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The GPR55 antagonist CID16020046 protects against intestinal inflammation

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Abstract

Background—G protein-coupled receptor 55 (GPR55) is a lysophospholipid receptor responsive to certain cannabinoids. The role of GPR55 in inflammatory processes of the gut is largely unknown. Using the recently characterized GPR55 inhibitor CID16020046, we determined the role of GPR55 in experimental intestinal inflammation and explored possible mechanisms of action.

Methods—Colitis was induced by either 2.5% dextran sulfate sodium (DSS) supplemented in the drinking water of C57BL/6 mice or by a single intrarectal application of trinitrobenzene sulfonic acid (TNBS).

Key results—Daily application of CID16020046 (20 mg kg⁻¹) significantly reduced inflammation scores and myeloperoxidase (MPO) activity. In the DSS colitis model, levels of TNF- α and IL-1 β , and the expression of cyclooxygenase (Cox)-2 and STAT-3 were reduced in colon tissues while in TNBS-induced colitis, levels of Cox-2, IL-1 β and IL-6 were significantly lowered. Evaluation of leukocyte recruitment by flow cytometry indicated reduced presence of lymphocytes and macrophages in the colon following GPR55 inhibition in DSS-induced colitis. In J774A.1 mouse macrophages, inhibition of GPR55 revealed reduced migration of macrophages and decreased CD11b expression, suggesting that direct effects of CID16020046 on macrophages may have contributed to the improvement of colitis. GPR55^{-/-} knockout mice showed reduced inflammation scores as compared to wild type mice in the DSS model suggesting a proinflammatory role in intestinal inflammation.

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Author contributions

RS, MS, AS, AH, R Schuligoi, GM designed the study and supervised the research
RS, AS, KJ, FR and CH participated in the *in vivo* studies, sample collection, score evaluation and leukocyte recruitment
AS and KJ conducted the *in vitro* assays and analyzed the results
RS, MS, AS, AH, R Schuligoi and GM wrote the manuscript
All authors were involved in data discussion and critical reviewing of the manuscript.

Disclosures

No competing interests declared

Conclusions and inferences—Pharmacological blockade of GPR55 reduces experimental intestinal inflammation by reducing leukocyte migration and activation, in particular that of macrophages. Therefore, CID16020046 represents a possible drug for the treatment of bowel inflammation.

Keywords

intestinal inflammation; DSS; TNBS; GPR55

Introduction

G protein-coupled receptor 55 (GPR55) was originally shown to respond to natural and synthetic cannabinoids as well as to endocannabinoids and was therefore labeled as a new cannabinoid receptor [1]. Data, however, converged on lysophosphatidylinositol (LPI) as the only consistent endogenous ligand [2,3]. The response of GPR55 to LPI can be effectively modulated by endocannabinoids which enhance LPI effects at low, but inhibit them at high concentrations [4]. Expression of GPR55 has been reported in many tissues, such as the CNS, adrenal glands, spleen and in the gastrointestinal (GI) tract [1,5], where it is found in the epithelium [6], cells of the lamina propria, and in the enteric nervous system of both small and large intestine [6-8]. Several types of leukocytes express GPR55, including neutrophils, monocytes, lymphocytes and macrophages [5,9]. Contrary to canonical cannabinoid (CB) receptors, GPR55 signals through Gα12/13 and Gq proteins [1,10] inducing Ca²⁺ release and activation of the downstream MAPkinases ERK1/2, and of small G proteins like RhoA [11,12]. In HEK293 cells overexpressing GPR55, LPI has been shown to activate the transcription factors NFκB and NFAT [13]. These signaling pathways were antagonized by the novel GPR55 inhibitor CID16020046 [13]. Therefore, unlike classical CB receptors, GPR55 initiates excitatory rather than inhibitory effects suggesting that GPR55 may promote functions that oppose the ones initiated by CB receptors. New findings on the heteromerization of CB receptors with GPR55 and their reciprocal modulation point out that GPR55 may be crucial for CB receptor signaling and its downstream effects [14-16]. The role of CB receptors in physiology and inflammation of the gut is well described (rev. in [17]), however, little information is available on the role of GPR55 in GI physiology. It has been established that GPR55 mediates relaxation of the gut through activation of enteric neurons and additionally through central mechanisms [7,18,19]. With regard to its pathophysiological role, it has been shown to be implicated in the development of neuropathic and inflammatory pain through modulation of proinflammatory cytokines [20]. GPR55 is also present in many types of cancer cells [21]. In a model of systemic inflammation induced by lipopolysaccharide, GPR55 expression was increased in the GI tract [8]. On the other hand, a recent study of DNBS colitis described that GPR55 was downregulated during intestinal inflammation [22]. Therefore, the role of GPR55 in GI inflammation warrants further investigation as it may be a possible target for new drugs fighting intestinal inflammation.

