Annals of Gastroenterology (2024) **37,** 333-340

Comparison of a selective STAT3 inhibitor with a dual STAT3/STAT1 inhibitor using a dextran sulfate sodium murine colitis model: new insight into the debate on selectivity

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Abstract

Background Recent advances in the treatment of inflammatory bowel disease include antitumor necrosis factor antibodies and the Janus kinase inhibitor tofacitinib, approved for ulcerative colitis. Janus kinase recruits signal transducers and activators of transcriptions (STAT), which are promising targets in inflammatory bowel diseases. However few inhibitors have been evaluated, and their selectivity with respect to STAT1 and STAT3 remains controversial. Here, we investigated the therapeutic potential of a selective inhibitor vs. a non-selective, closely related compound, in a dextran sulfate sodium (DSS) murine colitis model.

Methods Thirty Swiss/CD-1 male mice were used in this study. They were divided into a healthy control group, a colitis-DSS control group, a compound (cpd) 23-treated group, a cpd 46-treated group and an icariin-treated group. For the coadministration experiment with rutin, the cpd 46-treated group and the icariin-treated group were replaced by the oral rutin-treated group and the coadministration rutin/cpd 23-treated group. The effect of the tested inhibitors was also assessed by quantification of proinflammatory markers.

Results The selective inhibitor had a significantly greater effect than the dual inhibitor on the disease activity index. We also noticed in curative treatment a significant decrease in the most abundant proinflammatory biomarker present in neutrophilic granulocytes, myeloperoxidase and on proinflammatory cytokines, including tumor necrosis factor-α, interferon-γ, interleukins -6 and -23, with a mild synergy with rutin, the glycoside of quercetin.

Conclusion The current study shows how STAT3 selective inhibitors can exert a significant therapeutic effect in the treatment of experimental DSS-colitis.

Keywords Inflammatory bowel disease, ulcerative colitis, signal transducers and activators of transcription 3, dual inhibitor

Ann Gastroenterol 2024; 37 (3): 333-340

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Conflict of Interest: None

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Received 13 December 2023; accepted 26 March 2024; published online 25 April 2024

DOI: https://doi.org/10.20524/aog.2024.0880

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Introduction

Inflammatory bowel disease (IBD) includes all illnesses and pathological processes that affect the digestive system and lead to chronic inflammation [1]. Crohn's disease and ulcerative colitis are the main and most common forms of IBD. However, they differ in the affected area: Crohn's disease can affect parts of the entire digestive tract, while ulcerative colitis mainly affects the colon. Common symptoms include abdominal pain, diarrhea and bloody stool, which may lead to weight loss, anemia and a reduction in quality of life. Current pharmacological treatments [2], including aminosalicylates, corticosteroids and immunosuppressive agents such as thiopurine and cyclosporine, are often associated with side-effects and may be ineffective, while complete remission is rarely obtained. Antitumor necrosis factor (TNF) antibodies

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have represented great progress towards IBD therapy; however, there is significant variability in the disease response [3]. Therefore, alternative treatments and new targets aimed at promoting an endogenous anti-inflammatory response [4] and decreasing proinflammatory cytokines are currently under investigation [5].

Signal transducers and activators of transcription (STATs) are a family of transcription factors that play a crucial role in inflammation and the immune response [6,7]. Within this family, STAT3 is associated with inflammation of the intestinal epithelium [8] and has been found to be significantly elevated in IBD patients [9]. Trends in STAT1 expression are less pronounced [9]; however, an increased expression of STAT1 has been reported in ulcerative colitis and to a lesser extent in Crohn's disease [10]. STAT recruitment is mediated by Janus kinase (JAK) phosphorylation. The JAK inhibitor tofacitinib has been approved for ulcerative colitis, and new JAK inhibitors are in development [11,12], but few STAT3 inhibitors have been tested in IBD. Icariin [13] and metformin [14] improved IBD by STAT3/interleukin (IL)-17 inhibition, while C188-9 [15], a dual inhibitor of STAT3 and STAT1, was found to prevent IBD in dextran sulfate sodium (DSS) and 2,4,6-trinitrobenzene sulfonic acid models [16]. Therefore, the impact of the selectivity of STAT inhibitors in IBD is still in question.

