Published in final edited form as:

Inflamm Bowel Dis. 2011 August ; 17(8): 1651–1664. doi:10.1002/ibd.21538.

The atypical cannabinoid O-1602 protects against experimental colitis and inhibits neutrophil recruitment

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Abstract

Background—Cannabinoids are known to reduce intestinal inflammation. Atypical cannabinoids produce pharmacological effects via unidentified targets. We were interested whether the atypical cannabinoid O-1602, reportedly an agonist of the putative cannabinoid receptor GPR55, reduces disease severity of dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS)-induced colitis in C57BL/6N and CD1 mice.

Methods—DSS (2.5% and 4%) was supplied in drinking water for one week while TNBS (4 mg) was applied as a single intrarectal bolus.

Results—Both treatments caused severe colitis. Injection of O-1602 (5 mg/kg i.p.) significantly reduced macroscopic and histological colitis scores, and myeloperoxidase activity. The protective effect was still present in cannabinoid receptor 1 (CB₁) and 2 (CB₂) double knockout mice and mice lacking the GPR55 gene. To investigate a potential mechanism underlying the protection by O-1602 we performed neutrophil chemotactic assays. O-1602 concentration-dependently inhibited migration of murine neutrophils to keratinocyte-derived chemokine (KC), N-formyl-methionyl-leucyl-phenylalanine (fMLP) and the N-formyl-peptide receptor ligand WKYMVm. The

inhibitory effect of O-1602 was preserved in neutrophils from CB₁/CB₂ double knockout and GPR55 knockout mice. No differences were seen in locomotor activity between O-1602-treated and control mice indicating lack of central sedation by this compound.

Conclusions—Our data demonstrate that O-1602 is protective against experimentally induced colitis and inhibits neutrophil recruitment independently of CB₁, CB₂ and GPR55 receptors. Thus, atypical cannabinoids represent a novel class of therapeutics that may be useful for the treatment of inflammatory bowel diseases.

Keywords

atypical cannabinoids; colitis; inflammatory bowel disease

Introduction

Over the last decade, there has been a growing interest in the use of cannabinoids for the treatment of inflammatory processes and pain.^{1,2} Cannabinoids include plant-derived phytocannabinoids, synthetic cannabinoids and endocannabinoids,³ and exert their actions via the two G-protein coupled cannabinoid (CB) receptors, CB₁ and CB₂, which are part of the endocannabinoid system (ECS). The ECS comprises the components of endogenous cannabinoid metabolism, i.e. the enzymatic machinery for the production and degradation of endogenous cannabinoids, as well as the ligands (endocannabinoids) and the CB receptors. However, certain compounds that share structural similarities to cannabinoids are known as atypical cannabinoids and produce effects via non-CB₁/CB₂ targets.^{4,5} These targets may be novel CB receptors, such as the so-called abnormal cannabidiol (abn-CBD) receptor.^{6–8} Because of their overlapping pharmacology, these receptors can be regarded as part of an expanded ECS although their full biological roles remain to be elucidated.⁹ Abnormal cannabidiol (a synthetic analogue of the phytocannabinoid cannabidiol) and its derivative O-1602 represent two such atypical cannabinoids that lack significant binding affinity to CB₁ or CB₂,^{4,5} and cause non-CB₁/CB₂-mediated vasodilatation *in vitro*^{4,10} while also reducing mean arterial blood pressure *in vivo*.¹⁰

It has become increasingly evident that components of the ECS may play an important role in the regulation of gut inflammation, e.g. in inflammatory bowel diseases (IBD).^{11–13} Thus, agonism of CB receptors or blockade of fatty acid amide hydrolase (FAAH), the degrading enzyme of the endocannabinoid anandamide, provide significant protection against experimentally induced colitis in mice,^{14–17} whereas CB₁ receptor antagonism or absence of the CB₁ receptor gene worsens colitis.¹⁵ Endocannabinoid levels and expression of CB₁^{14,15,18} and CB₂ receptors^{13,14,17} increase in response to GI inflammation, a regulatory mechanism which is thought to be beneficial as it ameliorates colitis pathology.¹⁹ Pharmacological intervention of the ECS with exogenous cannabinoids has considerable potential, therefore, for the treatment of intestinal inflammation. However, their therapeutic use is significantly limited by unwanted psychotropic side effects.²⁰ On the other hand, certain phytocannabinoids, such as cannabidiol and Δ⁹-tetrahydrocannabivarin,²¹ and synthetically-derived abnormal cannabidiol,⁴ seem to lack these psychotropic effects, a feature that would give them a substantial advantage over CB₁ receptor agonists in the treatment of IBD. Cannabidiol, which has minimal affinity to CB₁ and CB₂,²² has already been proven protective in a model of trinitrobenzene sulfonic acid (TNBS)-induced colitis.²³

The aim of this study was to test the atypical cannabinoid O-1602 in two models of murine experimental colitis and to investigate whether it conferred a protective effect and, if so, a potential mechanism for its protection. O-1602 is of interest because it binds with high

selectivity to the new putative cannabinoid receptor GPR55,⁵ whose activation may account for some of the non-CB₁/CB₂ effects of cannabinoid ligands.^{10,24,25} In a recent study, O-1602 was shown to inhibit the migration of human neutrophils.²⁶ As accumulation and increased activity of neutrophils are hallmarks of colonic inflammation,^{27,28} we reasoned that reduction of neutrophil recruitment by O-1602 may be part of a potential protective effect in murine colitis and therefore also investigated the chemotactic response of murine neutrophils to O-1602.