We have recently characterized a highly selective GPR55 antagonist, CID16020046 [13] that has shown superior selectivity in ligand and binding pocket studies [23]. In an attempt to investigate the role of GPR55 in GI inflammation and to explore underlying mechanisms of

its action, we employed two models of experimental intestinal inflammation and applied CID16020046 to C57BL/6 mice. Experimental colitis models, such as DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis, show some features that are comparable to human inflammatory bowel disease (IBD). They are driven by lymphocyte and macrophage influx with specific cytokine release, typical of each of the models [24]. We report that antagonism of GPR55 using CID16020046 and genetic GPR55 knockdown ameliorates inflammation in the colon suggesting a proinflammatory role of GPR55 in models of intestinal inflammation.

Materials and methods

Pharmacological treatment and cell culture

DSS (reagent grade; 36-50,000 Da) was obtained from MP Biomedicals (Santa Ana, CA, USA) and TNBS from Sigma (St. Louis, MO, USA). The selective GPR55 inhibitor CID16020046 ((4-[4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo [3,4-c] pyrazol-5-yl] benzoic acid) was purchased from ChemDiv (San Diego, CA, USA) [13] and dissolved in DMSO. CID16020046 (or vehicle) was injected subcutaneously (s.c.) 30 min prior to onset of the colitis models at a dose of 20 mg kg⁻¹ and given once daily for 7 days in the DSS or for 3 days in the TNBS model. In a previous study, we showed that LPI-inhibited aggregation of platelets was completely reversed at a concentration of 10 µM CID16020046 [13]. For *in vitro* assays, we therefore used concentrations of 1, 5 and 10 µM of CID16020046. J774A.1 mouse macrophage cell line was obtained from Interlab Cell Line Collection, Genoa, Italy and cultured in DMEM medium (PAA Laboratories, Pasching, Austria) supplemented with 10% FBS and 1% PenStrep (PS). Cells were grown at 37°C in 5% CO₂-humidified atmosphere. In all assays, no passage higher than 10 was used. All assays were performed with the respective vehicles.

DSS- and TNBS-induced colitis

Male C57BL6/N mice (5-6 weeks old, 20-22g) from Charles River (Sulzfeld, Germany) were kept in house for 2 weeks prior to the experiments. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12-hr light-dark cycle with free access to standard laboratory chow and tap water. GPR55^{-/-} knockout mice (B6;129SGpr55tm1Lex/Mmnc) were generated by Lexicon Pharmaceuticals (The Woodlands, TX, USA) and obtained through the Mutant Mouse Regional Resource Center (MMRRC, Chapel Hill, NC, USA). Only male mice and age-matched littermates were used for the experiments. Experimental procedures were approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: 66.010/0101-II/3b/2013) and performed in strict accordance with international guidelines. All efforts were made to minimize suffering. For induction of DSS colitis, mice received 2.5% DSS supplemented in their drinking water for a period of 5 days. Colons were removed on day 7, scored in a blinded fashion, and tissue was collected for further use. Scoring was performed in an adapted form according to a system by Kimball et al. [25] and has been used before [6]. Loss of colon weight, shortening of colon length, stool consistency, and presence of fecal blood was scored from 0 to 4 with 0 depicting the normal and 4 the maximally affected state. Epithelial damage was considered as the amount of ulcers present and scored from 0

(normal mucosa) to 4 (more than five ulcers). The score index represents the sum of all subscores and had a maximum of 16. For induction of TNBS colitis, animals were lightly anesthetized with isoflurane, and TNBS (4 mg in 100 μ L of 30% ethanol) was infused into the colon using a gavage needle with a blunt end. Solvent alone (100 μ L of 30% ethanol) was administered in control experiments. Colonic damage was assessed by a semiquantitative scoring system 3 days after administration of TNBS, as previously described [26, 6]. Briefly, damage was scored according to the following scale, adding individual scores for ulcer, adhesion, colonic shortening, wall thickness, and presence of hemorrhage, fecal blood, or diarrhea. Ulcer: 0.5 points for each 0.5 cm; adhesion: 0 points = absent, 1 point = 1 adhesion, 2 points = 2 or more adhesions or adhesions to organs; shortening of the colon: 1 point = >15%, 2 points = >25% (based on a mean length of the untreated colon); wall thickness was measured in mm. The presence of hemorrhage, fecal blood, or diarrhea increased the score by 1 point for each additional feature. The macroscopic scoring, the collections of colon tissue for Western Blots, ELISAs, histology/immunohistochemistry and for the myeloperoxidase (MPO) assays were carried out from one set of DSS and TNBS experiments (n=4-16 animals), while colon tissue for the leukocyte recruitment assays was collected from a different set of DSS experiments (n=9 animals). Behavioral experiments were carried out separately with healthy C57BL/6N mice (n=8).

Myeloperoxidase (MPO) activity assay

MPO activity was determined as previously described with slight modifications [27]. Colon samples were placed into hexadecyltrimethyl ammonium bromide (HTAB) buffer (pH 6.0; Sigma) containing 50 mM KH_2PO_4 and 50 mM K_2HPO_4 (Merck, Darmstadt, Germany). Tissue was mechanically homogenized and afterwards centrifuged for 20 min at 9000 x g at 4°C. A solution of 200 μ L containing 10% phosphate buffer, 0.0167% o-dianisidine peroxidase substrate and 0.06% of H_2O_2 (Sigma) was added to 10 μ L of supernatant and measured in triplicates in a 96 well plate at 450 nm using a microplate spectrophotometer after 25 min (BioRad, Hercules, CA, USA).

