumulation of mast cells (MC) in various organs. In most patients, the disease is driven by the *KIT* D816V mutation, leading to activation of the KIT receptor and subsequent downstream signaling, including the JAK/STAT pathway. In recent years, KIT-targeting tyrosine kinase inhibitors (TKI) have emerged for the treatment of systemic mastocytosis; however, the overall response rate is often not sufficient. In this study, we investigated whether targeting the JAK/STAT pathway might be a novel treatment approach in mastocytosis. Using human MC lines carrying the *KIT* D816V mutation and human primary cord blood-derived MC, we examined the effects of different JAK inhibitors. Our findings revealed that the JAK inhibitors fedratinib and gandotinib decreased viability, reduced proliferation, and induced apoptosis in *KIT* D816V-positive MC lines (HMC-1.2 and ROSA$^{KIT\ D816V}$). In contrast, ruxolitinib, baricitinib, upadacitinib and abrocitinib failed to affect MC functions. Combinatorial treatment with fedratinib, gandotinib and the two TKI avapritinib and midostaurin was more effective than treatment with TKI alone. Fedratinib also induced apoptosis and enhanced the efficacy of TKI in primary cord blood-derived MC. These results indicate that fedratinib and gandotinib, but not the other JAK inhibitors used in this study, can suppress viability and induce apoptosis in *KIT* D816V-mutant and *KIT* WT MC and increase effects of TKI. These findings suggest to explore fedratinib and gandotinib as novel treatment option in mastocytosis.

Keywords: Avapritinib, fedratinib, JAK inhibitors, *KIT* mutation, *KIT* D816V, mast cells, mastocytosis, midostaurin, tryptase, tyrosine kinase inhibitors

Introduction

Mastocytosis is a clonal disease characterized by abnormal expansion and accumulation of neoplastic mast cells (MC) affecting various organs, such as the skin, bone marrow (BM) and gastrointestinal tract [1]. The classification of mastocytosis encompasses cutaneous mastocytosis, systemic mastocytosis (SM) and MC sarcoma. SM can be subdivided according to symptoms and prognosis into non-advanced SM, which includes indolent SM (ISM), BM mastocytosis (BMM) and smoldering SM (SSM), and advanced SM, which comprises aggressive SM (ASM), SM with an associated hematologic neoplasm (SM-AHN) and mast cell leukemia (MCL) [2, 3]. Patients with non-advanced forms suffer predominantly from MC mediator-related symptoms, whereas patients with advanced SM develop organ dysfunction and show a progressive course with poor prognosis [4, 5].

Regardless of the subtype, more than 90% of SM cases are driven by the *KIT* D816V mutation [6]. In addition, several non-*KIT* somatic mutations, such as *SRSF2*, *ASXL1*, *RUNX1* and others, can contribute to the pathophysiology of SM and are usually associated with more advanced forms of the disease and poor prognosis [7]. Treatment of SM includes tyrosine kinase inhibitors (TKI) like the *KIT* D816V-targeting TKI avapritinib [8, 9] and the multiki-

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nase inhibitor midostaurin [10, 11]. Furthermore, several clinical trials testing alternative *KIT* D816V-targeting TKI for the treatment of SM are currently ongoing [12, 13]. Allogeneic hematopoietic stem cell transplantation (ASCT) is the only potentially curative option; however, the advantages of ASCT in the era of modern KIT inhibitors remain to be defined [14, 15]. Despite these treatment options, many patients with SM are still not treated sufficiently or develop side effects, therefore, identifying novel therapeutic strategies for mastocytosis is of particular importance [5, 16].

The *KIT* D816V mutation leads to enhanced proliferation and survival of neoplastic MC by stem cell factor (SCF)-independent activation of the KIT receptor. This, in turn, activates several downstream signaling pathways such as Janus kinase (JAK)/signal transducers and activators of transcription (STAT), phosphatydylinositol triphosphate (PI3)-kinase, protein kinase C (PKC), Ras/mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mammalian target of rapamycin (mTOR) [17-24].

The JAK-STAT pathway is a highly conserved signaling network. It comprises four JAK (JAK1, JAK2, JAK3 and Tyk2), and seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) [25]. Dysregulation of the JAK/STAT pathway is associated with various diseases, especially cancers, allergic and autoimmune diseases. Therefore, several JAK inhibitors were developed, and are currently used in the therapy of atopic dermatitis, autoimmune diseases, myeloproliferative neoplasms and infectious diseases [26]. In diseases characterized by a hyperactivated JAK/STAT pathway, elevated proinflammatory cytokines in the serum were shown to respond well to JAK inhibitors. Also, JAK inhibitors were found to suppress MC activation and growth, suggesting their potential use in the setting of SM [27, 28]. Considering the high efficacy of JAK inhibitors in a wide range of diseases and their relatively favorable safety profile, we sought to investigate their potential in the treatment of mastocytosis.

In the current study, we analyzed the impact of different JAK inhibitors in *KIT* D816V-mutant human MC lines and primary human MC. We also explored the effect of combinatorial treatments with JAK inhibitors and TKI in these preclinical cell models of mastocytosis.

Material and methods

MC lines

The human MC lines HMC-1.1 (carrying the *KIT* V560G mutation) and HMC-1.2 (carrying both *KIT* V560G and *KIT* D816V mutations) were kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) [29]. They were maintained in Iscove's modified Dulbecco's medium (IMDM, Sigma-Aldrich, Irvine, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% (vol/vol) penicillin-streptomycin (BioConcept, Allschwil, Switzerland) and 1.2 mM 1-thioglycerol (Sigma-Aldrich, Irvine, United Kingdom). The human MC lines ROSA$^{KIT\ D816V}$ and ROSA$^{KIT\ WT}$ cells were generated and kept as previously described [30]. In brief, ROSA$^{KIT\ D816V}$ were cultured in IMDM supplemented with 10% FCS and 1% (vol/vol) penicillin-streptomycin. ROSA$^{KIT\ WT}$ and MCPV-1.1 [31], kindly provided by Prof. Dr. Peter Valent (Vienna, Austria), cells were cultured in IMDM supplemented with 10% FCS, 1% (vol/vol) penicillin-streptomycin and 10% SCF-containing supernatants of Chinese hamster ovary cells transfected with the murine s*cf* (kl) gene (CHO-Kl). CHO-Kl cells were kindly provided by Dr. Patrice Dubreuil (Marseille, France) [32]. Cells were expanded in DMEM enriched with 10% (vol/vol) FCS for the collection of SCF-containing supernatant, which was filtered via 0.22 µM syringe filters and stored at -20°C. Cells were tested for mycoplasma contamination by conventional PCR using the following specific primers: forward (GGG AGC AAA CAG GAT TAG ATA CCC T) and reverse (TGC ACC ATC TGT CAC TCT GTT AAC CTC).

