The impact of JNK inhibitor D-JNKI-1 in a murine model of chronic colitis induced by dextran sulfate sodium

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Purpose: The c-Jun N-terminal kinases (JNK) are involved in the activation of T cells and the synthesis of proinflammatory cytokines. Several studies have established the relevance of the JNK pathway in inflammatory bowel diseases. The present study analyzed the therapeutic effect of D-JNKI-1, a specific JNK-inhibiting peptide, in a low-dose dextran sulfate sodium (DSS) model of chronic colitis.

Methods: DSS colitis was induced in female C57/BL6 mice by cyclic administration using different concentrations of DSS (1.0% and 1.5%). Mice in the intervention groups received subcutaneous administration of 1 µg/kg D-JNKI-1 on days 2, 12, and 22. They were monitored daily to assess the severity of colitis, body weight, stool consistency, and the occurrence of occult blood or gross rectal bleeding using evaluation of the disease activity index. The animals were sacrificed after 30 days, and the inflamed intestine was histologically evaluated using a crypt damage score. Immunohistochemical quantification of CD4⁺ and CD8⁺ cells was also carried out.

Results: Administration of 1 µg/kg D-JNKI-1 resulted in a significant decrease in the disease activity index (P = 0.013 for 1.0% DSS; P = 0.007 for 1.5% DSS). As a mild form of colitis was induced, histological examination did not show any distinct damage to the mucosa and crypts. However, expression of CD4⁺ and CD8⁺ cells was reduced in mice treated with D-JNKI-1 (not significant).

Conclusion: Administration of D-JNKI-1 resulted in a clinical attenuation of chronic DSS colitis, and a therapeutic effect of D-JNKI-1 must therefore be assumed. The decrease in CD4⁺ and CD8⁺ cells may reflect the influence of D-JNKI-1 on T-cell activation, differentiation, and migration.

Keywords: c-Jun N-terminal kinase inhibitor, dextran sulfate sodium colitis, inflammatory bowel diseases, T cell, D-JNKI-1

Introduction

Inflammatory bowel diseases (IBDs) are regarded as disorders of unknown etiology with chronic relapsing inflammation affecting the gastrointestinal tract. Significant progress has been made in recent years toward understanding the pathogenesis of IBDs, although many aspects remain to be elucidated. Recent theories have emphasized the impact of genetic mutations in proteins linked to signaling pathways that influence the mechanisms of the innate and adaptive immune system.¹⁻⁴

In this context, recent studies have drawn attention to the impact of the mitogen-activated protein kinase (MAPK) pathway on IBD. MAPKs include various subgroups such as p42/44 extracellular signaling kinase (ERK), p38, and c-Jun.
N-terminal kinase (JNK). Immune response mediators activate intracellular signaling cascades such as MAPK and initiate inflammatory responses. Increased expression and activation of MAPKs – and of JNK in particular – has been described in patients with IBD.

Several studies have suggested that inhibition of JNK may alleviate intestinal inflammation. However, investigations of the effect of JNK on inflammation in intestinal inflammatory diseases have been limited due to a lack of specific inhibitors. Hollenbach et al. reported a reduction in inflammatory activity when the nonspecific p38 MAPK inhibitor SB203580 was administered in colitis induced by DSS and 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis in mice. The stress-activated protein kinase inhibitor CNI-1493, a guanylhydrazone, has been found to be clinically beneficial in patients with Crohn’s disease who did not respond to treatment with the anti-tumor necrosis factor-α (anti-TNF-α) antibody infliximab. The compound SP600125 was the first specific inhibitor of JNK to be identified that shows activity against all isoforms of JNK. SP600125 is a reversible adenosine triphosphate-competitive inhibitor that interferes with the phosphorylation of c-Jun. Initial results for this substance showed that it inhibited activation and differentiation of CD4 T cells. Assi et al. found that inflammation was alleviated and TNF-α levels were reduced in a mouse model of acute DSS colitis. Interactions with other pathways have also been observed at higher concentrations, however.