Histology and immunohistochemistry

Following macroscopic scoring, segments of the distal colon were stapled flat onto cardboard with the mucosal side up and fixed for 24 hrs in 10% neutral-buffered formalin. Tissue was then dehydrated, embedded in paraffin and standard hematoxylin staining was performed on 5 μ m thick sections. Colon sections were also prepared for antigen retrieval immunohistochemistry. To this end, sections were microwaved 2 x 5 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), and further processed by ABC method according to manufacturer's instructions (Vector Labs, Burlingame, CA, USA). Sections were incubated with anti-CD3 (1:1000; Abcam, Cambridge, UK) and anti-F4/80 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA), visualized with 3-3'-diaminobenzidine (Vector Labs) and counterstained with hematoxylin. The specificity of the antibodies was tested by omitting the primary antibody. Images were taken with an Olympus DP50 camera, and processed with Cell^A imaging software (Olympus, Vienna, Austria). Only brightness and contrast of the entire picture were adjusted. An imaging program (xcellence®; Olympus) was used for counting immunoreactive cells in colon sections from

DSS colitic mice and expressed per length ($10^2 \mu\text{m}$) of submucosa. For quantifying immunostained cells, we used 6-10 non-overlapping colon sections from 3 different mice of each treatment group (DSS+vehicle group and DSS+ CID16020046 group).

Western blots and ELISA

Protein concentrations of colon tissue samples were determined using BioRad Protein Assay Kit II and read on a microplate spectrophotometer according to the instructions of the manufacturers (BioRad). Protein extracts (30 μg) were diluted 1:1 with sample buffer containing 100 mM TRIS/HCl and 5% SDS, 15% glycerol, 0.004% bromophenolblue and 5% mercaptoethanol (all from Sigma). Samples were electrophorized on a 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) using iBlot device by Invitrogen (Waltham, Massachusetts, USA). Afterwards, the membrane was blocked for 1 hr in 5% blocking solution (1 mM CaCl_2 , 135 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCL, 0.1% [v/v] Tween20 and 5% milk powder) followed by incubation with the first antibodies, i.e. rabbit anti-Cox-2 (1:200; Abcam), anti-pSTAT3, and with anti-tSTAT3 (both 1:1000; Cell Signalling) or mouse anti- β -actin (Sigma) overnight at 4°C, and by 1 hr incubation with the HRP-conjugated antibodies (1:4000; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature. Bands were visualized with Pierce ECL Western blotting substrate (Thermo Scientific, Waltham, Massachusetts, USA), quantified with ImageJ Software (NIH, Bethesda, MD, USA) and normalized to β -actin or tSTAT3. Values represent group means of the normalized band densities. To determine the contents of cytokines in colon tissue, an ELISA (Ready-SET-Go!) from eBiosciences (San Diego, CA, USA) was used following the manufacturer's instructions.

Isolation of lamina propria leukocytes and flow cytometry

The method was conducted as previously described [27]. Briefly, after washing with HBSS, small pieces (5 mm) of colon were transferred into a 50 mL falcon containing HBSS, HEPES and PS and gently shaken for 4×10 min. The falcons were then rotated at 37°C 4×20 min each time in fresh HBSS/EDTA/PS to remove epithelial cells. After another wash, samples were transferred into complete RPMI medium (PAA Laboratories, Pasching, Austria) with 100 units/mL of collagenase type 2 (Thermo Scientific, Waltham, Massachusetts, USA) for 1 hr at 37°C. The cell suspension was then passed through a 40 μm cell strainer into a new falcon tube and centrifuged at 400 x g for 7 mins. The pellet was washed with PBS and leukocytes were stained for flow cytometric evaluation. Lamina propria leukocytes were stained with a PE-conjugated Siglec F antibody (1:100; BD Pharmingen), a PerCP- Cy5.5 conjugated Gr1 antibody (1:100, eBioscience) and Alexa Fluor 488-conjugated F4/80 antibody (1:20; BD Pharmingen) for 30 mins at 4°C. Samples were washed once in PBS, fixative solution was added and samples were kept on ice until analyzed on a FACSCalibur flow cytometer. Identification and selection of lamina propria leukocytes was accomplished on the basis of forward scatter (FSC) versus side scatter (SSC) parameters. Macrophages, monocytes and granulocytes were identified via their differential expression of F4/80 and Gr-1 while lymphocytes were selected via forward/side scatter appearance. The granulocyte population was further gated on the expression of Siglec F. Eosinophils were gated as Siglec F⁺ cells and neutrophils as Siglec F⁻ cells.

PCR

Cells were frozen in liquid nitrogen and lysed in RNA buffer. Total RNA was then extracted using QIAshredder and RNeasy Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. RT-PCR was performed with 1 µg of total RNA and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA) for cDNA transcription. Quantitative PCR (qPCR) was performed using SYBR® Green and GPR55 primers (#100-25636) from BioRad according to the manufacturer's instructions. Amplicons were electrophorized in 1% agarose gel and stained with ethidiumbromide.

