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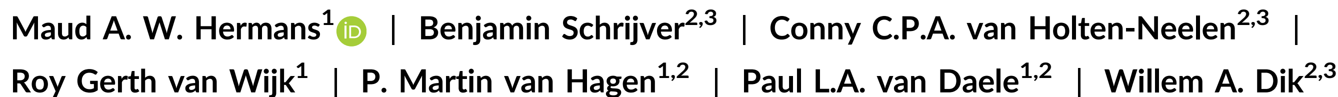
ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

The JAK1/JAK2- inhibitor ruxolitinib inhibits mast cell degranulation and cytokine release



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Abstract

Background: Mastocytosis is characterized by the accumulation of aberrant mast cells (MC). Patients suffering from mastocytosis suffer from a wide range of symptoms due to increased levels of MC mediators. It would therefore be of great benefit to inhibit MC mediator release. However, to date there are few drugs available that are known to effectively lower MC mediator levels. The evidence for the involvement of the janus kinase 2 (JAK2)—signal transducer and activation of transcription 5 (STAT5) signalling pathway in MC activation is slowly accumulating. Interference with the JAK2-STAT5 pathway might inhibit MC mediator release. Ruxolitinib, a JAK1/JAK2 inhibitor, indeed decreases symptoms like pruritus and fatigue in patients with myeloproliferative neoplasms. Yet, detailed studies on how ruxolitinib affects human mast cell activity are lacking.

Objective: To investigate the effect of JAK1/2-inhibition with ruxolitinib in the human mast cell lines LAD2 and HMC1.

Methods: LAD2 and HMC1 were stimulated with substance P, codeine or the calcium ionophore A23817. The effect of ruxolitinib on mast cell degranulation (via measurement of β-hexosaminidase, histamine release and CD63 membrane expression) and IL-6, IL-13, MCP-1 and TNF-α production was investigated. The involvement of STAT5 activation was explored using the selective STAT5 inhibitor pimozide.

Results: Ruxolitinib effectively inhibited codeine- and substance P-induced degranulation in a concentration-dependent manner. Ruxolitinib also significantly inhibited the production of IL-6, TNF-α and MCP-1 as induced by A23817 and substance P. Selective STAT5 inhibition with pimozide resulted in diminished degranulation and inhibition of cytokine production as induced by A23817 and substance P.

Conclusions & clinical relevance: This study demonstrates that the JAK1/JAK2 inhibitor ruxolitinib can inhibit MCactivity, possibly through prevention of STAT5 activation. This renders the JAK-STAT pathway as an interesting target for therapy to release symptom burden in mastocytosis and many other MC mediator-related diseases.

KEYWORDS

JAK, mast cells, pharmacology, pimozide, ruxolitinib, STAT

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1 | INTRODUCTION

Mast cell related diseases such as systemic mastocytosis or mast cell activation syndrome often cause debilitating symptoms mainly due to mast cell (MC)-derived mediators.[1-3] Currently, these symptoms are treated symptomatically with histamine antagonists, leukotriene antagonists, cromolyn acid and acetylsalicylic acid.[4] However, this regime is often insufficient to reduce symptoms to an acceptable level in the daily life of patients, especially anaphylaxis, gastrointestinal symptoms or flushing can greatly influence the quality of life.[5]

Most recent studies on the treatment of mastocytosis focus on the application of tyrosine kinase inhibitors (TKI) in the advanced forms of systemic mastocytosis (SM). These inhibitors mainly act through targeting of the KIT-receptor to inhibit MC growth.[4,6,7] Some of these TKI are only effective in MC with wild-type KIT while most patients with mastocytosis harbour the activating D816V KIT mutation.[8] Although newer TKI like midostaurin are also effective in patients with the D816V KIT mutation,its use is often hampered by gastrointestinal adverse effects.[6] Whereas the search for effective therapies for advanced mastocytosis has been going on for years, it is recognized only recently that TKI might also be of benefit in patients with indolent mastocytosis. The indolent subtype has an excellent prognosis regarding survival, but patients regularly experience symptoms caused by the released MC-mediators rather than from actual tissue invasion by MC. Clinical experience with KIT-targeting TKI in indolent systemic mastocytosis is limited. The multi-TKI masitinib was found to diminish MC mediator-related symptoms such as fatigue and pruritus in 8-25% of patients.[9] Next to well-known mediators such as histamine, leukotrienes and tryptase, MC produce numerous cytokines which are associated with constitutional symptoms in mastocytosis.[10-13] Therefore, medication that inhibits the release of these MC mediators would be a valuable addition to the, currently limited, therapeutic arsenal for indolent systemic mastocytosis.