D-JNKI-1 is the first chemically synthesized JNK inhibitor that satisfies both of the important criteria for specific inhibition of JNK pathways: high specificity and membrane permeability. D-JNKI-1 inhibits phosphorylation of the JNK substrate c-Jun and is more potent than the small-molecule inhibitor SP600125 mentioned previously. D-JNKI-1 is produced by linking the 20 amino acid terminal JNK-inhibitory sequence (JNK binding domain) of JIP1/IB1 to a ten amino acid HIV-Tat transporter sequence for cell penetration. Peptidergic JNK inhibition using D-JNKI-1 has recently demonstrated preclinical and clinical benefit without undesirable side effects in several degenerative diseases such as traumatic hearing loss, colitis, uveitis, diabetes, myocardial ischemia, and ischemic stroke.

The present study investigated for the first time the therapeutic effect of the specific inhibiting peptide D-JNKI-1 on the clinical course, intestinal inflammation, and T-cell expression in a murine model of mild chronic DSS colitis.

Materials and methods

Animals

The experiments were conducted in female C57/BL6 mice (Charles River Ltd, Sulzfeld, Germany). The mice were all 10–12 weeks of age at the start of the experiment (weight 20–25 g). They were housed five per cage in standard laboratory conditions (12-hour light/dark cycle, room temperature 24°C, humidity 50%–60%) and supplied with drinking water and food ad libitum (ssniff M-Z, 10 mm, ssniff Spezialitäten, Soest, Germany). One group of mice (n = 5) served as controls and were given drinking water without any DSS.

Induction of mild chronic DSS colitis

Oral administration of DSS causes clinical, histological, and pathogenic features of T-helper (Th)1/Th2 chronic colitis resembling those of human IBD. The DSS concentrations of 1.0% and 1.5% for inducing mild chronic colitis were established in previous studies (data not shown) and are suitable for experimental protocols.

The animals were randomized into two groups (n = 30). The mice received three cycles of treatment with DSS (molecular mass 36–44 kDa; MP Biomedicals, Aurora, OH, USA). The first group received 1.0% DSS and the second group received 1.5% DSS for 5 days in demineralized drinking water. Each cycle of DSS treatment was followed by a 5-day interval with distilled water only. At the end of the third DSS cycle (after 30 days), the animals were sacrificed by carbon dioxide overdose.

Administration of D-JNKI-1

D-JNKI-1 (also termed XG-102) was synthesized and delivered by Xigen Ltd (Epalinges, Switzerland). D-JNKI-1 was dissolved in a 0.9% sodium chloride solution for subcutaneous application. Each group (the 1.0% DSS group and the 1.5% DSS group) was randomly subdivided into an intervention group (n = 15) and a control group (n = 15). The mice in the intervention group received three subcutaneous nuchal administrations of 1 µg/kg D-JNKI-1 on days 2, 12, and 22. The mice in the control group received physiological saline (Braun, Melsungen, Germany) subcutaneously as a negative control at the same time points in a comparable stress situation.

Assessment of disease activity

Disease activity was quantified in all of the animals on a daily basis using the disease activity index (DAI), described elsewhere. The mice were weighed daily and visually inspected for stool consistency, diarrhea, and rectal bleeding.
Tissue samples
After the mice had been sacrificed, the entire colon was removed. It was rinsed with normal saline (Braun) and divided into four anatomic parts – the cecum and proximal, medial, and distal colon. All of the dissection steps were performed on ice, and the tissue was snap-frozen in liquid nitrogen and stored at \(-70\)°C.

Histological evaluation of inflammation
For light microscopy, 5 µm transverse cryostat sections from the four anatomic parts (cecum, proximal, medial, and distal colon) from each animal were cut on a rotary cryostat microtome (Leica CM1900; Leica Microsystems, Nussloch, Germany). Three consecutive sections per anatomic region and animal were mounted on coated glass slides and dried overnight at room temperature. The tissues were stained with hematoxylin and eosin (Vector Laboratories, Inc, Burlingame, CA, USA). Histological slides were examined under a light microscope (Leica DM4000 B microscope; Leica, Wetzlar, Germany). Mucosal damage in the entire colon was evaluated and graded independently by two blinded investigators (VB and IS). The degree of mucosal inflammation was graded semiquantitatively using the crypt damage score (CDS), as described previously.\(^{25}\) In addition, the score was slightly modified by taking immune cells in the mucosa and submucosa into account. With regard to intraobserver agreement, differences were noted in only 5% of the observations. Any differences in grading were resolved by a joint examination along with a third blinded examiner (SK).