Migration of J774A.1 mouse macrophages

Migration assays with J774A.1 cells were performed in 24-well Transwell plates with 8 µm membrane inserts (Corning Inc., Lowell, MA, USA). Cells were starved in DMEM containing 0.5% FBS (Life Technologies) overnight and then incubated with CID16020046. A suspension of 3×10^5 cells was placed in the upper compartment, and C5a (5 nM; Sigma) [28] was added to medium as a chemoattractant to the lower compartment. Cells were allowed to migrate for 2 hrs at 37°C and 5% CO₂ in a humidified incubator. Upon completion of migration, the upper sides of the filters were cleaned with a cotton swab, and filters were dried and fixed in formaldehyde for 30 mins. After a wash in PBS, filters were mounted and coverslipped in Vectashield® (Vector Labs). Cell nuclei were counted under a fluorescent microscope (Olympus IX 70). Each migration experiment was performed in duplicates. The average number of migrated cells was determined from at least three independent experiments.

CD11b expression in J774A.1 mouse macrophages

2×10^6 cells were transferred into 500 µL PBS and preincubated for 30 mins with 1, 5, and 10 µM CID16020046 or vehicle (DMSO). Cells were then stimulated with 1 nM of monocyte chemotactic protein 1 (MCP-1) [29] for another 30 mins at 37°C. Alexa Fluor 647 anti-mouse CD11b (15 µl; BD Biosciences) was added 15 min before the end of the incubation time. After adding the fixative solution, cells were measured on a FACSCalibur flow cytometer. Experiments were performed in triplicates and data were expressed as percentage change to the vehicle treatment.

Migration of human neutrophils

To study the migration of human neutrophils, a Boyden chamber migration assay was performed [30]. Blood was drawn from healthy donors after signing an informed consent. The procedures were approved by the Institutional Review Board of the Medical University Graz (protocol number 17-291 ex 05/06). Isolated human neutrophils were then resuspended in PBS (Ca²⁺ and Mg²⁺ free) at a density of 2×10^6 cells/mL. N-formyl-methionyl-leucylphenylalanine (fMLP; 100nM) was used as a chemoattractant and placed into the bottom wells of a 48-well microBoyden chamber with a 5-µm polycarbonate membrane. The cell suspension with the neutrophils were placed into the upper wells of the chamber and incubated at 37°C with different concentrations of CID16020046 (or DMSO as vehicle) for 1 hr. After incubation, the remaining cells were removed from the upper compartment of the

chamber and the cells of the lower compartment were transferred into polypropylene micro tubes. After adding 150 μ L of fixative solution, cells were analyzed on the FACSCalibur flow cytometer.

Open field test

An open field test with healthy C57BL/6 mice was carried out as previously described [31]. The experimental procedures were approved by the Austrian Federal Ministry of Science, Research and Economy (BMWF-66.010/0037-II/3b/2013). Briefly, mice received 20 mg/kg CID16020046 (or DMSO as vehicle) daily over a period of 6 days. On day 6, 30 mins after the last injection, they were placed in the middle of an opaque gray plastic box (50 $\times$ 50 $\times$ 30 cm, length $\times$ width $\times$ height) and their behavior during a 5-min test session was recorded and tracked by a video camera and the VideoMot2 tracking software (TSE Systems, Bad Homburg, Germany). The ground area of the box was divided into a 36 $\times$ 36 cm central area and a surrounding outer area. Time spent in the central area was used to assess anxiety-like behavior and total distance travelled was measured to evaluate locomotor activity.

Data analysis

All statistical analysis was performed using GraphPad Prism® 5.0 Software (GraphPad Software, La Jolla, CA, USA). Experimental data were analyzed either by one-way ANOVA and Tukey's multiple comparison post-hoc test or by Student's t-test. Statistical significance was set at $p < 0.05$.

Results

The GPR55 inhibitor CID16020046 improves inflammation in models of intestinal inflammation

Using the DSS model of intestinal inflammation, preliminary studies were conducted to assess an effective dose of CID16020046, which was determined by starting with a dose of 1 mg kg^{-1} and continued to a concentration of 20 mg kg^{-1} , the lowest dosage at which inflammation scores improved significantly (Fig. 1A). All the following *in vivo* experiments were then performed using a dosage of 20 mg kg^{-1} . In the DSS and TNBS colitis models, treatment with CID16020046 significantly reduced macroscopic scores (Figs. 1B and E) and MPO activity (Figs. 1G and H) as compared to vehicle-treated animals. Colon lengths in DSS colitis are shown as subscores (Fig. 1C) to demonstrate improvement of colon shortening. Subscores are part of the macroscopic score index, as described recently [6]. In colon sections of DSS- and TNBS-colitic mice treated with the GPR55 inhibitor, the architecture of the crypts appeared more preserved and infiltration of leukocytes to the lamina propria appeared to be reduced as compared to sections of vehicle-treated animals (Figs. 1D and F). For comparison, the colonic mucosa of a healthy animal (control) is also shown in Fig. 1D. Body weights typically dropped in DSS colitis after 5 days and after one day in the TNBS model, however, no significant differences were observed between the vehicle- and CID16020046-treated groups, which, at least for the DSS model, may have been due to the relatively low dose of DSS used (2.5%) (data not shown).