Janus kinase (JAK) molecules are involved in the intracellular transduction of signals from multiple receptors, leading to downstream activation of signal transducer and activation of transcription (STAT) molecules and subsequent cellular responses.[14] Of the different JAK- and STAT molecules and signaling pathways currently known, the JAK2-STAT5 pathway is considered the most important for growth and survival of MC.[15] STAT5 also plays a role in IgE-mediated MC degranulation, rendering the JAK2-STAT5 pathway an interesting target for the inhibition of MC activation.[16] In support of this, it has previously been shown that the JAK1/JAK2 inhibitor ruxolitinib attenuates ovalbumin-induced passive systemic anaphylaxis in mice, while another study demonstrated that several JAK2- and STAT5-inhibitors were able to inhibit activation of canine mastocytoma cell lines.[17,18] Furthermore, two recent case studies of patients with systemic mastocytosis reported that MC mediator-related symptoms decreased upon treatment with ruxolitinib.[19,20]

Although these data suggest that JAK2-STAT5 inhibition might effectively reduce the release of MC mediators, basal data on its actual effect on human MC are scarce. The next step towards safe implementation of JAK-STAT inhibitors in the treatment of mastocytosis would be to further explore their exact effects on human MC. To investigate this, we conducted in vitro studies with ruxolitinib, using two different human mast cell lines and several parameters of MC activation.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Two different human mast cell lines were used: HMC1 and LAD2. HMC1 cells grow independently of stem cell factor (SCF) as a result of an activating KIT mutation (kindly provided by dr. Butterfield, Mayo Clinic, Rochester, Minnesota).[21] DNA sequencing confirmed the presence of the G560V and D816V mutation in KIT. LAD2 is a SCF dependent mast cell line representing wild-type human MC (kindly provided by drs. Kirshenbaum and Metcalfe, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland).[22]

HMC1 cells were cultured in RPMI+ medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum, 50 μmol/L β-mercapto-ethanol (Sigma-Aldrich, St. Louis, Missouri), and antibiotics (penicillin/streptomycin, Lonza). LAD2 cells were cultured in Stem Pro-34 medium supplemented with 2.6% nutrient supplement (both Life Technologies, Grand Island, New York), 2 mmol/L ultraglutamine (Lonza), 100 ng/mL SCF (R&D systems, Abbingdon, UK), and antibiotics (penicillin/streptomycin).

2.2 | Beta-hexosaminidase release

MC degranulation was measured by β-hexosaminidase assay, as adopted from Rådinger et al.[23] In brief, 2×10^4 LAD2 cells in 100 μL per condition were transferred to a 96-well plate in calcium free phosphate-buffered saline (PBS). The cells were incubated for 30 minutes with varying concentrations of ruxolitinib (Apex Bio, Houston, Texas) or the STAT5-inhibitor pimozide (R&D systems) before further stimulation. Cells were stimulated with 40 μg/mL of codeine (Clinical pharmacy of Erasmus MC, Rotterdam, The Netherlands) or 5 μmol/L of substance P (R&D systems) for 15 minutes at 37°C. Hereafter, plates were centrifuged at 400 G for 4 minutes. Subsequently, 50 μL of supernatant was added to 100 μL of *p*-nitrophenyl *N*-acetyl- β-D-glucosaminide (p-NAG, Sigma-Aldrich) in citrate buffer (pH 4.5), while the cells were lysed by adding 150 μL 0.1% Triton X solution (Sigma-Aldrich). Fifty microlitres of the cell lysate was added to 100 μL of p-NAG in a different plate. After incubation for 90 minutes at 37°C, the reaction was stopped by adding 100 μL of glycine 400 mmol/L to each well. Optical density (OD) values were measured at 405 and 620 nm using a ELISA plate reader. The relative β-hexosaminidase release was calculated as follows: (2x Δsupernatant)/(Δsupernatant + (4*Δcell lysate)) = % β-hexosaminidase release. Δsupernatant = OD value supernatant—OD value blank condition.

2.3 | Histamine release

For the determination of histamine release, LAD2 cells were used. The cells were incubated with varying concentrations of ruxolitinib and subsequently stimulated with codeine for 15 minutes, according to the protocol as described above for the β-hexosaminidase assay. Histamine levels in the supernatant were measured by ELISA (IBL Solutions, Rorsachenberg, Switzerland).