Immunofluorescence of activated lymphocytes
Immunofluorescence analysis was carried out on 5 µm transverse cryostat sections cut from the medial colon using a rotary cryostat microtome at \(-18\)°C (Leica Microsystems). This location represents the segment of the colon with the highest inflammation. Four nonconsecutive sections were evaluated for each animal. The histological sections were mounted on poly-L-lysine slides (Super Starfrost, Menzel, Braunschweig, Germany), air-dried, and fixed in methanol at room temperature for 10 minutes. They were then blocked with Aurion BSA (Aurion BSA-c™; Aurion Immuno Reagents and Accessories, Wageningen, The Netherlands) for 45 minutes at room temperature.

The sections were subsequently incubated with the primary antibodies for 2 hours at room temperature: monoclonal rat anti-CD4 antibody (31.25 µg/mL; BD Biosciences, Heidelberg, Germany) diluted 1:200 in phosphate-buffered saline (PBS; Carl Roth, Karlsruhe, Germany) and monoclonal rat anti-CD8 antibody (0.2 mg/mL; Abcam, Cambridge, UK) diluted 1:50 in PBS. After washing in PBS, tissue-bound antibodies were detected using monoclonal goat DyLight549 antibody (0.5 mg/mL; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) diluted 1:200 in PBS.

Incubation with the secondary antibody was carried out for 2 hours at room temperature and then at 4° C overnight. The sections were then washed in PBS. Each section had its own control using the secondary antibody only. Finally, the sections were mounted and analyzed using a fluorescence microscope (Leica DM4000 B microscope; Leica). Quantification of CD4\(^+\) and CD8\(^+\) cells was performed by examining two randomly selected high-power fields (with a magnification of 40,000x).

Statistical evaluation
The results for the DAI and CDS were expressed as means ± standard error of the mean (SEM). DAI values were analyzed using repeated-measures analysis of variance (ANOVA) over all time points. Comparison of CDS values between the groups was carried out using one-way ANOVA; paired comparisons were made using Tukey’s test. The labeling density of CD4\(^+\) and CD8\(^+\) cells was evaluated by t-test. \(P\)-values ≤ 0.05 were considered statistically significant. Survival rates and hazard ratios were analyzed using a log-rank test and Cox regression analysis, with \(P\)-values ≤ 0.05 considered statistically significant; 95% confidence intervals were also indicated.

Animal care statement
All of the animal experiments were performed in accordance with German legislation for the protection of animals and the National Institutes of Health guidelines for the use and care of laboratory animals. The experiments were all approved by the Ministry of Agriculture and the Environment (Ministerium fuer Landwirtschaft und Naturschutz), Kiel, Germany (V 312-72241.121-22 [60-6/08]).

Results
Development of mild colitis using 1.0% and 1.5% DSS
Untreated mice did not show any abnormal symptoms. In a control group of untreated mice (\(n = 5\)), the mean DAI ranged between 0 and 0.5 over all time points.
All animals treated with DSS developed triphasic chronic colitis following cyclic administration of low-dose DSS (1.0% and 1.5%), with moderate intensity measured on the DAI (Figure 1).

Previous studies by our group\textsuperscript{26–28} have already shown that during the first few days of DSS colitis, the animals respond to DSS treatment at different rates. This variability among the animals explains the lower DAI in the D-JNKI-1 group on day 1 (Figure 1). The parameters of stool consistency and rectal bleeding, which represent a very significant proportion of the DAI, already change after the first day of DSS treatment in some animals, whereas in other animals two or

\textbf{Figure 1} Time course of the disease activity index (DAI) (means ± standard error of the mean) in the D-JNKI-1 group and control group after administration of three cycles of 1.0% dextran sulfate sodium (DSS) (A) and 1.5% DSS (B).

Notes: Administration of D-JNKI-1 on days 2, 12, and 22 resulted in clinical improvement, with significant differences in DAI scores between animals in the D-JNKI-1 group and those in the control group (*$P = 0.013$ for 1.0% DSS and **$P = 0.007$ for 1.5% DSS; statistical evaluation: analysis of variance over all time points).
three days of DSS treatment are necessary for colitis to start (Figures 2 and 3). During the subsequent course, all of the animals develop reproducible colitis.