Generation of human cord blood-derived MC

The generation of human primary cord blood-derived MC (CBMC) was performed as described previously [33]. Briefly, human CD34$^+$ hematopoietic progenitor cells were purified from cord blood mononuclear cells by density gradient separation (Density Diluent Medium mixed with High Density Spin Medium) (pluriSelect, Leipzig, Germany) followed by magnetic separation with CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions, and after informed consent and institutional review board approval. Isolated CD34$^+$ cells were cultured in serum-free medium (SFEM, STEMCELL™ Technologies, Vancouver, BC) supple-

mented with penicillin (100 units/mL), streptomycin (100 µg/mL), SCF-containing medium (3% vol/vol), interleukin-6 (IL-6, 50 ng/mL; PeproTech, Rocky Hill, USA) and interleukin-3 (IL-3, 10 ng/mL; PeproTech) for four weeks. Thereafter, cells were cultured for additional four to eight weeks in IMDM supplemented with insulin-transferrin-selenium, 2-mercaptoethanol, GlutaMAX-I (all from Gibco, New York, NY), penicillin-streptomycin, SCF (100 ng/mL) and IL-6 (50 ng/mL). Cell purity was confirmed by flow cytometry using anti-CD117 and -FcεRI antibodies (Table S1) on a CytoFLEX flow cytometry analyzer (Beckman Coulter, California, US).

JAK inhibitors and TKI

The JAK inhibitors ruxolitinib (INCB018424), baricitinib (LY3009104), upadacitinib (ABT-494), abrocitinib (PF-04965842), fedratinib (TG-101348) and gandotinib (LY2784544) and the TKI avapritinib (BLU-285) and midostaurin (PKC-412) were all purchased from MedChemExpress (Monmouth Junction, NJ, US). Ruxolitinib and baricitinib are known to target JAK1 and JAK2 (JAK1/2 inhibitors), while upadacitinib and abrocitinib predominantly target JAK1 (JAK1 inhibitors) and fedratinib and gandotinib predominantly JAK2 (JAK2 inhibitors).

XTT viability assay

HMC-1.2 and ROSA$^{KIT\ D816V}$ cells were seeded at a density of 3.5×10^5 cells/mL and treated with the indicated substances for 48 hours. After treatment, cell viability was determined with the CyQUANT XTT cell viability assay (ThermoFisher Scientific, Waltham, Massachusetts, US) following the manufacturer's protocol. Cell viability was detected by measuring absorbance at 450 nm and 660 nm (SynergyH1 Hybrid Reader, BioTek, Vermont, US) and normalized to DMSO-treated cells. IC_{50} for inhibitors were calculated by non-linear regression analysis (Prism 9.0).

Proliferation assay

HMC-1.2 and ROSA$^{KIT\ D816V}$ cells were seeded at a density of 10^5/mL and treated with the indicated substances for 48 hours. After treatment, cells were incubated with EdU (5-ethynyl-2'-deoxyuridine, EdU FC 488 Kit from Baseclick, Munich, Germany) for 90 minutes, followed by fixation and permeabilization. The click reaction with 6-FAM azide (488 nm) was performed according to the manufacturer's instructions. Proliferating cells (defined as EdU-positive cells) were evaluated by flow cytometry using a CytoFLEX flow cytometry analyzer.

Apoptosis assay

HMC-1.2 and ROSA$^{KIT\ D816V}$ cells were seeded at a density of 3.5×10^5 cells/mL and treated with the indicated substances for 48 hours. After treatment, cells were stained with the FITC Annexin V Apoptosis Detection Kit (BioLegend, San Diego, CA, US) according to the manufacturer's instructions, and analyzed by flow cytometry as described before. CBMC were seeded at a density of 5×10^5 cells/mL at week 8 of cultivation and treated with the indicated substances for 48 hours. After treatment, cells were incubated with antibodies against CD117 and FcεRI, then with annexin V and 7-AAD antibodies according to the manufacturer's instructions, and finally analyzed by flow cytometry. MC were identified as CD117$^+$ FcεRI$^+$. Apoptotic MC were identified as annexin V- and 7-AAD-positive cells within the MC population (CD117$^+$ FcεRI$^+$ cells).

Western blotting

Technical details are described in the 'Methods' Section of the Supplementary Materials.

Statistical analysis

Results are shown as mean ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey post-hoc multiple comparison test or two-way ANOVA with Šidák post-hoc multiple comparison test (Prism 9.0). *P* values <0.05 were considered significant. No samples were excluded from the analysis.

Results

Fedratinib and gandotinib decrease viability in human neoplastic MC lines

To investigate the overall impact of JAK inhibitors on functions of human neoplastic MC, we conducted a pharmacological screening using the human neoplastic MC line HMC-1.2, which carries the *KIT* D816V and *KIT* V560G mutations (**Figure 1A-C**) [29]. First, we assessed the viability of HMC-1.2 cells after the treatment with various JAK inhibitors, including those predominantly targeting JAK1 (upadacitinib, ab-