STAT3 inhibitors bind to the dimerization interface of the Src homology 2 (SH2) domain and selectivity towards STAT1 is challenging because of the high homology of STAT3 and STAT1 [17]. Molecular dynamics and ensemble docking simulations have shown that STAT inhibitors exhibit non-specific statistical binding to a large part of the protein surface, and that selectivity is determined by the equilibrium statistical partitioning of binding between the SH2 domain and the DNA-binding and coil–coil protein domains [18]. A few years ago, we developed a series of vanillin-derived STAT3 inhibitors [19]. Among these, compound 23 (cpd 23) and compound 46 (cpd 46) are aminotetrazole- and aldehyde-bearing analogs, respectively. A deep structure–activity relationship study supported by molecular modeling reveals that the aminotetrazole moiety deviates from binding to the SH2 domain of STAT1. Therefore, cpd 23 and cpd 46 display very similar inhibition of STAT3 (with an IC_{50} of 25.7±2.2 and 23.7±1.8 μM, respectively). While compound 46 shows an equivalent effect on both STAT3 and STAT1, compound 23 has a minimal effect on STAT1. Cpd 23 was the best compromise between selectivity and activity, and we demonstrated that cpd 23 prevents lymphocyte T polarization into Th17 and Treg without affecting their differentiation into Th1 lymphocytes.

Based on the above-mentioned observations, we saw an opportunity to investigate the potential therapeutic use of these closely related compounds with an opposite selectivity towards STAT1 in murine experimental colitis induced by DSS. The effects of cpd 23, a selective STAT3 inhibitor, vs. cpd 46, a dual STAT3 and STAT1 inhibitor, were investigated [20,21]. The chemo-preventive properties of an association with rutin were also assayed. Rutin is a bioflavonoid present in many fruits and vegetables. It is known to have anti-inflammatory effects, but may also play a role in different signaling pathways involved in inflammatory responses, such as JAK-STAT, p38 MAPK and JNK. In view of these findings, this study investigated the effect of cpd 23 and cpd 46 on the production of proinflammatory cytokines (tumor necrosis factor [TNF]-α, interferon [IFN]-γ, IL-17A, IL-23, and IL-6) and myeloperoxidase (MPO) activity.

Materials and methods

Materials

Mouse cytokine ELISA kits were purchased from eBioscience (Paris, France). Dimethylsulfoxide (DMSO) and DSS were obtained from Carlo Erba Reagents (Val-de-Reuil, France) and MP Biomedicals (Illkirch, France), respectively. Icariin, rutin and all other chemical components were obtained from Sigma Aldrich (Deisenhofen, Germany). All reagents were of analytical grade. Compound 23 (cpd 23), *N*-(3-chloro-5-methoxy-4-((4-nitrobenzyl)oxy)benzyl)-2H-tetrazol-5-amine, and compound 46 (cpd 46), 3-chloro-5-methoxy-4-((4-nitrobenzyl)oxy)benzaldehyde were synthetized as previously described [18].

Animals

Experiments were performed using Swiss/CD-1 mice (male 4-6 weeks, Janvier, France). All mice were maintained under specific pathogen-free conditions. Experiments were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, USA). Experiments were carried out in compliance with the French legislation on animal experiments under the experimentation authorization no. A-25-48. Mice were group-housed under controlled temperature (25°C) and photoperiod (12:12-hour light–dark cycle) conditions and given unrestricted access to standard diet and tap water. Mice were allowed to acclimate to these conditions for at least 7 days before inclusion in the experiments.

DSS-induced colitis model

Experimental colitis was induced according to the protocol described by Chassaing *et al* [20]. Swiss/CD-1 mice were given 3% DSS in drinking water for 10 days. Healthy control mice were given drinking water without DSS. The experimental mice were divided into a healthy control group, a colitis-DSS control group, a cpd 23-treated group, a cpd 46-treated group and an icariin-treated group (n=6 per group). For the coadministration experiment with rutin, the cpd 46-treated group and the icariin-treated group were replaced by the oral rutin-treated group and the coadministration rutin/cpd 23-treated group. Disease severity was evaluated using a point score system consisting of stool consistency (pellets, 0; loose, 1;

and watery, 2) rectal bleeding (no bleeding, 0; slight bleeding, 1; and bloody, 2) and body weight loss (±1% of original weight, 0; ≤5% loss, 1; ≤10% loss, 2; and >10% loss, 3). Thus, the maximal severity in an individual scored 4 and this score was lower in less severe cases. Six mice were included in each group.