In the experimental trial with 1.0% DSS, the DAI reached maximum scores after 14 days in the D-JNKI-1 group (3.60 ± 0.29) and after 28 days in the control group (DAI 3.93 ± 0.15). Peak DAI values were detected at the end of each DSS treatment interval (Figure 1A). In the experimental trial with 1.5% DSS, the DAI reached maximum scores after 15 days in the D-JNKI-1 group (4.00 ± 0.38) and after 17 days in the control group (DAI 4.13 ± 0.47). Peak DAI values were detected at the end of each DSS treatment interval (Figure 1B). None of the animals in either experimental trial died (mortality 0%). Subcutaneous injection of D-JNKI-1

![Figure 2](https://www.dovepress.com/)

**Figure 2** Time course of stool consistency (means ± standard error of the mean) in the D-JNKI-1 group and control group after administration of three cycles of 1.0% dextran sulfate sodium (DSS) (A) and 1.5% DSS (B).

**Notes:** Quantification of stool consistency: solid stool = 0 points; soft stool = 2 points; diarrhea = 4 points. Administration of D-JNKI-1 on days 2, 12, and 22 in the 1.5% DSS group resulted in a significant difference in stool consistency (**P = 0.006**). In the 1.0% DSS group, only a trend toward a different stool consistency was demonstrated (not significant n.s.; analysis of variance over all time points).
significantly attenuated the clinical colitis parameters in both experimental trials. Differences in DAI values between the D-JNKI-1 and control groups were statistically significant, as evaluated using repeated-measures ANOVA over all time points (1.0% DSS, \( P = 0.013 \); 1.5% DSS, \( P = 0.007 \); D-JNKI-1 vs control group).

With regard to the DAI, D-JNKI-1 also tends to affect the parameters of stool consistency and rectal bleeding that compose the DAI. Significant results were observed in the 1.0% DSS group with regard to rectal bleeding (\( P < 0.05 \)) and in the 1.5% DSS group with regard to stool consistency (\( P < 0.01 \)) (Figures 2 and 3).

Figure 3 Time course of rectal bleeding (means ± standard error of the mean) in the D-JNKI-1 group and control group after administration of three cycles of 1.0% dextran sulfate sodium (DSS) (A) and 1.5% DSS (B). Notes: Quantification of rectal bleeding: no blood = 0 points; occult blood = 2 points; macroscopic blood = 4 points. Administration of D-JNKI-1 on days 2, 12, and 22 in the 1.0% DSS group resulted in significantly (*\( P = 0.012 \)) less rectal bleeding. In the 1.5% DSS group, only a trend toward less rectal bleeding was observed (not significant (n.s.); analysis of variance over all time points).

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No significant differences in body weight changes between the D-JNKI-1 groups and the control groups were observed after treatment with 1.0% and 1.5% DSS, probably because only mild colitis was induced.

**Histological scoring of the degree of inflammation**

DSS administration in both trial groups (1.0% DSS and 1.5% DSS) caused moderate inflammation, characterized by infiltration of inflammatory cells and loss of goblet cells. As a further change characteristic of chronic inflammation, the submucosa was thickened, with pronounced edema. Tissue damage was scored by quantifying the length of crypts and lymphocyte infiltration.

After DSS treatment with 1.0% DSS, histological scoring using the CDS developed by Egger et al.\(^3^2\)\(^-\)\(^29\) showed increased crypt damage in the medial colon (CDS in the medial colon: D-JNKI-1 8.53 ± 0.82 and saline 8.27 ± 1.11). However, these differences in CDS did not reach statistical significance (Figure 4A). Cyclic administration of 1.5% DSS also led to the highest inflammation in the medial colon. Additionally, no significant differences were observed between the group treated with D-JNKI-1 and the control group (CDS in the medial colon: D-JNKI-1 7.47 ± 1.02 and saline 7.93 ± 0.77) (Figures 4 and 5).

**Activated lymphocytes in the inflamed colon**

Immunofluorescence analysis of CD4\(^{+}\) and CD8\(^{+}\) cell expression was carried out in tissue from the medial colon in the 1.5% DSS experimental trial group, as this part of the colon developed the highest grade of inflammation. The total number of these cells was counted in two random sight fields. In both the D-JNKI-1 group and the control group, the number of CD4\(^{+}\) cells was lower than the total number of CD8\(^{+}\) cells. After induction of chronic DSS colitis, CD8 staining was lower in the D-JNKI-1 group in comparison with the control group (15.8 ± 1.55 and 20.72 ± 1.81). CD4 staining was also lower in the D-JNKI-1 group than in the control group (25.23 ± 3.15 and 28.60 ± 3.29). However, neither the CD4 nor CD8 counts showed significant differences (Figures 6 and 7).