The GPR55 inhibitor CID16020046 reduces proinflammatory cytokines

To investigate whether CID16020046 exerted its anti-inflammatory action by changing cytokine production, we evaluated the pro-inflammatory cytokine profile in both models by ELISA. TNF- α , IL-1 β and IL-6 levels were measured in colonic tissue of both models. In the DSS model, CID16020046-treated animals showed reduced levels of TNF- α and IL-1 β but no changes in IL-6 when compared to the vehicle-treated mice (Figs. 2A, C, and E). In TNBS colitis, levels of IL-1 β and IL-6 were significantly lower in CID16020046-treated animals compared to vehicle treatment, whereas TNF- α did not change significantly (Figs. 2B, D, and F).

CID16020046 decreases Cox-2 expression

We further analyzed the effects of CID16020046 on inflammation markers and measured Cox-2 and phosphorylation of transcription factor STAT3, which is also upregulated in IBD patients [32], in both models. GPR55 antagonism decreased Cox-2 expression significantly in both models (Figs. 3A-C) but revealed opposite effects on STAT3 phosphorylation. While the level of pSTAT3 was lowered after GPR55 inhibition in the DSS model (Figs. 3D and F), it was significantly increased in the TNBS colitis model (Figs. 3E and F).

Effects of CID16020046 on infiltration of leukocytes into the lamina propria of the colon

Based on the immunohistochemical findings that treatment with CID16020046 may have an impact on the presence of immunocytes in the colonic submucosa we measured and quantified leukocyte recruitment into the lamina propria of the colon in the DSS model. Treatment with CID16020046 led to an almost 50% decrease in the number of infiltrated macrophages and lymphocytes as compared to the vehicle group (Fig. 4A), whereas no significant changes were observed in the amount of monocytes and eosinophils (Fig. 4A). Interestingly, the number of neutrophils was increased (Fig. 4A). To visually demonstrate the leukocyte populations that were found reduced in the recruitment experiment, immunohistochemical staining for the T lymphocyte marker CD3 and the macrophage marker F4/80 was employed and revealed significantly decreased immunoreactivity in the colon submucosa after GPR55 inhibitor treatment (as compared to treatment with vehicle) (Fig. 4B).

Effects of GPR55 inhibitor CID16020046 on the migration and activation of the macrophage cell line J774A.1 and on the migration of human neutrophils

To investigate actions of CID16020046 treatment at a cellular level and because of the prominent effect of CID16020046 on the macrophage recruitment, we determined effects of CID16020046 on the migration and inflammatory activity of the mouse macrophage cell line J774A.1. This cell line showed high expression of GPR55 mRNA as compared to RAW264.7 macrophages and were therefore used in the *in vitro* assays (Fig. 5A). C5a (5nM)-induced migration [28] of J774A.1 cells was concentration-dependently reduced after incubation with 1 and 5 μ M CID16020046 (Fig. 5B and C). Similarly, expression of CD11b, a β 2 integrin that is rapidly activated and upregulated on the leukocyte membrane upon activation [29], was concentration-dependently decreased almost back to control levels after incubation with 1-10 μ M CID16020046 (Fig. 5D). Treatment with 1 and 2.5 μ M

CID16020046 also strongly diminished migration of human neutrophils when using 100 nM of fMLP as a chemoattractant (Fig. 5E). Concentrations of CID16020046 used in the study did not change cell viability (data not shown).

GPR55 knockout mice exhibit less severe inflammation than wild type mice during DSS colitis

To evaluate the role of GPR55 in intestinal inflammation in a genetic knock out model, GPR55^{-/-} knock out and GPR55^{+/+} wild type mice were subjected to experimental DSS colitis. In line with our data obtained with the GPR55 inhibitor, inflammatory scores and MPO activity were significantly reduced in GPR55^{-/-} knock outs as compared to and GPR55^{+/+} wild types (Fig. 6A-D).

CID16020046 does not change the locomotor and anxiety behavior of healthy mice

GPR55 is expressed in the brain and may be linked to motor behavior [33]. As GPR55 agonists have been also shown to exert central effects [7], we used an open field test to investigate whether CID16020046 would change the locomotor and anxiety behavior of mice. Daily treatment of healthy mice with 20 mg/kg of CID16020046 (6 x s.c.) did not alter their locomotor activity (Fig. 6E) (measured as total travelling distance) or anxiety behavior (Fig. 6F) (measured as time spent in the center of the box), as compared to vehicle-treated animals. Additionally, mean body weights and body temperatures did not differ between the treatment groups (Figs. 6G, H) suggesting that CID16020046 does not induce central activity or sickness behavior.