Therapeutic efficiency

After colitis induction, the animals were treated with different drugs. During the treatment period (curative or preventive treatment), all animals received by intraperitoneal route 20 µL of either cpd 23 or cpd 46 solutions in DMSO (10 mg/kg body weight), or icariin once daily for 3 consecutive days. For the coadministration with rutin suspension (20 mg/kg body weight), this was administered by the oral route. The control groups received saline only (colitis and healthy controls). The animals were sacrificed 24 h after the last drug administration and their colons were resected. The degree of inflammation was quantified using a disease activity index that assesses weight loss, stool consistency and rectal bleeding, as described above.

Resected colon tissue samples were opened longitudinally and rinsed with cold phosphate buffer to remove luminal content. MPO and cytokine activity was measured to quantify the severity of the colitis. Enzyme or cytokine activity is a reliable index of the severity of inflammation caused by the infiltration of activated neutrophils into inflamed tissue. MPO activity was analyzed according to a standard method [21], and cytokine activities were analyzed according to the manufacturer's instructions provided with the eBiosciences Mouse ELISA Assay Kit.

Statistical analysis

The results are expressed as mean values ± standard deviation For the analysis of statistical significance, analysis of variance (ANOVA) followed by Dunn's test for all pairwise comparisons in case of multiple comparisons, or by the Tukey test when normality and equal variance applied. Student's *t*-test was applied to study the significance of differences between 2 treatment groups; however, if normality and/or equal variance were not found, the Mann-Whitney *U* test was applied. In all cases, $P<0.05$ was considered significant.

Results

Selectivity towards STAT3 over STAT1

After the induction of experimental colitis, the animals' clinical activity indices increased within 10 days in response to intestinal inflammation. Following the initiation of treatment, a decrease in clinical activity became apparent on day 3 for both cpd 23 and icariin. Indeed, the results obtained showed that within 3 days of treatment, cpd 23 and icariin clearly reversed the clinical activity score curve, whereas cpd 46 did not, and even seemed to have a deleterious effect (Fig. 1A). The systemic inflammatory marker MPO was significantly decreased by cpd 23 and icariin, from 250 U/g without treatment to 110 U/g and 60 U/g, respectively, while its level was only moderately reduced by cpd 46, from 250 U/g without treatment to 200 U/g (Fig. 1B). In contrast, TNF-α was not discriminatory, as it was significantly reduced from 7200 U/g without treatment to 2700 U/g, 3200 U/g and 2500 U/g with cpd 23, cpd 46 and icariin, respectively (Fig. 1C). IFN-γ, IL-6 and IL-23 levels were in line with the clinical scores. IFN-γ was slightly reduced by cpd 23 and icariin from 3000 U/g without treatment to 2000 U/g and 1700 U/g, respectively (700 U/g for the healthy group), and there was no significant difference between them. IL-6 and IL-23 were clearly reduced by cpd 23 and icariin. IL-6 decreased from 750 U/g to 370 and 200 U/g with cpd 23 and icariin, respectively, while IL-23 decreased from 500 U/g without treatment to 290 U/g and 190 U/g with cpd 23 and icariin, respectively. It is worth noting that IFN-γ, IL-6 and IL-23 activity remained always high when mice were treated with cpd 46 (Fig. 1D-F). All products tested had a weak influence on IL-17A levels (Fig. 1G).

A positive curative effect but a disparate preventive impact

To evaluate the preventive effect of cpd 23, after the acclimation days animals were treated with cpd 23 during 3 consecutive days before the start of the DSS-colitis induction. Animals were sacrificed 24 h after the last day of induction and their colons were resected and treated as previously described. The curative treatment was administered during the 3 days after the last day of DSS-colitis induction. Preventive and curative effects of cpd 23 against DSS-colitis were evaluated more objectively by measurement of the clinical activity score, MPO activity, and concentration of the proinflammatory cytokines TNF-α and IL-6 (Fig. 2). The disease activity index showed no effect of preventive treatment. The curative administration confirmed the previous result, with an overall effect on MPO, TNF-α and IL-6. In this experiment, administration of cpd 23 from the third day led to a return to normal production of TNF-α and IL-6. Preventive administration had a positive effect on MPO production, but TNF-α and IL-6 remained high and even appeared to show a deleterious effect.