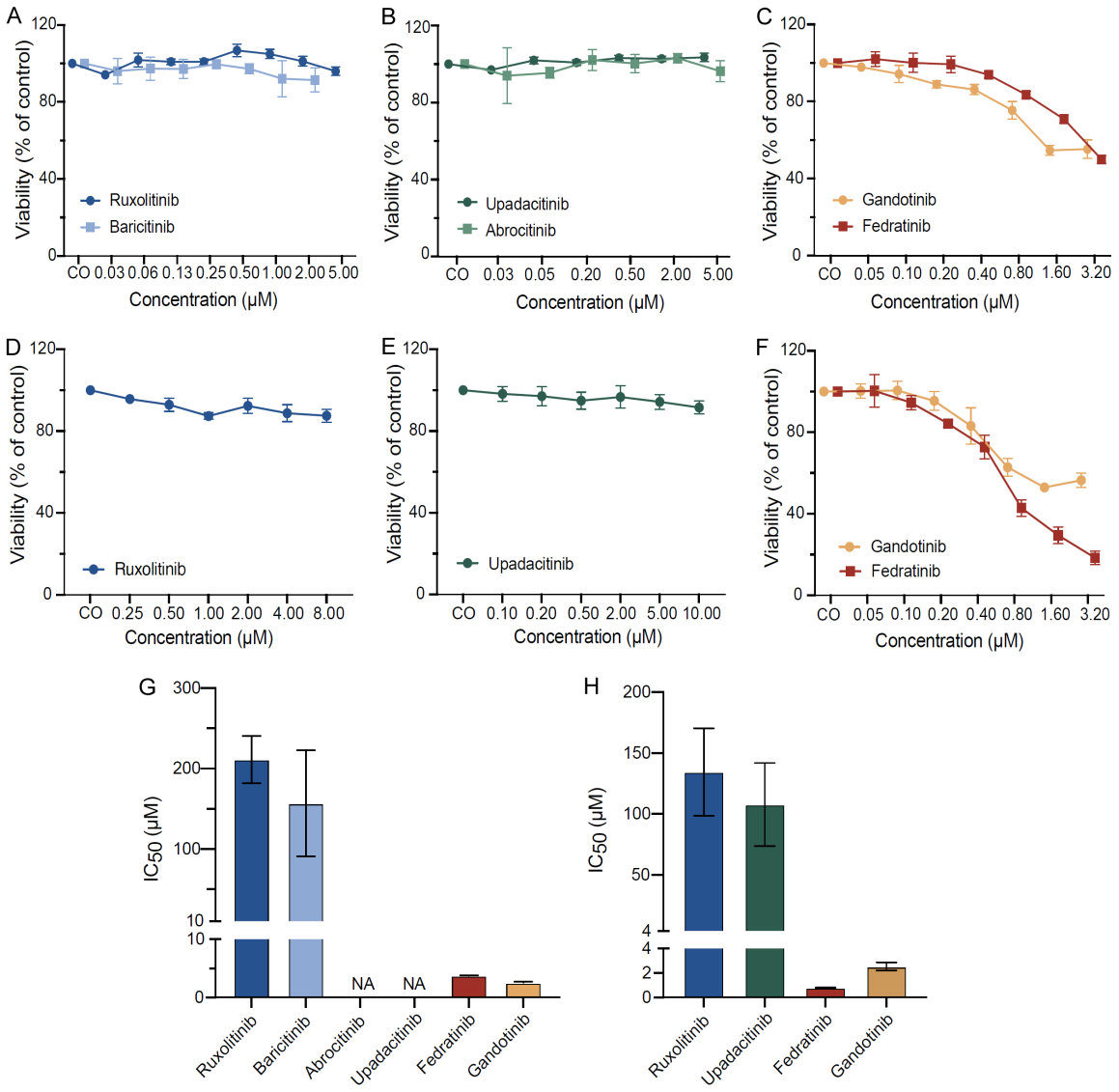

Figure 1. Fedratinib and gandotinib decrease viability in human neoplastic MC lines. HMC-1.2 (A-C) and ROSA$^{KIT\ D816V}$ cells (D-F) were incubated in DMSO control medium (CO) or various concentrations of ruxolitinib, baricitinib (A, D), abrocitinib, upadacitinib (B, E), fedratinib and gandotinib (C, F) for 48 h. MC viability was evaluated by CyQUANT™ XTT Cell Viability Assay and normalized to DMSO-treated cells. IC_{50} of JAK inhibitors in HMC-1.2 (G) and ROSA$^{KIT\ D816V}$ cells (H) were calculated by nonlinear regression analysis. Results represent the mean and SEM of three to five independent experiments.

rocitinib), JAK2 (fedratinib, gandotinib) or both JAK1/2 (ruxolitinib, baricitinib). Upadacitinib, abrocitinib, ruxolitinib and baricitinib failed to affect MC viability (**Figure 1A** and **1B**, respectively). In contrast, fedratinib and gandotinib decreased viability of HMC-1.2 (**Figure 1C**), with IC_{50} values of 3.74 μM for fedratinib and 2.65 μM for gandotinib (**Figure 1G**).

To validate these findings, the same set of experiments was performed using a second human MC line carrying the *KIT* D816V mutation, ROSA$^{KIT\ D816V}$ (**Figure 1D-F**). Similarly, in ROSA$^{KIT\ D816V}$ cells, ruxolitinib (**Figure 1D**) and upadacitinib (**Figure 1E**) did not affect MC viability at concentrations up to 8-10 μM, whereas fedratinib and gandotinib decreased viability with IC_{50} values of 0.8 μM for fedratinib and 2.32 μM for gandotinib (**Figure 1F** and **1H**).

In the human MC lines HMC-1.1 and MCPV-1.1, a decrease in MC viability was observed only with fedratinib and gandotinib, consistent with the results obtained for the *KIT* D816V-carrying MC lines (data not shown). In ROSA$^{KIT\ WT}$ cells, fedratinib and gandotinib exhibited 15-25 ti-

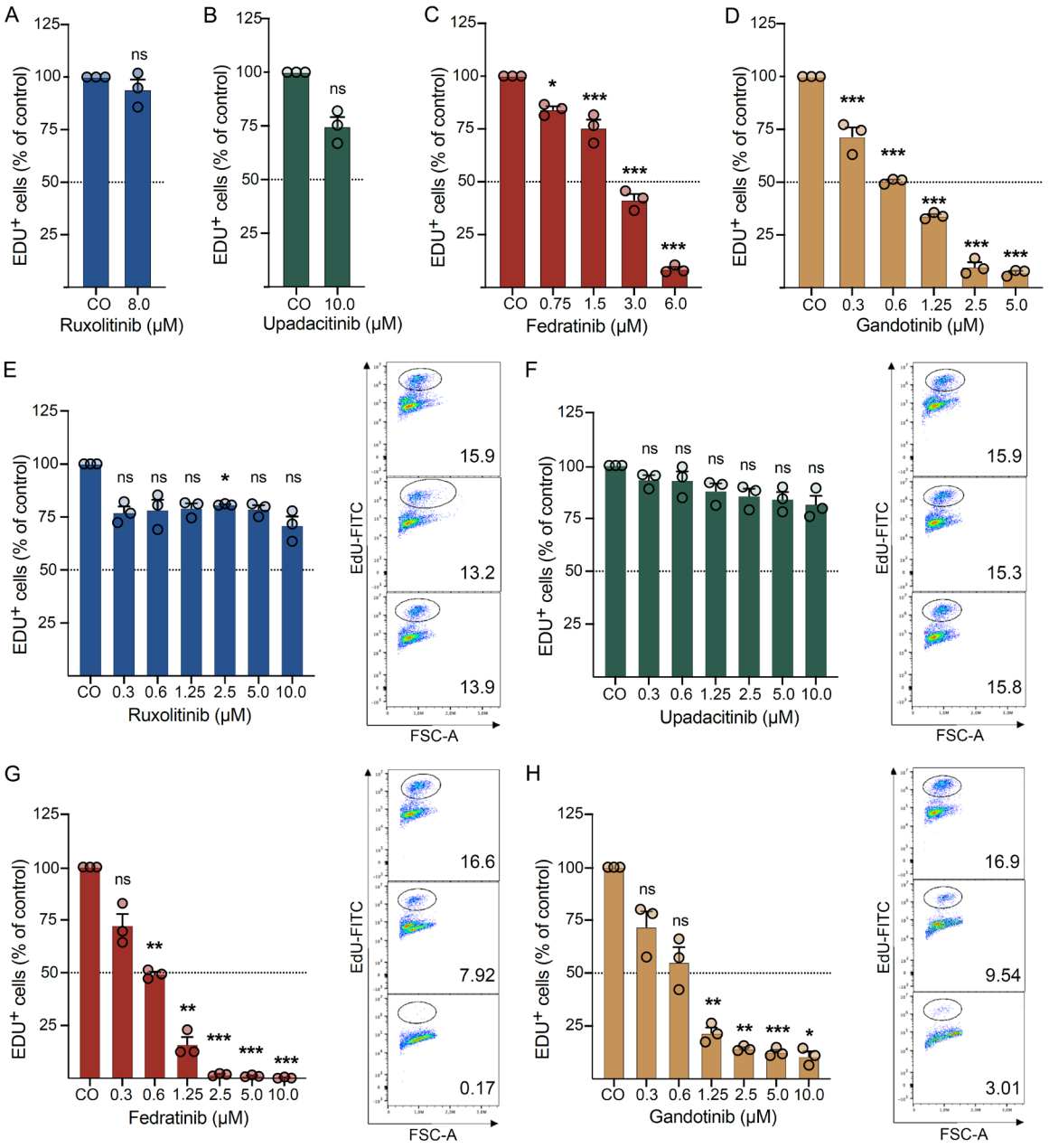

Figure 2. Fedratinib and gandotinib decrease proliferation in human neoplastic MC lines. HMC-1.2 (A-D) and ROSA$^{KIT\ D816V}$ (E-H) cells were incubated in a DMSO control medium (CO) or various concentrations of ruxolitinib (A, E), upadacitinib (B, F), fedratinib (C, G) and gandotinib (D, H) for 48 h. Proliferating cells were considered as EdU$^+$ (Edu-FITC) and normalized to DMSO-treated cells. Results represent the mean and SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. (E-H) Right panel. Representative flow cytometry plots for corresponding JAK inhibitors are also shown (from top to bottom): CO, 0.6 μM, 2.5 μM.

mes lower IC_{50} compared to the other JAK inhibitors (data not shown).