**Discussion**

In IBD, perpetual production of inflammatory mediators leads to inflammation of intestinal tissue.\(^3^0\)\(^-\)\(^3^3\) Although many forms of medical treatment have now been developed in order to control immune reactions and T-cell responses, relapsing intestinal inflammation continues to be a major problem. New insights into the pathophysiology of IBD indicate that specific and targeted inhibition of inflammatory processes appears to be a promising treatment option. In this context, the modulation of cytokines such as interleukin (IL)-10, IL-11, and IL-12 has shown initially first positive effects on the progression of IBD.\(^3^4\)\(^-\)\(^3^7\) Treatment with infliximab, a monoclonal antibody against TNF-α, is now a recognized and well-established therapy option in patients with severe courses of Crohn’s disease.\(^3^8\)\(^-\)\(^3^9\) However, an important drawback in many current treatment options for IBD is the fact that the drug effect is often unspecific and associated with serious
adverse side effects. Further research is therefore necessary in order to optimize the treatment of patients with IBD.

In this context, the JNK pathway represents one possible target for intervening in the complex inflammatory mechanisms involved in IBD. As JNKs regulate various inflammatory reactions of the innate and specific immune response, promote inflammatory responses, and control the expression of proinflammatory cytokines such as IL-2, IL-6, and TNF-α and the activation of inflammatory cells and apoptotic pathways, interventions in JNK signal transduction may provide new therapeutic opportunities for patients with IBD. However, the development of anti-JNK therapies has so far been limited due to a lack of appropriate inhibitors. Current JNK inhibitors provide poor specificity and cell permeability.

The present study investigated for the first time the effect of the highly specific JNK inhibitor D-JNKI-1 on mild chronic DSS colitis, a disease model with pathophysiological properties similar to those of ulcerative colitis. As a result of the toxic effect of the polysaccharide DSS on epithelial cells, the substance induces enteritis in the colon after oral administration, with clinically measurable symptoms such as weight loss and bloody diarrhea. At an early stage, mucosal permeability is increased and the intestinal barrier function is destroyed. Intestinal antigens reach deeper layers of the intestinal wall and provoke an acute inflammatory response. In this model, the early inflammatory reaction is directed by macrophages and granulocytes. As the colitis continues, particularly in this model of chronic colitis, the influence of T lymphocytes in the Th1/Th2 cytokine profile increases. Further consequences include mucosal damage, which shows a linear relationship to the DSS concentration administered, with an increase in the proportion of typical Th1 cytokines: ie, TNF-α, IL-12, and IL-1. In addition, progressive shortening of the intestinal crypt structure leads to the development of inflammatory infiltration.

Two different intensities of chronic DSS colitis (1.0% DSS and 1.5% DSS) were induced in the present study to analyze the influence of D-JNKI-1 on mild colitis. The clinical and histological features of inflammation were assessed.
scored using the DAI and CDS, as described previously.\textsuperscript{25,26} In both experimental trials (1.0% DSS and 1.5% DSS), D-JNKI-1 clearly attenuated the disease activity and showed a therapeutic effect on the clinical course of DSS colitis. This observation is in line with recent studies in which less specific inhibitors of the MAPK/JNK pathway were used, such as SB203580 and CNI-1493.\textsuperscript{5,9,10}

D-JNKI-1 also tends to affect the parameters of stool consistency and rectal bleeding that compose the DAI. Significant results were observed with regard to rectal bleeding in the 1.0% DSS group ($P < 0.05$) and with regard to stool consistency in the 1.5% DSS group ($P < 0.01$) (Figures 2 and 3). The reason for the lack of significance in the other groups and in relation to weight loss may be that only mild colitis was induced and only a few animals developed macroscopically apparent bloody diarrhea (four points in the DAI calculation). The majority of animals presented only with a smooth stool consistency (two points in the DAI for calculation).