Synergistic effect in combination with rutin

Rutin, a glycoside of quercetin, displays moderate clinical action in IBD [22]. Rutin is known to have radical scavenging properties and potential anti-inflammatory properties, as reactive oxygen species are a common vector of damage and inflammation. The synergistic effect of cpd 23 in combination with rutin was quantified as previously by measuring MPO and cytokine activities (TNF-α and IL-6), but also by evaluating the disease activity index. Rutin and cpd 23 clearly improved the

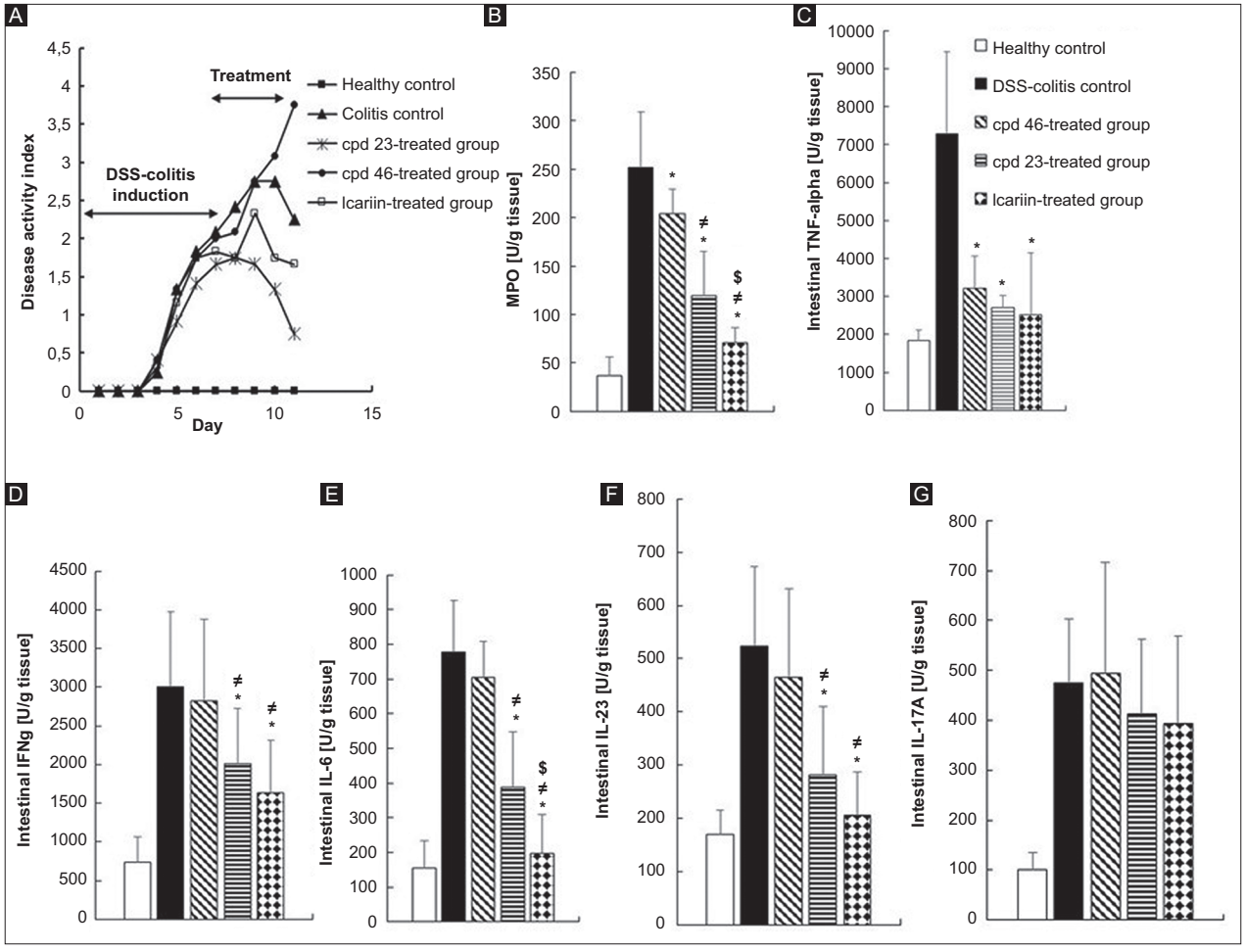

Figure 1 Evaluation of compound (cpd) 23 and icariin, selective inhibitors of STAT3 vs. cpd 46, inhibitor of both STAT1 and STAT3. (A) Disease activity index, (B) myeloperoxidase activity, C) TNF-α, D) IFN-γ, E) IL-6, F) IL-23, G) IL-17 (n=6 each group)
*P<0.05 compared with colitis group; ≠P<0.05 compared with cpd 46; $P<0.05 compared with cpd 23. Colitis was induced with DSS (dextran sodium sulfate 3 mg/mL in drinking water). Animals were treated for 3 days by intraperitoneal administration of 10 mg/kg of cpd 23, cpd 46 and icariin
TNF, tumor necrosis factor; IFN, interferon; IL, interleukin