Fedratinib and gandotinib decrease proliferation in human neoplastic MC lines

In line with what was observed for viability, ruxolitinib (**Figure 2A** and **2E**) and upadacitinib (**Figure 2B** and **2F**) failed to affect proliferation in the two MC lines HMC-1.2 and ROSA$^{KIT\ D816V}$ up to 8-10 μM concentrations. On the contrary, fedratinib and gandotinib demonstrated dose-dependent inhibition of proliferation in both HMC-1.2 (**Figure 2C**, **2D**) and ROSA$^{KIT\ D816V}$ (**Figure 2G**, **2H**) cells. In HMC-1.2 cells, a significant reduction in proliferation was observed already at 0.75 μM for fedratinib and 0.3 μM for

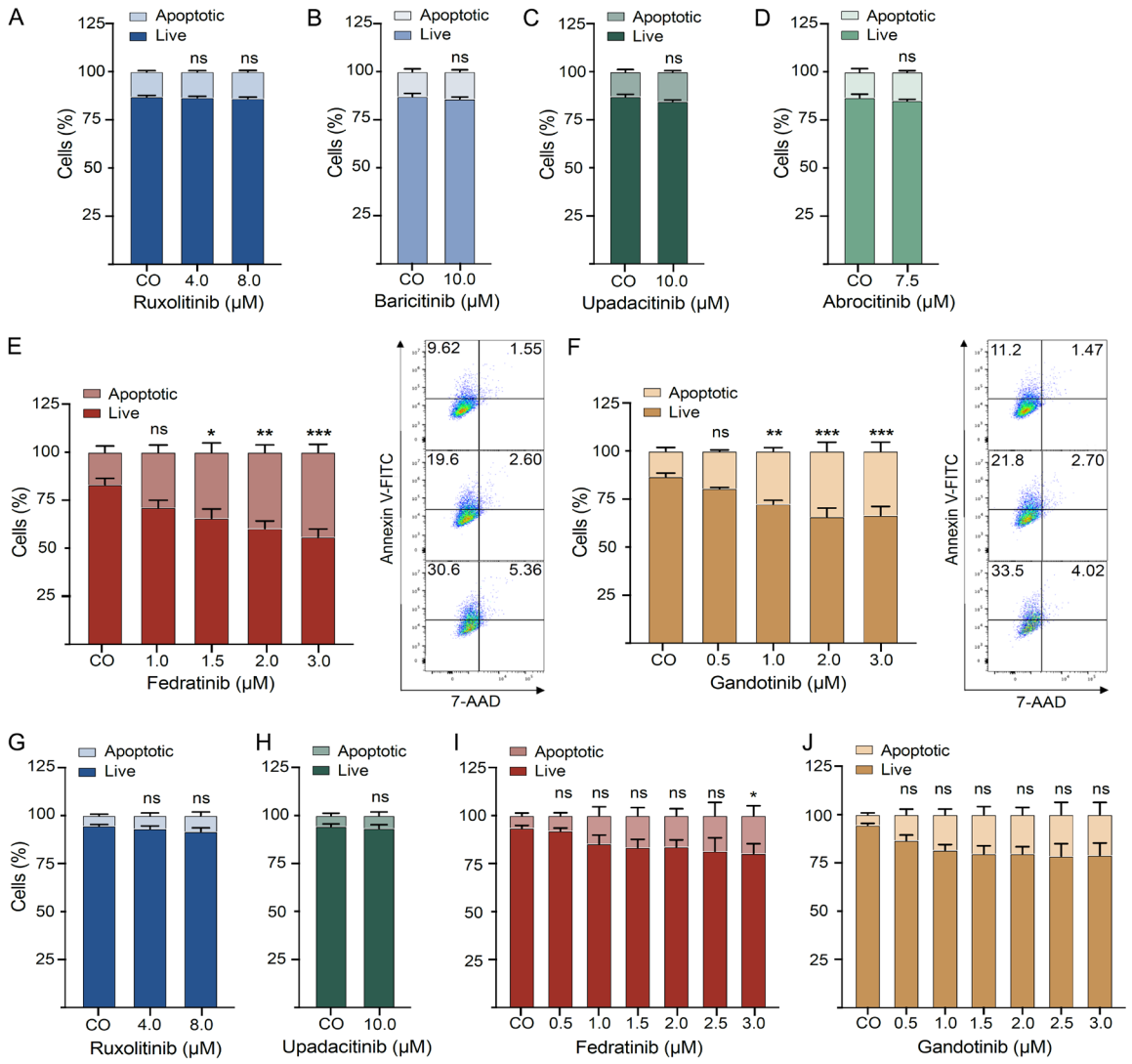

Figure 3. Fedratinib and gandotinib induce apoptosis in human neoplastic MC lines. HMC-1.2 (A-F) and ROSA$^{KIT\ D816V}$ (G-J) cells were incubated in a DMSO control medium (CO) or various concentrations of ruxolitinib (A, G), baricitinib (B), upadacitinib (C, H), abrocitinib (D), fedratinib (E, I) and gandotinib (F, J) for 48 h. Apoptotic cells were defined as annexin$^+$ and annexin$^+$7-AAD$^+$ cells by flow cytometry as described in 'Materials and Methods'. Results represent the mean and SEM of three to five independent experiments. *P<0.05, **P<0.01, ***P<0.001. Representative flow cytometry plots for corresponding JAK inhibitors are shown (from top to bottom): (E) CO, 1.5 μM, 3.0 μM; (F) CO, 1.0 μM, 3.0 μM.

gandotinib, while in ROSA$^{KIT\ D816V}$ cells, significant anti-proliferative effects were observed at 0.6 μM for fedratinib and 1.25 μM for gandotinib.

Fedratinib and gandotinib induce apoptosis in human neoplastic MC lines

To evaluate how JAK inhibitors affect the survival of human neoplastic MC, the number of apoptotic cells was evaluated in HMC-1.2 cells after treatment with different JAK inhibitors (**Figure 3A-F**). Ruxolitinib, baricitinib, upadacitinib and abrocitinib (**Figure 3A-D**) failed to affect apoptosis of HMC-1.2 cells up to concentrations of 7.5-10 μM. By contrast, fedratinib and gandotinib significantly induced apoptosis in a dose-dependent manner at pharmacologically meaningful concentrations (1.5 μM for fedratinib and 1.0 μM for gandotinib), as evidenced by flow cytometry (**Figure 3E**, **3F**).