2.4 | Surface membrane CD63 expression

MC degranulation is accompanied by the movement of CD63 to the external surface of the membrane, and CD63 expression can therefore be used to measure MC degranulation.[24] LAD2 cells were suspended in PBS to a concentration of 3×10^5 cells in 500 μL per tube. The cells were first incubated with ruxolitinib or pimozide for 30 minutes, and subsequently stimulated with codeine at a concentration of 40 μg/mL for 15 minutes at 37°C. The cells were stained with FITC-conjugated anti-CD63 antibody (ThermoFisher, Waltham, Massachusets). Membrane CD63 expression was analysed on a flowcytometer (LSRII, Becton Dickinson, Franklin Lakes, New Jersey).

2.5 | Cytotoxicity assay

Cytotoxicity of ruxolitinib and pimozide was tested by several methods. Firstly, HMC1 and LAD2 cells were cultured for up to 24 hours in the presence of varying concentrations of ruxolitinib (from 50 nmol/L to 50 μmol/L) and pimozide (2 -100 μmol/L). The cells were assessed by microscopy and trypane blue exclusion. Secondly, LDH levels in supernatant of cell cultures of 24 hours were measured by a standard colorimetric assay kit (Roche, Basel, Switzerland).

2.6 | Cytokine production

For the measurement of cytokine and chemokine production, HMC1 cells were seeded in a 96-well plate at 2×10^5 cells in 200 μL culture medium per condition. Subsequently, the cells were incubated for 30 minutes with ruxolitinib (or pimozide) before stimulating them with 1 μmol/L of A23187 or 5 μmol/L of substance P. After testing several time periods of stimulation ranging between 6 and 24 hours for the optimal duration, TNF-α levels in the supernatant were measured after 6 hours of stimulation, and MCP-1 and IL-6 levels were measured after 24 hours of stimulation. See the results section for further details. Cytokines were measured by ELISA (all R&D systems, except for IL-6 which was from ThermoFisher). The choice of cytokines was based on previous research on cytokine levels in patients with mastocytosis and other myeloproliferative diseases.[25-27]

2.7 | Statistical analysis

Graphpad Prism 5 (San Diego, California) was used to analyse most data except for the flow cytometry data, for which FlowJo (Ashland, Oregon) was used. One-way Analysis of Variance (ANOVA) and subsequent Dunnett post-hoc tests were used to determine the statistical significance between the ruxolitinib- or pimozide-treated conditions and the positive control conditions. IC_{50} values were calculated for the inhibitory effects on degranulation and cytokine production of both compounds. The results of β-hexosaminidase, histamine and cytokines/chemokine measurements are depicted in bar graphs as mean values with standard error of the mean (SE). CD63 expression is depicted in histograms as mean fluorescent intensity.

3 | RESULTS

3.1 | Compound control

Ruxolitinib was not cytotoxic to either HMC1 as LAD2 cells up to a concentration of 50 μmol/L as determined by microscopy, trypane blue exclusion and by LDH release assay for up to 48 hours of incubation. Pimozide was cytotoxic to both cell lines from a concentration of 50 μmol/L and higher as determined by LDH release assay. The vehicle, DMSO, did not affect degranulation up to a concentration of 5%, as measured by β-hexosaminidase release and CD63 expression (data not shown).

3.2 | Degranulation

First, titration of the stimuli was performed to find the optimal duration and concentration of stimulation for the β-hexosaminidase assay (Figure S1) and flow cytometry (Figure S2). HMC1 cells appeared unsuitable for these degranulation assays because their degranulation could not be further enhanced by stimulation with codeine, as measured by β-hexosaminidase release (Figure S3). For HMC1 cells, there also was no significant upregulation of CD63 expression upon stimulation with codeine (data not shown). Therefore, only LAD2 cells were used for all degranulation assays. Optimal concentrations to stimulate degranulation were 40 μg/mL for codeine, and 5 μmol/L for substance P, for a duration of 15 minutes.

Ruxolitinib effectively inhibited substance P- and codeine-induced degranulation of LAD2 cells, in a concentration-dependent manner reaching statistical significance at 50 and 10 μmol/L, (*P*: 0.028 and *P*: 0.006, respectively; Figure 1A,B). This corresponded to an IC_{50} of 7.9 μmol/L (95% CI 2.3-27.3) for substance P-induced degranulation, and an IC_{50} of 10 μmol/L (95% CI; 8.9-12.6) for codeine-induced degranulation. Ruxolitinib also inhibited codeine-induced histamine release and CD63 expression of LAD2 cells, however, without reaching statistical significance (Figure 1C and D, respectively).