Discussion

A clinical study recently demonstrated that patients with Crohn's disease experienced benefit from treatment with *Cannabis* [34], suggesting that cannabinoids may be potential candidates for the pharmacotherapy of intestinal inflammation. However, those findings also imply that knowledge of the cannabinoids' anti-inflammatory actions and their impact on the endocannabinoid system is a crucial prerequisite before embarking on a cannabinoid-based therapy. For instance, it is still unclear how GPR55 and other non-CB1/CB2 receptors, which are activated by endo- and exogenous cannabinoids, fit into this system. With respect to the recent findings that GPR55 modulates CB receptor signaling and forms heteromers with CB receptors [4,14,16], it is important to explore the pathophysiological role of GPR55 in intestinal inflammation in more detail. In our study, we demonstrate that pharmacological inhibition of GPR55 offers protection in two mouse models of intestinal inflammation. In addition, GPR55^{-/-} knockout mice revealed reduced inflammation in the DSS model. The GPR55 inhibitor CID16020046 caused reduced influx of macrophages and lymphocytes into the colon in the DSS model and was able to decrease migration and activation of J774A.1 mouse macrophages, suggesting that GPR55 drives inflammation through influx and activation of immunocytes in the gut. The inhibitor did not induce central activity or signs of sickness. Therefore, unlike the anti-inflammatory role of CB1 and CB2 in colonic inflammation [35-37], GPR55 may play a pro-inflammatory role in intestinal inflammation, and it can be blocked with a specific antagonist with no central activity.

The GPR55 antagonist CID16020046 has been characterized in a previous study by our group and has shown potent *in vitro* effects on platelet functions by completely reversing LPI-inhibited aggregation [13]; however, no data yet existed on the *in vivo* efficacy of this antagonist. Firstly, we found that, as a marker of inflammation, Cox-2 expression was reduced by the GPR55 inhibitor. Cox-2 is largely increased in intestinal inflammation [38, 39] and responsible for the production of pro-inflammatory mediators [40]. Elevated expression of Cox-2 in DSS and TNBS models has been localized to a multitude of cells within the colon, such as to immunocytes and epithelial cells [41,42], and even to the enteric nervous system [43]. However, both preventive [41] and destructive effects [44] of Cox-2 inhibitors have been reported in DSS colitis lending support to the concept that Cox-2-derived mediators exert dual roles in inflammation. Due to their cell specific actions, therefore, pro- and/or anti-inflammatory effects are sometimes difficult to distinguish [45]. Irrespective of this discrepancy, several reports including ours demonstrate that improvement of experimental intestinal inflammation correspond with a decrease in Cox-2 expression [41,42,46,47].

Secondly, we found that, following GPR55 blockade, the DSS colitis model displayed a reduction in the pro-inflammatory cytokines IL-1 β and TNF- α , while in the TNBS model, only IL-1 β was significantly reduced. However, both colitis models revealed different effects on IL-6. DSS-induced colitis is known to be strongly upheld by the innate immune system and dominated by Th2 cell and macrophage influx with release of TNF- α , IL-1 β , IL-6, and other cytokines [24,48-50]. Conversely, TNBS-induced colitis is more dominated by the presence of Th1 cells and macrophages with TNF- α , IL-1 β , and MIP-1 α release, reminiscent of Crohn's disease [24,51]. The reduction in inflammation by the GPR55 inhibitor involved a decreased Cox-2 and IL-1 β expression in both models, but decreased IL-6 expression was only observed in the TNBS model. The data thus indicate that common and distinct model-specific mechanisms exist in the process of mucosal damage repair.

Since we observed opposing effects of CID16020046 at the levels of IL-6, we wanted to investigate the IL-6 signaling pathway in more detail. We, therefore, determined STAT3 phosphorylation which is also thought to play an important role in intestinal inflammation; however, its function is still unclear [52]. Similar to the observations on IL-6, STAT3 phosphorylation was differentially regulated in the DSS and TNBS colitis model. IL-6 is regarded as a cytokine that promotes Th17 lymphocyte development [53] but also exerts mucosal protection during colitis [54]. STAT3 has been implicated in both protection and damage of intestinal inflammation depending on its cellular source. In particular, STAT3 produced in T-cells has shown damaging effects on the colonic mucosa [52] while STAT3 produced in epithelial cells is crucial for mucosal protection [54]. Whereas IL-6 stayed elevated after GPR55 inhibitor treatment in our experiments, most likely contributing to mucosal protection, pSTAT3 levels were decreased compared to vehicle treatment. Conversely, in TNBS colitis, levels of pSTAT3 increased while its upstream inducer, IL-6, decreased upon treatment with CID16020046, indicating that the IL-6/STAT3 response to the treatment with the GPR55 inhibitor depends on the experimental model employed. Although the responses are different, they finally result in the promotion of mucosal homeostasis. In addition, we have to consider that, by the end of the colitis experiments, mucosal repair mechanisms most likely have set in. It therefore seems that, depending on the

colitis model used, molecular mechanisms of mucosal restitution operate at different timepoints which could explain the different levels measured for pSTAT3 and IL-6.