In ROSA$^{KIT\ D816V}$ cells, apoptosis-inducing activity was less prominent, but also reached statis-

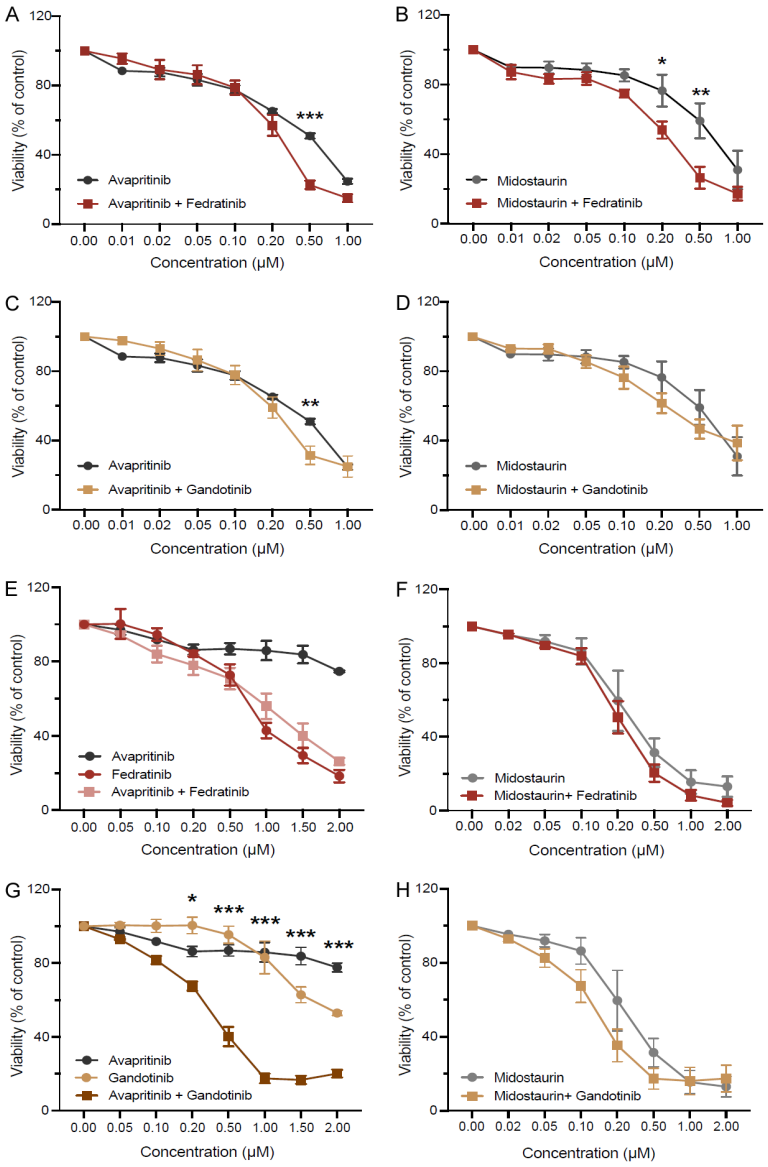

Figure 4. Fedratinib and gandotinib enhance the viability-reducing effect of TKI in human neoplastic MC lines. HMC-1.2 (A-D) and ROSA$^{KIT\ D816V}$ (E-H) cells were incubated in a DMSO control medium, various concentrations of the TKI avapritinib (A, C, E, G) and midostaurin (B, D, F, H), either alone or in combination with fedratinib (A, B, E, F) and gandotinib (C, D, G, H) for 48 h. MC viability was evaluated by CyQUANT™ XTT Cell Viability Assay and normalized to the DMSO control medium. Results represent the mean and SEM of three to five independent experiments. As ROSA$^{KIT\ D816V}$ cells did not respond to avapritinib (E, G), the two-way ANOVA statistical test was performed between the JAK inhibitor group and their combinations with avapritinib. *P<0.05, **P<0.01, ***P<0.001.

tical significance in cells treated with fedratinib at a concentration of 3 μM (**Figure 3I**). Notably, ruxolitinib (**Figure 3G**) and upadacitinib (**Figure 3H**) failed to exert any impact on MC survival. Although treatment with gandotinib showed a slight increase in the apoptotic cell fraction, this effect was not statistically significant (**Figure 3J**).

Fedratinib and gandotinib enhance the viability-reducing effect of TKI in human neoplastic MC lines

In mastocytosis, especially in advanced forms, the use of drug combinations may be an effective approach to increase the response rate to treatment, overcome resistance to TKI and minimize side effects. Currently, two TKI, namely the *KIT* D816V-selective inhibitor avapritinib and the multikinase inhibitor midostaurin, are used in clinical routine for the treatment of SM. Therefore, we explored the combination of TKI with fedratinib and gandotinib (**Figure 4**), since the latter were found to impact viability, proliferation, and apoptosis of neoplastic MC in single-treatment experiments (**Figures 1-3**).

In HMC-1.2 cells, avapritinib in combination with either fedratinib (**Figure 4A**) or gandotinib (**Figure 4C**) was significantly more efficient in reducing MC viability at a concentration of 0.5 μM compared to single treatment with avapritinib. Similarly, the combinatorial treatment of midostaurin with fedratinib at concentrations of 0.2 μM and 0.5 μM was significantly more effective in reducing MC viability compared to single treatment with midostaurin (**Figure 4B**). The combination of midostaurin with gandotinib did not demonstrate a significant effect on viability compared to single treatment with midostaurin (**Figure 4D**).

ROSA$^{KIT\ D816V}$ cells did not respond to single treatment with avapritinib, at least at concentrations up to 2 μM (**Figure 4E** and **4G**). How-

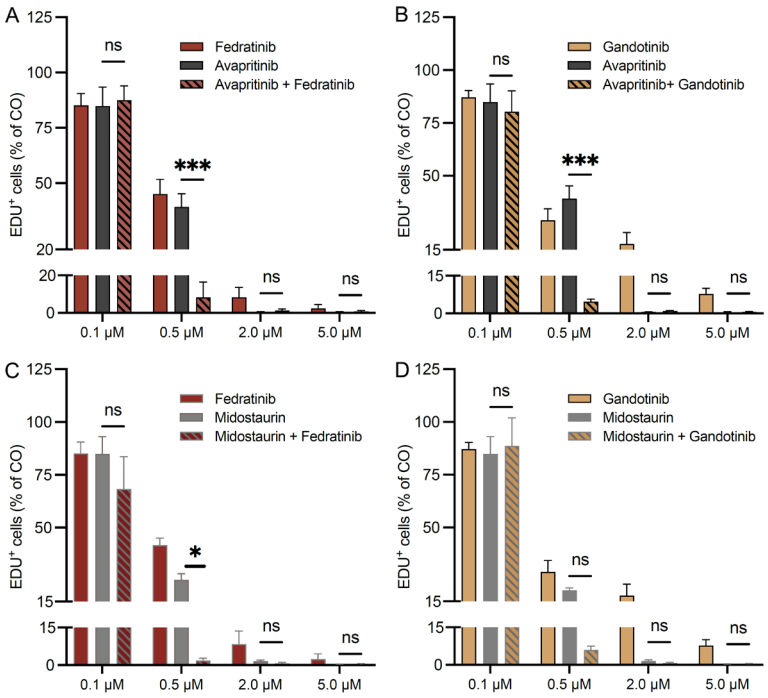

Figure 5. Fedratinib and gandotinib enhance the growth-inhibitory effect of TKI in human neoplastic MC lines. HMC-1.2 cells were incubated in a DMSO control medium or various concentrations of avapritinib (A, B) and midostaurin (C, D), alone and in combination with fedratinib and gandotinib, for 48 h. Proliferating cells were considered as EdU$^+$ and normalized to DMSO control as described in the 'Materials and Methods' section. Results represent the mean and SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

ever, when avapritinib was combined with gandotinib, cell viability was significantly reduced compared to single treatment with gandotinib (**Figure 4G**). Combinations of midostaurin with either fedratinib (**Figure 4F**) or gandotinib (**Figure 4H**) did not show an additional decrease in viability of ROSA$^{KIT\ D816V}$ cells compared to single treatment.