disease activity index. This improvement was slightly better with coadministration (Fig. 3). A synergistic effect on MPO was observed. However, a decrease in TNF-α was antagonized by rutin and the effect of coadministration on IL-6 was similar and probably due to cpd 23.

Discussion

Animal models that accurately recapitulate the *in vivo* disease state of human patients with ulcerative colitis are potentially very useful in the development of new therapeutic agents. Using an animal model to target the disease tissue and assess efficacy is a key step in this process. In this study, the DSS-colitis mouse model was used to investigate the effect of STAT3 vs. STAT1 selectivity on the *in vivo* efficacy of STAT inhibitors. Given that DSS disrupts the intestinal epithelial barrier without a primary effect on immune regulation, DSS colitis is an appropriate model for studying acute IBD and the mechanisms involved in the development of intestinal inflammation [23]. Moreover, previous studies showing that STAT3 is increased in the DSS-colitis mice model [8,13] support the choice of this model to evaluate the anti-inflammatory effects of STAT inhibitors.

Previously, we demonstrated that cpd 23 inhibits the STAT3-induced luciferase activity with an IC_{50} of 26 μM [18] and that cpd 46 is an analog of cpd 23 without the aminotetrazole moiety responsible for STAT3 selectivity [18]. The inhibition and binding affinity of cpd 23 and cpd 46 to the STAT3 Sh2 domain were very similar. However, while cpd 23 displayed low inhibition of STAT1, cpd 46 had a significant binding affinity for the STAT1 Sh2 domain and showed inhibition of STAT1 and STAT3 at the same level [18].

Globally, the therapeutic assessment of cpd 23 and cpd 46 showed a therapeutic effect on the inflamed tissue, with substantial mitigation of the DSS colitis model, especially for the selective cpd 23, as evidenced by the improvement in the