Previously studies by our group demonstrated that cyclic administration of 1.0% DSS induces mild chronic colitis, whereas a further increase in the DSS dosage to 1.7% triggers severe colitis that is associated with high mortality rates (>30%).\textsuperscript{26–28} Following this observation, the dosages of 1.0% DSS and 1.5% DSS were chosen in the present study to induce a mild form of chronic histology. Histological analysis of the colon in the DSS-treated mice showed moderate inflammation, with slight immune cell infiltration and ulcerative tissue destruction. Unfortunately, no significant differences with regard to histological inflammation were seen after administration of D-JNKI-1. One possible reason for this might be the experimental protocol used, in which an apparently very mild form of chronic colitis was induced, which makes it difficult to assess the therapeutic effect of D-JNKI-1 on the histopathological structure of the colon mucosa. Prospective studies of severe colitis (with a DSS dosage of 1.7% or 2.0%) and higher D-JNKI-1 dosage will be needed in order to investigate the therapeutic effect of D-JNKI-1 at the histological level.

In human IBD, pathological inflammation is associated with a proliferation of CD8\textsuperscript{+} and CD4\textsuperscript{+} cells.\textsuperscript{49} It has been demonstrated that JNK1 and JNK2 play different roles during CD8\textsuperscript{+} and CD4\textsuperscript{+} cell activation.\textsuperscript{50} Perhaps again due to the only mild form of colitis induced, the present results did not reach statistical significance. Nevertheless, they show a trend toward reduced T-cell infiltration after administration of D-JNKI-1, particularly with CD8\textsuperscript{+} cells but also with CD4\textsuperscript{+} cells.

A comparable anti-inflammatory effect of D-JNKI-1 was previously described by our group\textsuperscript{19} in an acute form of severe TNBS colitis, which is an acute model of IBD with pathophysiological properties similar to those of Crohn’s disease.\textsuperscript{51–53} Simultaneous subcutaneous application of D-JNKI-1 significantly reduced the DAI. After 72 hours – ie, at the end of the study – the colon was removed and analyzed immunohistochemically. D-JNKI-1 significantly reduced pathological changes such as ulceration and crypt deformation, immune cell pathology such as infiltration and the presence of CD3\textsuperscript{+} and CD68\textsuperscript{+} cells, activation of caspase-3 and expression, activation of the JNK substrate and transcription factor c-Jun, and production of TNF-\textalpha. In TNBS colitis, a single administration of subcutaneous D-JNKI-1 was at least as effective as the daily oral administration of sulfasalazine used in the treatment of IBD.

With regard to the DSS model, previous studies by our group showed that JNK-dependent expression of the inflammatory cytokines TNF-\textalpha, IL-6, and transforming growth factor-\beta1 (TGF\textbeta1) apparently do not play a major role in the present model of mild chronic DSS colitis. In JNK1 knockout mice (MAPK\textalpha––/–), JNK2 knockout mice (MAPK\textbeta––/–), and wild-type controls (WT1, WT2), calibrator-normalized quantitative real-time polymerase chain reaction did not show any significant differences with regard to the expression of TNF-\textalpha, TGF\textbeta1, or IL-6.\textsuperscript{78} This finding is in line with observations by Alex et al,\textsuperscript{54} who reported an effect of these cytokines in acute DSS colitis but not in chronic DSS colitis. No cytokine analysis was therefore carried out in the present study.

Although there are no animal models that completely reflect all the pathophysiological aspects of human IBD, the successful alleviation of the clinical course of acute TNBS colitis and chronic DSS colitis in mice supports the hypothesis that inhibition of JNK may represent a promising therapeutic principle in IBD.

**Conclusion**

The present study shows that the JNK pathway represents a possible target for intervening in the complex inflammatory mechanisms involved in IBD. The therapeutic potential of the highly specific JNK inhibitor D-JNKI-1 was tested for the first time in a mild form of chronic DSS colitis in mice. It was found that in addition to being effective in the model of TNBS-induced colitis,\textsuperscript{19} D-JNKI-1 is also effective in another model of colitis, DSS-induced colitis. The results, showing an improvement in the clinical course, support the view that D-JNKI-1 has potential as a new form of treatment in patients with IBD.
Disclosures

All authors declare no conflicts of interests. We do not have any paid or unpaid consulting, employment royalty, stock, patent agreement, position, or other financial relationship with any individual, company, or organization with a vested interest in the subject matter mentioned in the manuscript.

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