Fedratinib and gandotinib enhance the proliferation-inhibiting effect of TKI in human neoplastic MC lines

Given the previous finding that fedratinib and gandotinib increased the effect of TKI on viability of MC lines, we next tested whether combined JAK and KIT targeting had a similar effect on MC proliferation (**Figure 5**). Here, we found that fedratinib (**Figure 5A**) and gandotinib (**Figure 5B**) both showed cooperative effects with avapritinib on proliferation in HMC-1.2 cells, with a statistically significant decrease of proliferation at a concentration of 0.5 μM, compared to single treatment with avapritinib. Similarly, combining fedratinib with midostaurin significantly reduced proliferation at 0.5 μM compared to single treatment with midostaurin (**Figure 5C**), while the combination of midostaurin with gandotinib did not demonstrate a significant effect on proliferation (**Figure 5D**).

Fedratinib inhibits phosphorylation of signaling molecules downstream of KIT in human neoplastic MC lines

To explore the mechanism of decreased MC functions in response to JAK inhibitors, we next performed Western blotting to analyze the expression of several signaling molecules downstream of KIT, including STAT5, STAT3 and AKT, after incubation with fedratinib, avapritinib and combined fedratinib and avapritinib (Figure S1). In these experiments, we observed that the *KIT* D816V-targeting TKI avapritinib, as expected, suppressed phosphorylation of pKIT in both HMC-1.2 (Figure S1A) and ROSA$^{KIT\ D816V}$ (Figure S1B) cells. In addition, avapritinib decreased phosphorylation of pSTAT5 and pSTAT3 in HMC-1.2, but not in ROSA$^{KIT\ D816V}$ cells. Fedratinib was also found to reduce phosphorylation of pSTAT5 and pSTAT3 in both cell lines. Furthermore, combinatorial treatment with fedratinib and avapritinib decreased phosphorylation of pKIT, pSTAT5 and pSTAT3 in both cell lines and pAKT in HMC-1.2 cells.

Fedratinib enhances apoptosis induced by TKI in human cord blood-derived MC

To further expand our findings, we analyzed the effect of various JAK inhibitors, TKI and their combination on apoptosis of primary human MC (**Figure 6**). In 8-week cultured CBMC, ruxolitinib (**Figure 6A**, **6B**) and upadacitinib (**Figure 6C**, **6D**) did not affect the number of apoptotic cells. The TKI avapritinib (**Figure 6A**, **6C**, **6E**) and midostaurin (**Figure 6B**, **6D**, **6F**) both

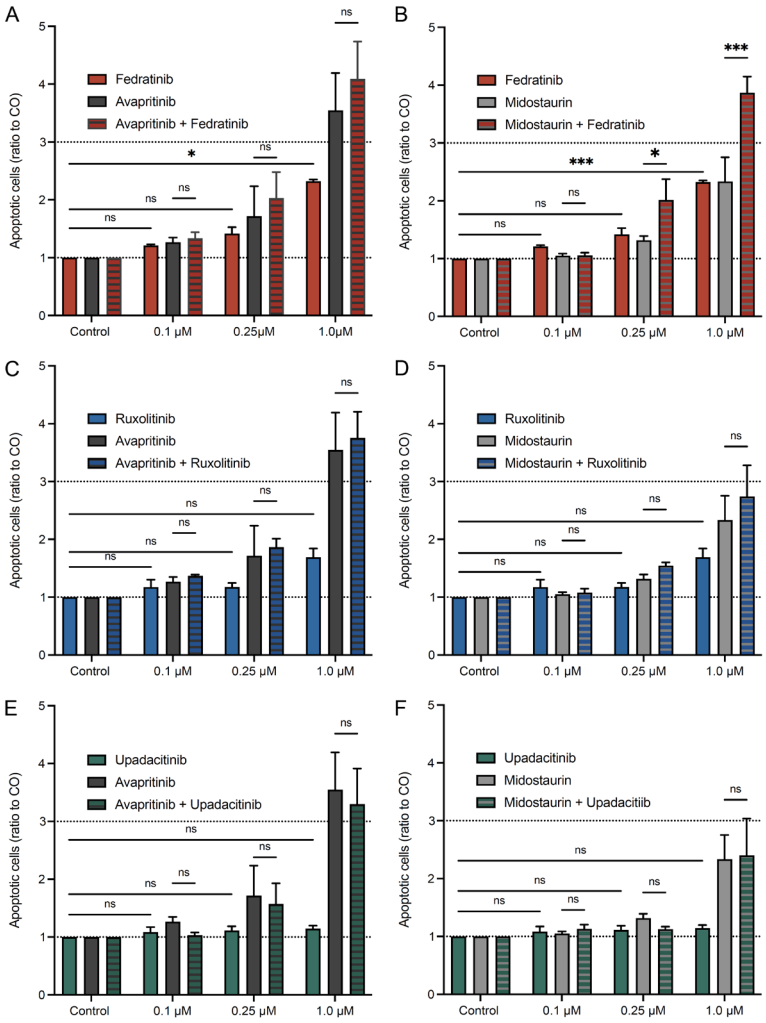

Figure 6. Fedratinib induces apoptosis and increases the effect of the TKI midostaurin in human CBMC. Primary human CBMC, cultured for eight weeks, were incubated in DMSO control medium or medium containing various concentrations of ruxolitinib (A, B), upadacitinib (C, D), fedratinib (E, F), avapritinib (A, C, E) and midostaurin (B, D, F) as well as combinations of JAK inhibitors and TKI for 48 h. CBMC were identified as $CD117^+FceRI^+$ cells, and apoptotic CBMC were defined as $annexin^+$ and $annexin^+7\text{-}AAD^+$ cells by flow cytometry, as described in the section 'Materials and Methods' of this manuscript. Results represent the mean and SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.

induced apoptosis at higher concentrations (1.0 µM) in CBMC. There was no increase in apoptosis when treatments with either ruxolitinib or upadacitinib were combined with TKI. In contrast, treatment with fedratinib showed a trend, albeit not statistically significant, for inducing apoptosis in CBMC (**Figure 6E**, **6F**). Moreover, we observed a significant increase in apoptosis upon combinatorial treatment with fedratinib and midostaurin at 1.0 µM in CBMC (**Figure 6F**).

Discussion

In the present study, we show that fedratinib and gandotinib, but not the other JAK inhibitors tested in our study (ruxolitinib, baricitinib, upadacitinib and abrocitinib), reduce viability and proliferation and induce apoptosis in human *KIT* D816V-mutant MC lines. We also found that fedratinib and gandotinib induce apoptosis in primary human CBMC. Furthermore, we demonstrate that fedratinib and gandotinib enhance the anti-proliferative and cytotoxic effects of TKI, both in human neoplastic MC lines as well as in CBMC. These findings provide a rationale for exploring the potential of JAK inhibitors, especially fedratinib and gandotinib, in MC-associated diseases. In particular, our data on *KIT* D816V-mutant MC suggest the potential of using these JAK inhibitors, either alone or in combination with TKI, in the treatment of mastocytosis.