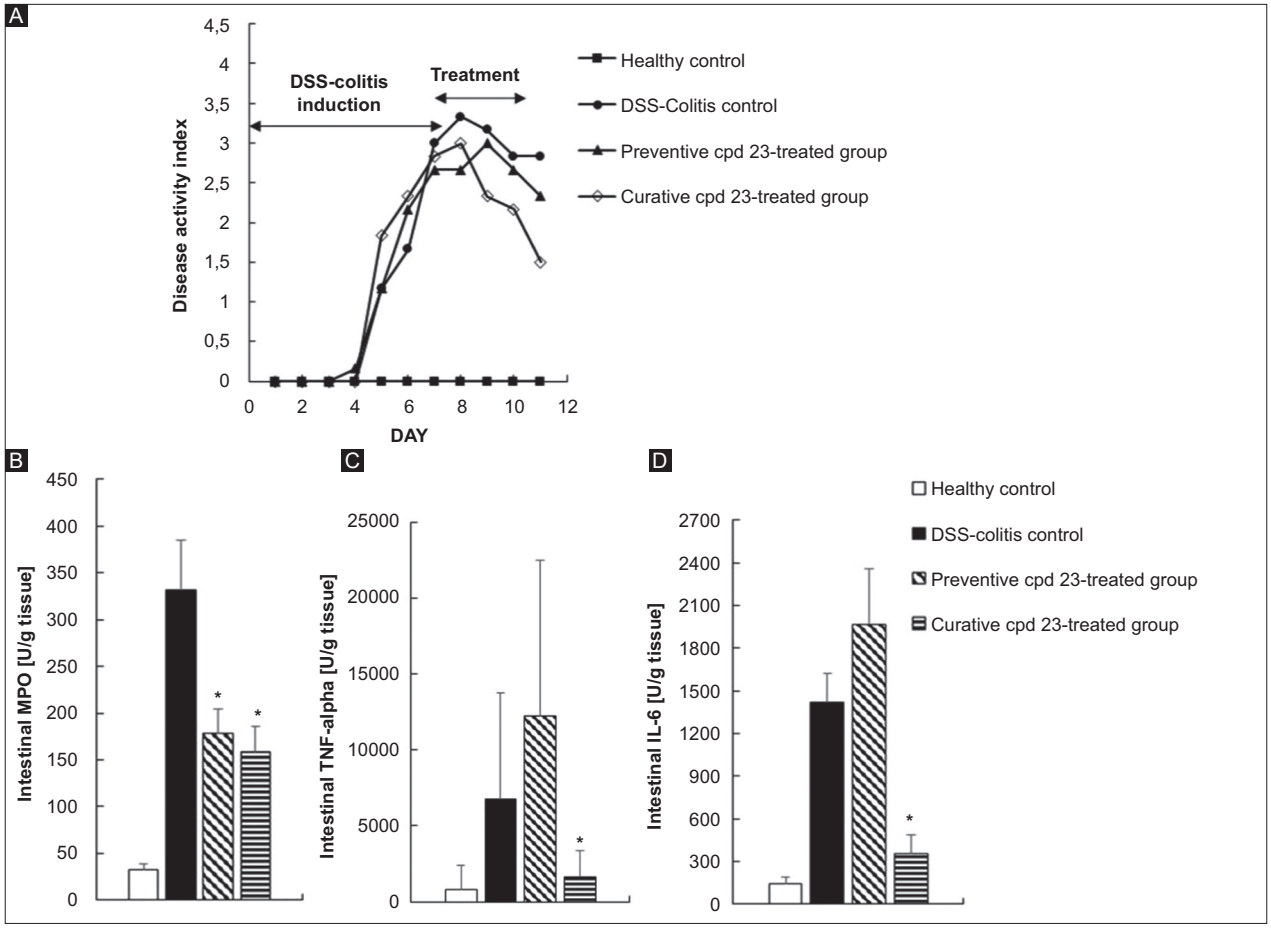

Figure 2 Comparison of curative and preventive effect of compound (cpd) 23. (A) Disease activity index, (B) myeloperoxidase activity, (C) TNF-α, (D) IL-6 (n=5 each group)

*P<0.05 compared with colitis control group. Colitis was induced with DSS (dextran sodium sulfate: 3 mg/mL in drinking water). Animals were treated by intraperitoneal administration of 10 mg/kg of cpd 23 for 3 days before induction (preventive group) and 3 days after induction (curative group)

TNF, tumor necrosis factor; IL, interleukin

disease activity index and a remarkable reduction in MPO, as well as various proinflammatory cytokines including TNF-α, IL-6, IL-23 and IFN-γ. In contrast, cpd 46, a dual inhibitor of STAT1 and STAT3, exhibited ambiguous activity. Indeed, after administration of cpd 46 we noted an increase in clinical symptoms, and a non-significant effect on inflammatory markers, except for TNF-α. Usually, the dual inhibition of these 2 transcription factors, greatly involved in the colitis mechanism [24,25], is considered as a potential strategy to enhance anti-inflammatory activity. Thus, it was quite surprising that cpd 46, which acts on both STAT1 and STAT3, was less effective than cpd 23.

First, this observation is not yet completely clear. The effect on STAT1 and STAT3 transcription factors is time-limited in an independent way. Thus the *in vivo* selectivity of compounds may be less obvious. Nevertheless, the outcome of colitis treatment with the selective STAT3 inhibitor cpd 23 at the concentrations used was comparable to that achieved with the reference STAT3 inhibitor icariin. Icariin is a natural flavonoid glucoside with a predominant effect on STAT3 rather than STAT1. As shown by Tao *et al* [13], icariin has a significant effect on STAT3 phosphorylation at 3 μM, whereas STAT1 phosphorylation is only slightly inhibited at 10 μM. In addition, it has been shown that STAT3 inhibition by icariin alone leads to significant regulation of Th17 activity and alleviation of inflammatory effects in a murine model [26]. These results suggest that the selectivity towards the STAT3 pathway vs. STAT1 has an impact on efficacy.