Our leukocyte recruitment assays and the immunohistochemical data showed that CID16020046 inhibited influx of lymphocytes and macrophages in the DSS model. Also F4/80 and CD3 staining of colonic sections revealed that macrophage and T cell influx was reduced following blockade with CID16020046. These cell populations are elevated in the inflamed colon and known to drive intestinal inflammation [36,55,56]. The reduced influx of these cells following the treatment with CID16020046 may have played an important role in the alleviation of colitis. To explore a potential direct effect of CID16020046 on mononuclear cells, the mouse macrophage cell line J774A.1 was chosen to study whether migration and activation depended on GPR55. This cell line showed high levels of GPR55 transcripts compared to others (Fig. 5A). The GPR55 antagonist reduced migration to C5a in our Transwell migration assays and reduced MCP-1-induced expression of CD11b, a marker that is increased in the inflamed colonic mucosa [57] suggesting that the reduced influx of macrophages may be due to a direct effect of CID16020046 on macrophages. Our data are corroborated by a recent report demonstrating that GPR55 is highly expressed in human leukocytes and that proinflammatory activity was dependent on GPR55, attributing this receptor a possible role in leukocyte function during inflammation [9]. CID16020046 also inhibited migration of human neutrophils which confirms the finding by Balenga et al. that activation of GPR55 in human neutrophils induces migration [30]; however, the *in vitro* anti-migratory effect on neutrophils was not mirrored in our *in vivo* recruitment assay suggesting that other mediators, generated *in vivo*, may have interacted with GPR55 pathways altering the neutrophil migration. For instance, CB2 pathways have been shown to interfere with GPR55 signaling at the level of small GTPases in human neutrophils thus altering the cell's migratory behavior [30].

In summary, we elucidated the role of GPR55 in intestinal inflammation by pharmacologic and genetic interference. The data suggest that GPR55 may exert proinflammatory actions which can be antagonized by a novel GPR55 inhibitor, CID16020046, with no central activity. In particular, we found that the inhibitor protected against intestinal inflammation by interfering with lymphocyte and macrophage recruitment to the colon. The inhibitor thereby exerts direct effects on macrophages, reducing migration and CD11b activation. These findings are important with respect to the role of the endocannabinoid system in the inflamed gut, because GPR55, which can be activated by cannabinoids, displays functions distinct to other CB receptors in intestinal inflammation. GPR55 may be an important modulator of CB receptor-mediated actions during intestinal inflammation and could represent an interesting drug target. This may be helpful in the future treatment of human IBD.

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Key messages

The aims of this study were to investigate the unknown role of GPR55 in intestinal inflammation and to explore its mechanisms of action by using a specific GPR55 antagonist and GPR55^{-/-} knockout mice.

Colon tissue of mice subjected to different models of intestinal inflammation were biochemically and immunohistochemically investigated for severity of inflammation.

The GPR55 antagonist improved intestinal inflammation macroscopically, decreased levels of proinflammatory cytokines and reduced leukocyte recruitment to the colon. GPR55^{-/-} knockout mice were less susceptible to intestinal inflammation than their wild type littermates.

GPR55 is an atypical cannabinoid receptor likely involved in proinflammatory actions during intestinal inflammation which is important for future cannabinoid-based treatment of bowel inflammation.

DSS colitis - Macroscopic scores

TNBS colitis - Macroscopic scores

Myeloperoxidase (MPO) assays

Fig. 1. The GPR55 inhibitor CID16020046 improves macroscopic scores in DSS and TNBS colitis and reduces MPO activity

Macroscopic evaluation of DSS colitis (presented as score index) was performed using different doses of CID16020046 to determine an effective dosage (A). A dose of 20 mg kg⁻¹ was afterwards used for all *in vivo* experiments. Macroscopic scores for both colitis models are shown in B and E. For DSS colitis, colon lengths are also presented as subscores (C) but are also part of the score index. MPO activity was determined to evaluate the severity of inflammation in both colitis models (G, H). Representative hematoxylin staining of colon

sections from animals with DSS colitis (**D**) and TNBS colitis (**F**) revealed reduced damage of the mucosa and less submucosal infiltrate after treatment with GPR55 inhibitor. In (**D**), a colon section from a healthy animal is shown. Calibration bars: 50 μ m; (n=4-16, one-way ANOVA; Tukey's multiple comparison *p<0.05; ***p<0.001).

Expression of cytokines in DSS and TNBS colitis

Fig. 2. Treatment with GPR55 inhibitor CID16020046 reveals a reduction of proinflammatory cytokines levels

Images show levels of TNF-α (A, B), IL-1β (C, D) and IL-6 (E, F) in colon tissue of DSS and TNBS mice after treatment with the GPR55 inhibitor CID16020046. In the DSS model, TNF-α and IL-1β levels were significantly reduced in CID16020046-treated mice (A, C) while IL-6 levels stayed unaltered (E). In the TNBS model, IL-1β (D) and IL-6 (F) were significantly decreased, while TNF-α levels (B) were also lowered, however not

significantly, after treatment with CID16020046. (n=4-15, one-way ANOVA; Tukey's multiple comparison * $p<0.05$).