Numerous studies have shown antineoplastic effects of JAK inhibitors in various cancer cell lines (summarized e.g. by Qureshy *et al.*) [34]. JAK1 inhibitors (AZD4205, itacitinib, filgotinib), JAK1/2 inhibitors (ruxolitinib, momelotinib) as well as JAK2 inhibitors (fedratinib, pacritinib, WP1066) have been found to have growth-inhibitory effects in preclinical solid tumor models. Furthermore, fedratinib and ruxolitinib have demonstrated antineoplastic effects in myeloproliferative neoplasms (MPN) in both preclinical models and clinical trials, and are currently FDA-approved for the treatment of MPN patients [34]. Emerging data suggest that JAK inhibitors increase the efficacy of chemotherapeutic and other targeted agents, mostly by preventing STAT3 activation, which is involved in chemotherapy insensitivity [35]. In

addition, Mathew and colleagues described that itacitinib improved clinical and immune responses to anti-PD1 treatment via promotion of CD8 T cell plasticity and therapeutic responses of exhausted and effector memory - like T cell clonotypes [36].

To date, suppressive effects of JAK inhibitors have furthermore been demonstrated in various types of cells secreting pro-inflammatory cytokines and involved in the pathogenesis of allergic and autoimmune diseases. Nyireida *et al.* showed that tofacitinib, ruxolitinib and AG490 (tyrphostin) decreased the production of proinflammatory cytokines by cytokine-activated T cells (Tck) and Tck cell-activated macrophages. De Vries *et al.* found that tofacitinib and a JAK1 inhibitor affected macrophage activation and skewed polarization towards M2-like macrophages [37]. In addition, Kurowski *et al.* showed that the JAK1/2 inhibitor baricitinib suppressed basophil activation and degranulation in vitro [38].

However, there are only a few studies that investigated the impact of JAK inhibitors on MC functions. Hermans *et al.* showed that ruxolitinib does not have a cytotoxic effect on HMC-1.2 cells and the human MC line LAD2 at concentrations up to 50 μM. On the other hand, they observed that ruxolitinib inhibited codeine- and substance P-induced degranulation and the production of IL-6, TNF-α and MCP-1 in the two MC lines [28]. Tobio *et al.* demonstrated that the *KIT* D816V mutation is associated with increased secretion of IL-6 in both cells from mastocytosis patients and HMC-1 cells, and that ruxolitinib and fedratinib were able to inhibit the IL-6 production from HMC-1.2 cells [28, 39]. Furthermore, Lasho *et al.* reported that fedratinib was able to reduce phosphorylation of proteins downstream of JAK/STAT in HMC-1.1 and HMC-1.2 cells and that fedratinib acted synergistically with the TKI dasatinib to inhibit proliferation of HMC-1.2 cells, however, the effect of fedratinib on other cellular functions and on primary MC was not explored [27]. In addition, Keller *et al.* found that several JAK inhibitors, including fedratinib, decreased proliferation and induced apoptosis in canine *KIT*-mutant mastocytoma cell lines [40].

Taken together, these studies indicate that JAK inhibitors may not only exert direct cytostatic and cytotoxic effects on human neoplastic MC, but could also have prominent anti-inflammatory effects in mastocytosis. Patients with SM are known to show constitutive activation of functionally impaired blood monocytes and increased plasma levels of IL-1β, IL-6, IL-8, TNFα and IL-10 secreted by blood monocytes, indicating a broad activation of the innate immune response [41]. Recently, an altered distribution of leukocyte subsets and a proinflammatory proteome were described in two cohorts of patients with ISM [42]. Furthermore, increased plasma levels of IL-6 in patients with ISM were found to be associated with a high risk of later developing advanced forms of SM [43]. It was also demonstrated that TNF promotes the expansion of neoplastic MC via suppression of normal myeloid cells, and high levels of TNF correlated with inferior survival in SM [44]. Thus, patients with mastocytosis, particularly those with advanced SM, are also characterized by inflammation, which could be targeted by JAK inhibitors. In line, a recent study showed that the JAK2 inhibitor AG490 reduced TNF-induced IL-18 bioactivity by blocking caspase-1 in fibroblasts [45]; and IL-18 was found to also play an important role in the generation and maturation of MC and basophils from murine bone marrow progenitors [46].

Data on the usage of JAK inhibitors for the treatment of mastocytosis are limited to the description of a few clinical cases. In one case of ASM associated with myelofibrosis and a rare *KIT* K509I mutation, ruxolitinib was added to imatinib for better control of constitutional symptoms [47]. Another clinical report described a patient with SM-AHN (SM with refractory cytopenia with multilineage dysplasia) without *KIT* D816V mutation that achieved a good response upon ruxolitinib treatment and successfully underwent allogeneic stem cell transplantation [48]. However, in both cases, ruxolitinib was used off-label for the treatment of *KIT* D816V-negative forms of SM and never in combination with TKI. To our knowledge, previous pre-clinical studies mainly focused on ruxolitinib, while studies with other JAK inhibitors are very limited. As a result, the potential effects of other JAK inhibitors may have been overlooked, and they were therefore not considered for either single-agent or combinatorial treatment approaches in patients with mastocytosis. Moreover, topical JAK inhibitors have been demonstrated to be effective and rela-

tively safe for treating various skin diseases such as atopic dermatitis [49-51] and vitiligo [52]. However, their use has not been explored yet in cutaneous mastocytosis, where they might have potential, particularly in the treatment of mastocytoma.

In summary, our data show that fedratinib and gandotinib inhibit viability and proliferation and induce apoptosis in neoplastic MC, when used alone and in combination with TKI currently used for the therapy of SM. Hence, our findings suggest to explore whether this treatment is beneficial in patients with SM. In such study, it will also be of particular interest to investigate the anti-inflammatory potential of JAK inhibition, in addition to their direct cytotoxic effects on neoplastic MC, as a proinflammatory phenotype is associated with disease progression and poor survival in patients with SM.

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Disclosure of conflict of interest

MA has consulted for and received honoraria from AB Science, Blueprint Medicines and Thermo Fisher. KH has consulted for and received honoraria from ALK, Allergopharma, BioCryst, Blueprint, Cogent, Galderma, KalVista, Leo, Menarini, Novartis, Pfizer, Sanofi, Takeda and Thermo Fisher.

Abbreviations

advSM, advanced systemic mastocytosis; ASCT, allogeneic hematopoietic stem cell transplantation; ASM, aggressive systemic mastocytosis; BM, bone marrow; BMM, bone marrow mastocytosis; BSA, bovine serum albumin; CBMC, cord blood-derived mast cells; FCS, fetal calf serum; FcεRI, high-affinity receptor for IgE; ISM, indolent systemic mastocytosis; MC, mast cells; MCL, mast cell leukemia; MNC, mononuclear cells; MPN, myeloproliferative neoplasms; SCF, stem cell factor; SM, systemic mastocytosis; SM-AHN, systemic mastocytosis with an associated hematologic neoplasm; SSM, smoldering systemic mastocytosis; TKI, tyrosine kinase inhibitors.