3.3 | Cytokine and chemokine production

HMC1 cells were used for all experiments performed to evaluate cytokine and chemokine production. First, experiments were performed to determine which substance and duration of stimulation

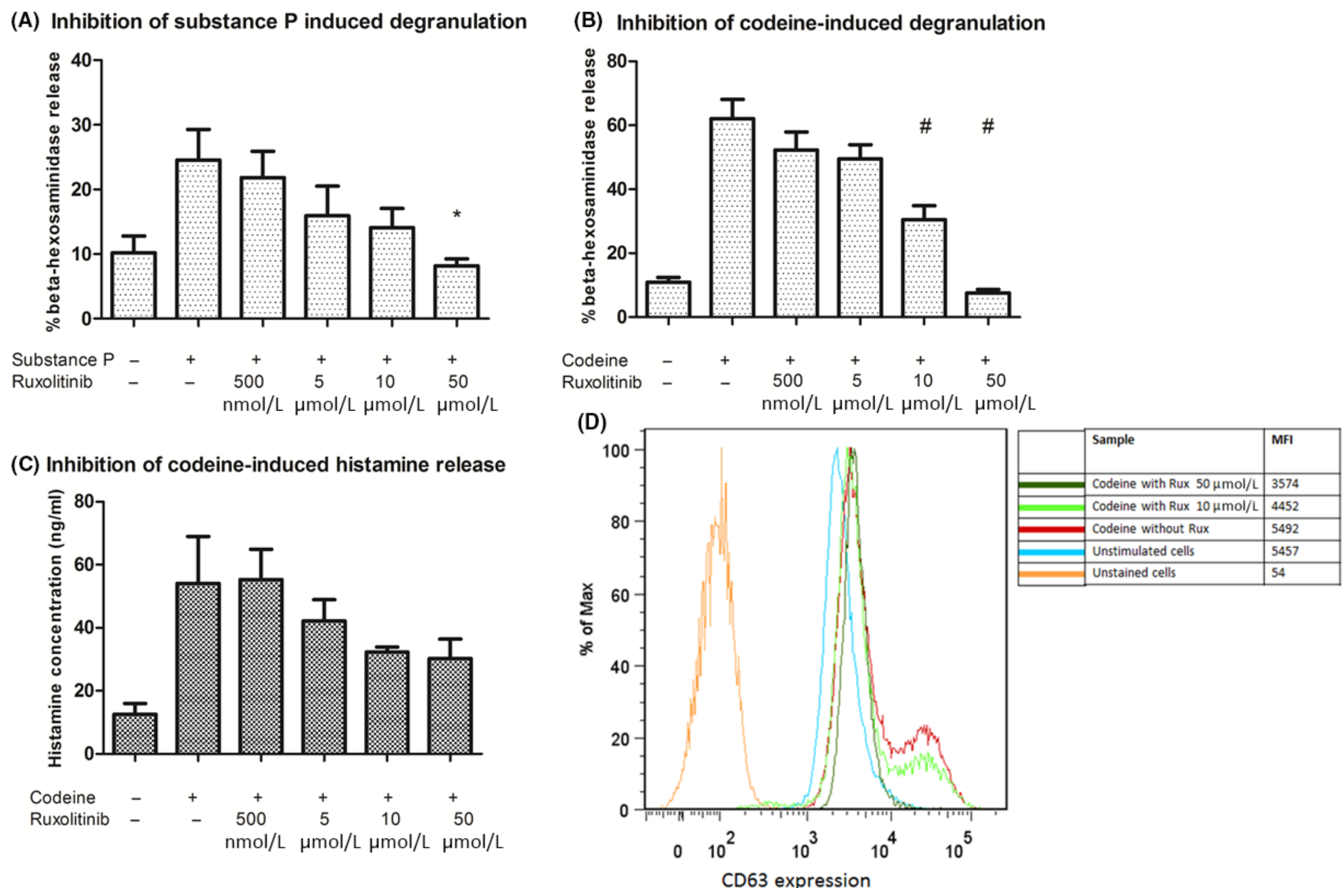


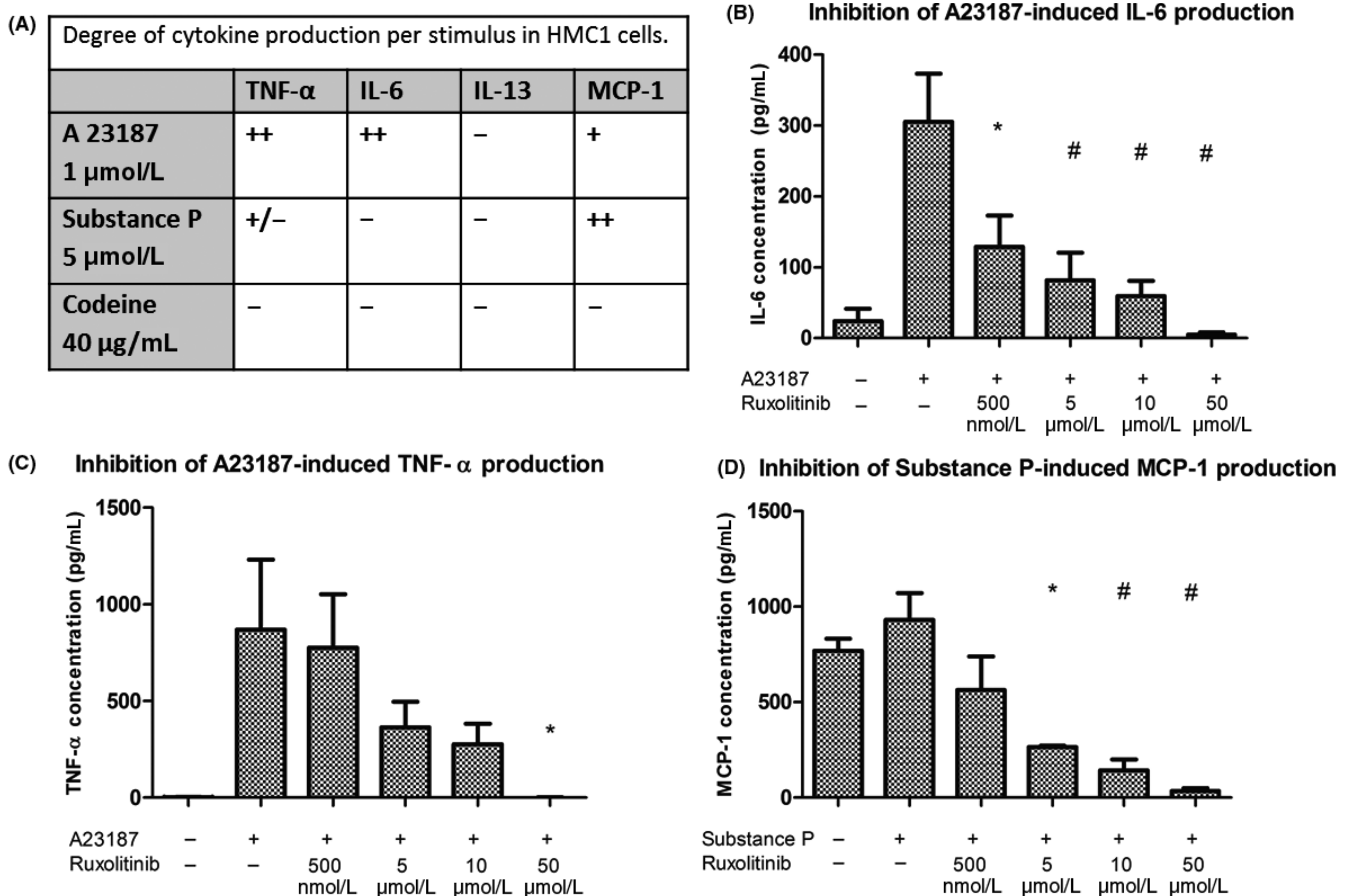
FIGURE 1 The Effect of Ruxolitinib on Degranulation of LAD2 Cells, as Measured by Beta-Hexosaminidase Release, CD63 Expression and Histamine Release. *Legend:* Ruxolitinib effectively inhibits degranulation of LAD2 cells in a dose-dependent manner. A, Beta-hexosaminidase assay for substance P induced degranulation. Concentration of Substance P: 5 µmol/l. B, Beta-hexosaminidase assay for codeine induced degranulation. Concentration of codeine: 40 µg/mL. C, Inhibition of histamine release after stimulation with codeine 40 µg/mL. D, Inhibition of CD63 expression after stimulation with codeine 40 µg/mL. MFI = mean fluorescent intensity. $^*P < 0.05$ and $^{\#}P < 0.01$ as compared with positive control. All bars are depicting the mean of 3 repeated experiments with SE