Expression of Cox-2 and pSTAT3 in DSS and TNBS colitis

Fig. 3. GPR55 inhibitor CID16020046 reduces Cox-2 levels but has opposing impact on pSTAT3 expression in DSS and TNBS colitis

Western blots of whole colon tissue demonstrated reduced Cox-2 expression after treatment of DSS (A) and TNBS colitis (B) with CID16020046 (CID). Representative blots of DSS and TNBS colitis show bands of Cox-2 in colon tissue from four animals (C). Band densities of samples were normalized to actin (veh, vehicle; n=6-12, Student's t-test; *p<0.05; ***p<0.001). pSTAT3 levels were reduced in the DSS (D) but increased in the TNBS colitis model treated with CID16020046 (E). Representative blots of DSS and TNBS colitis show

bands of pSTAT3 in colon tissue from four animals (**F**). Band densities of samples were normalized to total STAT3 (tSTAT3) (n=6, Student's t-test; *p<0.05; **p<0.01).

Fig. 4. Leukocyte recruitment into the colon in DSS colitis

CID16020046 inhibited lymphocyte and macrophage infiltration into the lamina propria of the colon in DSS-colitic mice, while the number of monocytes and eosinophils stayed unaltered. Neutrophils showed increased influx upon CID16020046 treatment (**A**) ($n=9$, Student's t -test; $*p<0.05$). Immunohistochemistry for CD3 to identify T lymphocytes and for F4/80 to identify macrophages is shown in the inflamed colon during DSS colitis (**B**). CD3 and F4/80 staining is reduced in the submucosa of GPR55 inhibitor-treated mice, as compared to the vehicle-treated mice (images are representative of sections from 3 different

mice from each group). Low (x10) and high magnification (x40) images of the colon are presented (*e*, epithelium; *s*, submucosa; *ml*, muscle layer). For cell counting, 6-10 sections from non-overlapping areas (from 3 different mice each group) were used. * $p < 0.05$; student's t-test.

Fig. 5. Effects of GPR55 inhibitor CID16020046 on the migration and activation of mouse J774A.1 macrophages, and on the migration of human neutrophils

PCR gels shows amplification of GPR55 transcripts in the mouse macrophage cell lines RAW264.7 and J774A.1 (A). GPR55 cDNA obtained from the manufacturer served as a positive control (#100-29101; BioRad). Transwell migration assays using J774A.1 cells and 5nM of C5a as a chemoattractant showed a reduction in migration after incubation with 1 and 5 μM of GPR55 inhibitor CID16020046 (n = 4; ANOVA; Tukey's post-hoc test) (B). Representative images from cells that have migrated to the lower side of the migration filter

are shown in (C); for visualization and counting, cell nuclei were stained with DAPI; size bar: 200 μm (C). CD11b expression was concentration-dependently reduced by CID16020046 in J774A.1 cells (D); data are from three independent experiments; ANOVA; Tukey's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (control=no treatment; vehicle=DMSO). Migration assays in human neutrophils, using 100 nM of fMLP as a chemoattractant, showed a potent decrease in the migration after treatment with 1 and 2.5 μM CID16020046 (vehicle=DMSO); $n = 3-5$; ANOVA; Tukey's post hoc test; *** $p < 0.001$ (E).

DSS colitis in GPR55 wild type and knock out mice

Fig. 6. GPR55^{-/-} knock out mice exhibit less severe inflammation than GPR55^{+/+} wild type mice during DSS colitis

Macroscopic evaluation (presented as score index) was performed on GPR55^{+/+} wild type (GPR55 WT) and GPR55^{-/-} knock out mice (GPR55 Ko) and compared with their respective controls (GPR55 wild type and knock out mice with no DSS treatment). GPR55 knock out mice are less susceptible to intestinal inflammation in the DSS model (A). The colon lengths are presented as subscores but are also part of the score index. Colon lengths of GPR55 wild type and knock out mice were both set at 100% (B). Myeloperoxidase

(MPO) activity was determined to evaluate the severity of inflammation. MPO values from GPR55 wild type and knock out mice were both set at 100% (C). Representative hematoxylin staining of colon sections from GPR55 wild type (WT) and knock out (Ko) showed slightly less damaged mucosa and less submucosal infiltrate (D). Calibration bar: 50 μ m; (n=5-9, one-way ANOVA; Tukey's multiple comparison *p<0.05; **p<0.01; ***p<0.001).

GPR55 inhibitor CID16020046 does not change locomotor and sickness behavior in healthy mice. Locomotor behavior of CID16020046 (20 mg/kg)- and vehicle (DMSO)-treated healthy mice was determined in an open field test as distance travelled within the plastic box (in meters; *m*) (E). Anxiety behavior was measured as time spent in the center of the box (in seconds; *sec*) (F). Body weight of healthy mice receiving CID 16020046- or vehicle (DMSO) was also measured daily and showed no change of weight (G). In addition, a rectal thermometer was used to daily monitor the body temperature of mice receiving GPR55 inhibitor or vehicle. No differences in body temperature were observed between these two groups (H).