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Supplementary Materials

Methods

Western blotting

HMC-1.2 and ROSA$^{KIT\ D816V}$ cells were seeded at a density of 10^6 cells/ml and treated with the indicated substances for 4 hours. Whole-cell protein extracts were prepared by cell lysis in Cell Lysis Buffer (Cell Signaling Technology, Cambridge, UK) supplemented with protease inhibitor and phosphatase inhibitor 2 (Calbiochem, Darmstadt, Germany). Cell lysates were incubated for 30 minutes on ice and insoluble particles were removed by centrifugation at 14,000 × g for 15 minutes. After normalizing for protein content, lysates were supplemented with Laemmli buffer, boiled for 5 minutes at 95°C, subjected to polyacrylamide gel electrophoresis using mPAGE™ 4-12% Bis-Tris gels (Millipore, Burlington, MA) and transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad, Hercules, CA). The membrane was blocked in 5% bovine serum (BSA) or 5% milk in tris-buffered saline (TBS) with 0.5% Tween20 solution (TBS-T) and incubated with primary antibodies to phosphorylated proteins (Table S1) overnight at 4°C. Secondary horseradish peroxidase (HRP)-coupled antibodies were purchased from Cell Signaling Technology (Cambridge, UK) and used according to the manufacturer's recommendations. After incubation with SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, Massachusetts, US), signals were detected on a FUSION FX system (Vilber, Paris, France). The membrane was then stripped using Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific, Waltham, Massachusetts, US) and reprobed with primary antibodies against total proteins, followed by secondary antibody and chemiluminescence detection.

Table S1. Antibodies applied for Western blotting and flow cytometry

	Clone	Source	Isotype	Dilution/concentration	Application	Company
p-KIT (Y703)	D12E12	Rabbit	IgG	1:3000	WB	Cell Signaling Technology
pSTAT3 (Y705)	D3A7	Rabbit	IgG	1:3000	WB	Cell Signaling Technology
pSTAT5 (Y694)	C11C5	Rabbit	IgG	1:3000	WB	Cell Signaling Technology
pAKT (S473)	D9E	Rabbit	IgG	1:5000	WB	Cell Signaling Technology
c-KIT	D13A2	Rabbit	IgG XP	1:3000	WB	Cell Signaling Technology
STAT3	7907	Rabbit	IgG	1:7000	WB	Cell Signaling Technology
STAT5	D206Y	Rabbit	IgG	1:3000	WB	Cell Signaling Technology
AKT (pan)	C67E7	Rabbit	IgG	1:5000	WB	Cell Signaling Technology
β-actin	13E5	Rabbit	IgG	1:10000	WB	Cell Signaling Technology
GAPDH	D16H11	Rabbit	IgG XP	1:5000	WB	Cell Signaling Technology
CD117	104D2	Mouse	IgG1	1.25 µl/0.25*10^6 cells	FACS	Invitrogen
FcεRI	AER-37/CRA-1	Mouse	IgG2b	1.25 µl/0.25*10^6 cells	FACS	BioLegend
CD63	H5C6	Mouse	IgG1	1.25 µl/0.25*10^6 cells	FACS	Biolegend
CD107a	H4A3	Mouse	IgG1	1.25 µl/0.25*10^6 cells	FACS	BioLegend
CD203c	NP4D6	Mouse	IgG1	1.25 µl/0.25*10^6 cells	FACS	BD

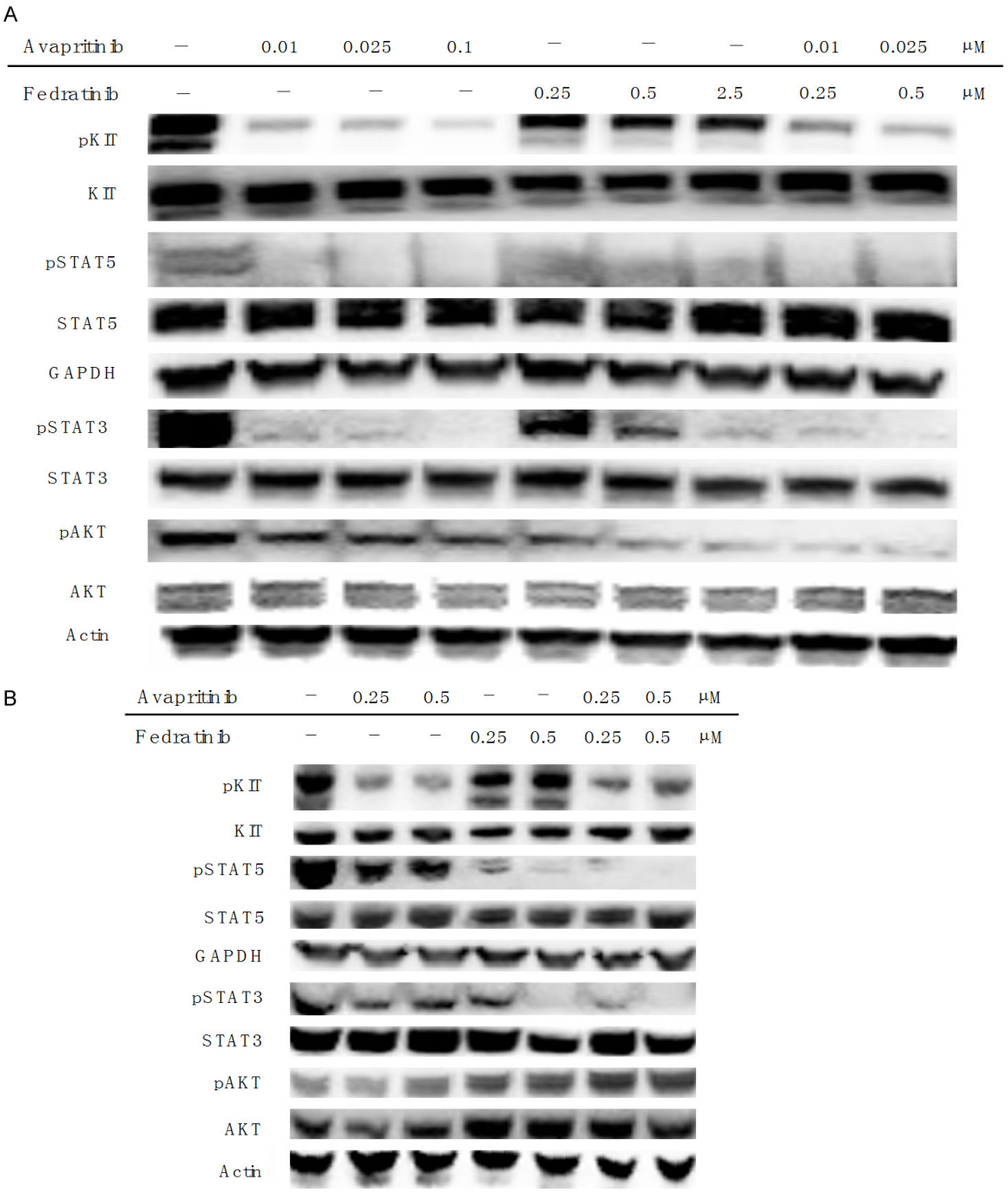

Figure S1. Fedratinib and avapritinib decrease phosphorylation of KIT and KIT-downstream molecules in human neoplastic MC lines. HMC-1.2 cells (A) and ROSA$^{KIT\ D816V}$ cells (B) cells were incubated in DMSO control medium or medium containing various concentrations of avapritinib, fedratinib or their combinations, as indicated, at 37 °C for four hours. Western blotting was performed using antibodies directed against phosphorylated (p) KIT, total KIT, pSTAT5, STAT5, pSTAT3, STAT3, pAKT and AKT. GAPDH and β-actin were used as loading controls. Technical details are described in the section 'Materials and Methods' of the manuscript. Data are representative of at least three independent experiments, of which three showed similar results.