was optimal for the induction of IL-6, TNF- α, MCP-1 and IL-13 production in HMC1 cells. This revealed that the optimal stimulation time to induce production of IL-6 and MCP-1 was 24 hours, and that a stimulation time of 6 hours was optimal for TNF-α. This trend was seen regardless of which stimulus was used. A23187 was more potent stimulus for IL-6 and TNF-α production, whereas Substance P was the best stimulus for MCP-1 production (summarized in Figure 2A). Since the IL-13 production was low, regardless of the stimulus used, this was not further pursued. Codeine did not induce the production of detectable levels of any of the tested cytokines.

Ruxolitinib effectively inhibited the production of IL-6, TNF-α, and MCP-1 by HMC1 cells in a concentration-dependent manner (Figure 2B-D). For IL-6 production, a statistically significant effect was reached at 500 nmol/L of ruxolitinib and higher (*P:* 0.047 for 500 nmol/L compared with positive control). For TNF-α, the highest level of ruxolitinib (50 µmol/L) displayed significant inhibition (*P:* 0.038). MCP-1 production was significantly inhibited by ruxolitinib 5 µmol/L and higher (*P:* 0.021). The IC_{50} for the inhibition of IL-6 production by ruxolitinib was 7.6 µmol/L (95% CI 6.0-9.7), and the IC_{50} for the inhibition of TNF-α production was 1.3 µmol/L (95% CI 0.2-9.1). The IC_{50} for MCP-1 production was 4.1 µmol/L, however with a wide 95% CI (0.4-43).

3.4 | Pimozide

To determine the contribution of STAT5 to MC degranulation and cytokine production, we investigated the effect of pimozide, a selective STAT5 inhibitor. Pimozide partly inhibited substance P- and codeine-induced degranulation of LAD2 cells in a concentration-dependent manner, however, the obtained decrease in beta-hexosaminidase release was not statistically significant (Figure 3). Pimozide had more potent inhibitory effect on cytokine production by HMC1 cells. The A23187 induced production of IL-6 and TNF- α by HMC1 cells was already significantly reduced at the lowest concentration of pimozide of 5 µmol/L (*P:* 0.002 and *P:* 0.029, respectively, Figure 4A, B). Similarly, the substance P-induced MCP-1 production was also inhibited, reaching statistical significance at a concentration of 20 µmol/L (*P:* 0.002; Figure 4C). Of note, the spontaneous



(A)

Degree of cytokine production per stimulus in HMC1 cells.				
	TNF-α	IL-6	IL-13	MCP-1
A 23187 1 μmol/L	++	++	–	+
Substance P 5 μmol/L	+/–	–	–	++
Codeine 40 μg/mL	–	–	–	–

FIGURE 2 The Effect of Ruxolitinib on Cytokine and Chemokine Production by HMC1 Cells. *Legend:* After establishing the effect of A23187, codeine and substance P on the production of various cytokines and the cytokine MCP-1 (A), experiments were performed with the optimal stimulus for each cytokine. Incubation with ruxolitinib lead to decreased production of IL-6 (B) and TNF-α (C) after stimulation with A23187 for 24 or 6 hours, respectively. The production of MCP-1 after stimulation with substance P for 24 hours was also inhibited (D). $*P < 0.05$ and $^{\#}P < 0.01$ as compared with positive control. All bars are depicting the mean of 3 repeated experiments with SE

production of MCP-1 appeared to be relatively high in repeated experiments. The IC_{50} for the inhibition of IL-6 production by pimozide was 7.4 μmol/L (95% CI 3.1-17.9), the IC_{50} for TNF-α production by pimozide was 8.0 μmol/L (95% CI 3.7-17.1). The IC_{50} for MCP-1 production was 13.9 μmol/L (95% CI 7.1-27.2).

4 | DISCUSSION

Here, we provide evidence that the JAK1/JAK2 inhibitor ruxolitinib can efficiently inhibit MC degranulation as well as the production of cytokines. Since substance P and codeine are ligands for G-protein coupled receptors (GPCRs),[28,29] this work implies a link between the JAK2-STAT5 pathway and GPCRs. These stimuli were chosen because it is well-known that codeine can cause MC degranulation, as is confirmed by the fact that many patients with mastocytosis experience MC mediator related symptoms after ingestion of codeine.[30] However, since codeine is an exogenous stimulus, we repeated most experiments with substance P and/or A23187.