Accumulating evidence suggests that several cytokines play a major role in the pathogenesis of IBD [27,28]. Many of these cytokines act as ligands for cell surface receptors that activate STAT3 [29,30]. STAT3 has been shown to be activated in the actively inflamed colon of IBD patients [31]. Furthermore, increased STAT3 in T-cells, as well as macrophages and epithelial cells, has been shown to directly correlate with histological degrees of inflammation [9,32]. Thus, the effect of cpd 23 can be explained by the blockade of IL-6 trans signaling, but also that of IL-23, TNF-α and IFN-γ, which suppress T-cell

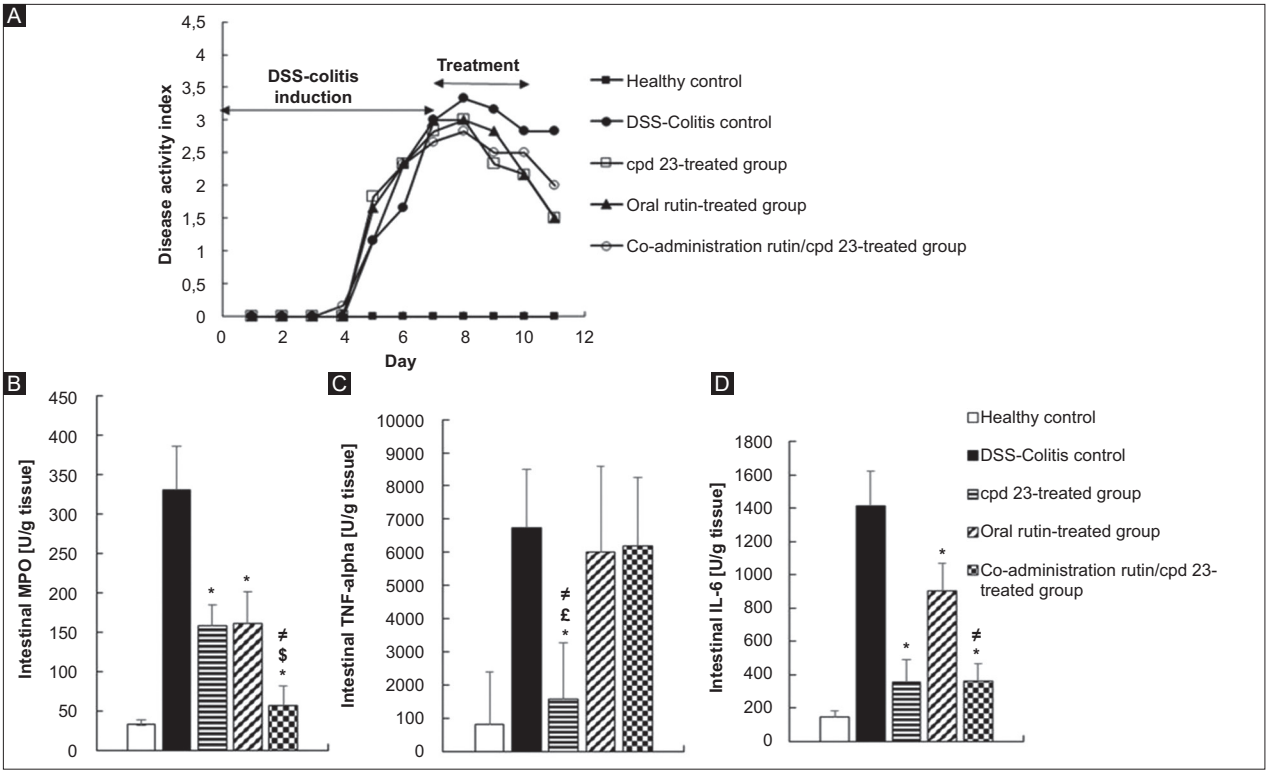

Figure 3 Coadministration of compound (cpd) 23 and rutin. (A) Disease activity index, (B) myeloperoxidase activity, (C) TNF-α, (D) IL-6 (n=5 each group)

*P<0.05 compared with colitis control group; $P<0.05 compared with cpd 23; ≠P<0.05 compared with oral rutin; £P<0.05 compared with coadministration rutin/cpd 23. Colitis was induced with DSS (dextran sodium sulfate: 3 mg/mL in drinking water). Animals were treated by intraperitoneal administration of 10 mg/Kg of cpd 23, 20 mg/kg of rutin or a combination of both

TNF, tumor necrosis factor; interleukin

resistance to apoptosis, reducing intestinal inflammation by selective inhibition of STAT3, as previously shown [33,34].