In our study, ruxolitinib clearly inhibited MC activity, but its exact intracellular mechanism is not fully elucidated yet. JAK2, and subsequently STAT5, are situated downstream of KIT in MC,[16] and thereby involved in the proliferation and survival of MC.[15] The involvement of the JAK2-STAT5 pathway in MC degranulation is less well-described, although there is some evidence available. The role of STAT5 in IgE-mediated MC degranulation has been investigated by several groups, and it is now accepted that STAT5 is activated downstream of FcεR1.[16,31,32] In line with this, previous work has proven that JAK2 is involved in IgE-mediated leukotriene production in mice.[33] Our current data confirm the role of JAK1 and/or JAK2 in MC degranulation and even more so in cytokine and chemokine production. We also found that selective inhibition of STAT5 with pimozide inhibited MC activity, although this did not reach statistical significance for degranulation. Nevertheless, our data support the theory that STAT5 is important for MC activation, although possibly in a lesser extent than JAK2.

Substance P and codeine are ligands to GPCRs.[28,29] Ruxolitinib and pimozide both effectively blocked MC activation as induced by

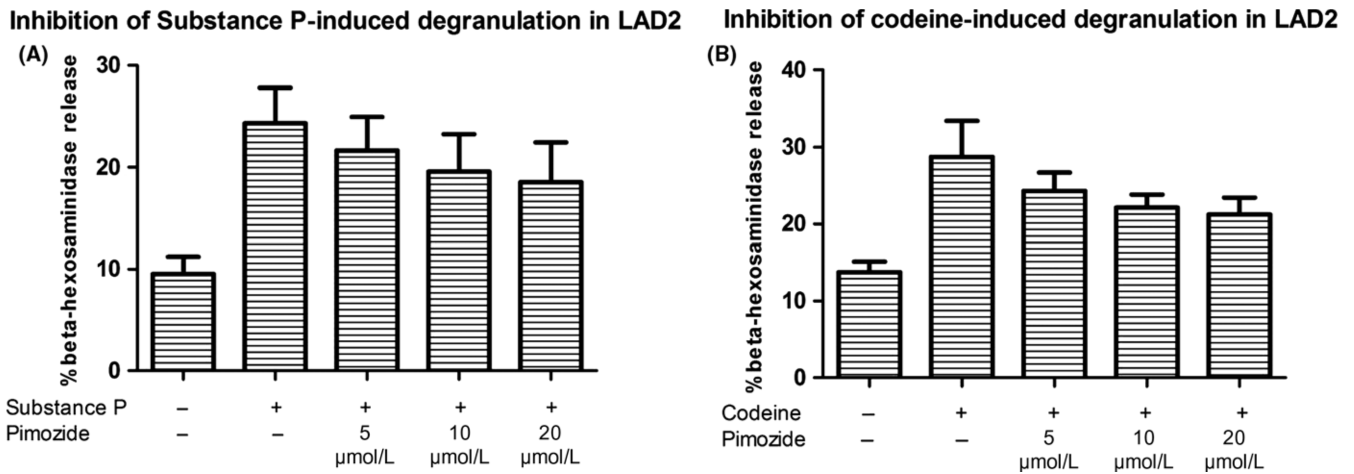


FIGURE 3 The Effect of Pimozide on Degranulation of LAD2 Cells. *Legend:* As measured by beta-hexosaminidase release assay, pimozide partly inhibits degranulation of LAD2 in a dose-dependent manner. This effect was similar for stimulation with substance P 5 μmol/L (A) and codeine 40 μg/mL (B). All bars are depicting the mean of 3 repeated experiments with SE

FIGURE 4 The Effect of Pimozide on Cytokine and Chemokine Production by HMC1 Cells. *Legend:* Incubation with pimozide lead to decreased production of IL-6 (A) and TNF-α (B) after stimulation with A23187 for 24 or 6 hours, respectively. The production of MCP-1 after stimulation with substance P for 24 hours was also inhibited (C). * $P < 0.05$, and ** $P < 0.001$ as compared with positive control. All bars are depicting the mean of 3 repeated experiments with SE

these stimuli. These points at a role for JAK2-STAT5 signalling in GPCR-mediated MC activation. Associations between GPCRs and JAK2-STAT5 signalling have been described in other hematopoietic cell types, for instance for the C-C motif chemokine receptor 5 expressed by T-cells and the platelet activating factor receptor expressed by monocytic cells[34,35] But to our knowledge, our study is

the first to suggest an association between GPCRs and a JAK-STAT pathway in MC.

The most important signaling pathway downstream of GPCRs involves phosphoinositide 3-kinase (PI3K), which is a versatile tyrosine kinase that can integrate signals from different receptors.[36-38] There is some evidence for crosstalk between PI3K and STAT5, specifically in neoplastic MC.[39,40] Possibly, JAK1/JAK2 inhibition by ruxolitinib ultimately interferes with the crosstalk between PI3K and STAT5, although this hypothesis obviously needs further testing.

Ruxolitinib is a rather specific inhibitor of JAK1 and JAK2, yet at high concentrations it is known to inhibit JAK3 as well.[41,42] The IC_{50} of ruxolitinib for JAK3 inhibition was reported to be 438 ± 243 nmol/L in an in vitro model for myeloproliferative neoplasms (MPN), which was ~150-200 times higher than the IC_{50} for JAK1 or JAK2 inhibition.[41] The IC_{50} values for ruxolitinib are much higher in MC: they ranged between 1.3 and 10 μmol/L in our study. These values are comparable to a study that investigated the effect of ruxolitinib on murine MC.[17] Since higher concentrations of ruxolitinib are necessary to inhibit MC activation compared with the inhibition of myeloid progrenitor cells, it cannot be formally excluded that the observed effect of ruxolitinib on MC also involves inhibition of JAK3 activity.

Regardless of any (exogenous) stimuli, neoplastic MC constitutively exhibit high levels of phosphorylated STAT5.[43] Additional evidence for the continuous auto-activation of MC in patients with mastocytosis comes from the elevated levels of MC mediators at random measurements in blood and urine of these patients.[11,12,44] The inhibition of the release and/or production of various MC mediators can potentially reduce debilitating symptoms including pruritus, flushing, diarrhea and anaphylaxis, thereby improving the quality of life of these patients. Excessive MC activity also plays a role in many other diseases, including mast cell activation syndrome, chronic spontaneous urticaria, allergies and fibrotic disease.[45,46] The reduction of MC mediator levels via the inhibition of JAK-STAT signaling might therefore be of therapeutic interest for these diseases as well.

Ruxolitinib is currently only approved for the treatment of the classical myeloproliferative neoplasms (MPN). In patients with MPN, treatment with ruxolitinib leads to a reduction of daily symptoms like abdominal discomfort, pruritus and fatigue.[47,48] In line with our findings, the COMFORT-II trial showed a decrease in the levels of IL6, TNF-α and VEGF in patients with MPN that were treated with ruxolitinib.[49] Given the increased number and activity of MC in bone marrow of patients with MPN it was hypothesized that ruxolitinib exerted its beneficial effect in MPN by inhibition of MC mediator release/production.[50,51] This hypothesis is corroborated by our current study that indeed demonstrates an overall inhibiting effect of ruxolitinib on MC activity. In line with this, two recent case studies showed a convincing decrease in MC mediator related symptoms in systemic mastocytosis upon ruxolitinib treatment[19,20]

Based on our data, combined with other available (pre-)clinical evidence as discussed above, JAK1/JAK2-STAT5 inhibition might represent a promising new therapeutic strategy for patients with mastocytosis and many other MC mediator related diseases. Ultimately, randomized clinical trials are necessary.

5 | CONCLUSION

We have demonstrated that the JAK1/2 inhibitor ruxolitinib effectively attenuates degranulation and cytokine production by human MC. The fact that pimozide also partly inhibited degranulation, and substantially inhibited cytokine production, suggests a role for STAT5 in MC activation. JAK2-STAT5 inhibition thus emerges as a new, highly effective, method to lower MC mediator levels. Ruxolitinib, and JAK-STAT inhibition in general, are interesting therapeutic options to reduce debilitating symptoms in mastocytosis and a wide range of other MC mediator-related diseases such as mast cell activation syndrome, chronic spontaneous urticaria and even fibrotic disease.

CONFLICTS OF INTEREST

PvD has received an occasional consultant fee from Novartis for advising on clinical aspects of mastocytosis. MH has received an occasional speaker fee from Novartis.

AUTHORS CONTRIBUTIONS

MH, Pvd and WD created the concept of the study. MH, BS and WD designed the experiments. BS, JvH and MH executed the experiments. MH performed data analysis and wrote the first draft of the study. PvD, RGvW and PMvH advised on the interpretation of the data. All authors critically revised the manuscript.

ETHICS COMMITTEE

This study did not require approval from an ethics committee because no patient material was used.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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