The acute mitigating effect of the STAT3 selective cpd 23 may also be related to its ability to decrease MPO activity. MPO is released from infiltrated polynuclear neutrophils and macrophages in the setting of inflammatory bowel disease [35]. MPO is a key vector of oxidative damage and has been proposed as a therapeutic target to protect the intestine from inflammation. Thus, modulation of T-cell homeostasis via downregulated STAT3 signaling by cpd 23 contributes to a decrease in neutrophil infiltration. This results in a decrease in MPO with an improved anti-inflammatory effect. In addition, neutrophils can produce many different cytokines, such as IL-1β or TNF-α, under different stimulations [36,37], and the action on neutrophils allows TNF-α downregulation that explains the low level of this cytokine after treatment with cpd 23. Interestingly, coadministration with rutin shows a synergistic effect on MPO levels, probably due to the antioxidant potential of rutin, which regulates a signal transduction pathway [38].

Finally, a mechanistic consensus has emerged concerning the dysregulation of T-helper 17 (Th17) lymphocytes in IBD [39]. It contributes to the initiation of inflammation and avoids a return to the steady state. STAT3 is associated with Th17 polarization, while STAT1 signaling prevents Th17 polarization [40] and is associated with Th1 polarization [41]. We have already demonstrated [18] that cpd 23 prevents the polarization of lymphocytes T CD4+ into Th17 and Treg, without affecting their differentiation into Th1 lymphocytes; it therefore does not affect STAT1 signaling. Cpd 23 and rutin have a significant effect on IL-23 that induces Th17 cells. However, there seems to be an unexpectedly negligible effect of the 3 compounds (cpd 46, cpd 23, icariin) on IL-17 levels, reflecting a weak effect on Th17 function that increases the inflammatory process in IBD [26].

The question of a preventive effect has been raised to improve the effect on proinflammatory markers, and we noted a preventive effect of cpd 23 on MPO, translating into an action on reactive oxygen species. However, a deleterious effect was observed on the other parameters, suggesting a preponderant effect after the development of the inflammatory response, and explaining the reduced *in vivo* effect. Finally, synergism studies with oral rutin revealed interesting points, notably an effect on neutrophil cells characterized by a downregulation of MPO activity. On the other hand, no synergistic effects were observed on the other proinflammatory markers (IL-6, TNF-α). For this reason, further studies with targeted anti-inflammatory molecules associated with cpd 23 should be carried out to properly characterize this synergistic effect.

STAT3 inhibitors studied in colitis models may not properly reveal their pharmacological effect, given their low selectivity.

By comparing the similar compounds 23 and 46, of which only compound 23 is selective, the current study focuses on demonstrating a difference in effect due to selectivity.

The current study shows how STAT3 inhibitors, and especially STAT3-selective inhibitors, can exert a significant therapeutic effect in the treatment of experimental DSS-colitis. This work contributes to the debate on the therapeutic efficacy of selective inhibitors and gives a mechanistic overview of how selective STAT3 inhibitors can substantially ameliorate various aspects of such an inflammatory disease. The therapeutic potential of compounds like cpd 23 seems to originate from a combination of effects, i.e., downregulation of proinflammatory cytokines and control of MPO activity. STAT3 inhibitor-based approaches represent a valid alternative approach to the development of new molecules for IBD therapy. Our findings indicate that cpd 23, a selective STAT3 inhibitor, may be a potential treatment option for IBDs such as colitis. In addition, the difference in activity between 2 similar compounds highlights the need to investigate the selectivity of compounds under investigation in IBD models.

Acknowledgment

Thanks to Dr Mona ABDEL MOTTALEB for her help with the English proof-reading.

Summary Box

What is already known:

- Signal transducers and activators of transcriptions (STAT) STAT1 and STAT3 are associated with inflammatory bowel disease (IBD)
- STAT recruitment is mediated by Janus kinase (JAK) phosphorylation
- JAK inhibitors are effective in the treatment of ulcerative colitis

What the new findings are:

- Development of novel STAT inhibitors: compound cpd 23 (STAT3 inhibitor) and cpd 46 (dual STAT3/STAT1 inhibitor), which are aminotetrazole and aldehyde-based analogs, respectively
- Cpd 23, a selective STAT3 inhibitor, showed better anti-IBD efficacy than cpd 46, a dual STAT3/STAT1 inhibitor
- Cpd 23 coadministered with rutin, a bio-flavonoid, induced a downregulation of myeloperoxidase activity in a dextran sulfate sodium (DSS) mouse colitis model
- Selective STAT3 inhibitors may have a significant therapeutic effect in the treatment of experimental DSS colitis

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