Materials and methods

C57BL/6 (males, 5–9 weeks old, 20–26g) and CD1 mice (males, 3–5 weeks old, 20–24g) were purchased from Charles River (Saint-Constant, Quebec, Canada) and kept in house for 2 weeks prior to experiments. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12-h light–dark cycle with free access to standard laboratory chow and tap water. Cannabinoid receptor double knockouts were imported from the University of Bonn, Germany, and bred in our own facility at the University of Calgary. To generate CB₁^{−/−}/CB₂^{−/−} mice, breeding pairs of CB₁^{−/−} and CB₂^{−/−} single knockout mice, backcrossed to C57BL/6 mice for 6 generations, were used. Only homozygous animals deficient in the two cannabinoid receptor genes (CB₁^{−/−}/CB₂^{−/−}) were included in the study.^{4,29} GPR55 knockout (GPR55^{−/−}) mice were acquired from the Texas Institute of Genomic Medicine (TIGM, Houston, TX) and bred at the animal facilities of the Department of Psychological and Brain Sciences, Indiana University, Bloomington, USA where all experiments with the GPR55^{−/−} mice were conducted.³⁰ Animals were matched by age and body weight. Experimental procedures were approved by the University of Calgary Animal Care Committee (protocol number M07102) and the Animal Care and Use Committee of Indiana University, Bloomington (protocol number 09–044) and performed in accordance with international guidelines.

Induction of DSS and TNBS colitis

Colitis was induced in CD1 mice by adding 4% (wt/vol) of dextran sulfate sodium (DSS) to the drinking water (tap water) while control animals received tap water only. This dose of DSS produced a high reproducibility of colonic inflammation in preliminary experiments. For C57BL/6, CB₁^{−/−}/CB₂^{−/−} and GPR55^{−/−} mice (C57BL/6 background) a lower dose of DSS (2.5% wt/vol) was used which had previously proved to be optimal in terms of mortality and reproducibility in this mouse strain. All mice were kept on DSS over a 7-day period. Body weights were measured daily. DSS-containing drinking water was also monitored daily to ensure consumption of DSS. TNBS colitis was induced in male C57BL/6 mice as described before.¹⁷ In brief, animals were lightly anesthetized with isoflurane and TNBS (4 mg in 100 µL of 30% ethanol) was infused into the colon through a catheter (outside diameter 1 mm) inserted 3 cm proximally to the anus in mice. Solvent alone (100 µL of 30% ethanol) was administered in control experiments. The dose of TNBS was found to induce reproducible colitis with mortality rates in the published range (0%–25%).

Drugs and pharmacological treatments

Dextran sulfate sodium salt (DSS; reagent grade; 36–50,000 Da) and trinitrobenzene sulfonic acid (TNBS) were purchased from MP Biomedicals (Solon, Ohio, USA) and Sigma-Aldrich (Oakville, Ontario, Canada), respectively. O-1602 (5-Methyl-4-[(1R, 6R)-3-methyl-6-(1-cyclohexen-1-yl)-1, 3-benzenediol], dissolved in methyl acetate, and WIN 55,212-2 were obtained from Tocris Bioscience (Bristol, UK). Because of its toxicity, methyl acetate was evaporated prior to the *in vivo* experiments and ethanol was used instead as a solvent. O-1602 was then further diluted in Tween 80 and sterile saline. Vehicle consisted of ethanol, Tween 80 and sterile saline (1:1:8). O-1602 (or vehicle) was injected

intraperitoneally (i.p.) 30 min prior to onset of DSS or TNBS treatment at a dose of 5 mg/kg and given once daily or twice daily for 6 days. For the chemotactic assays, a stock solution of 10 mM O-1602 in dimethyl sulfoxide (DMSO, 99.7%) was prepared. For the locomotor assay experiments, WIN 55,212-2 (1 mg/kg) and O-1602 (5 mg/kg) were dissolved in 2% DMSO, 4% Tween 80 and sterile saline. All assays were also performed with the respective vehicles.

Macroscopic scoring and damage assessment

At the end of the DSS and TNBS colitis experiments, mice were killed by cervical dislocation. The colon was immediately removed, rinsed gently with saline solution, opened longitudinally along the mesenteric border and examined. In DSS colitis, colonic damage was assessed 7 days after start of DSS exposure according to a scoring system by Kimball et al,³¹ adapted for the present study. Loss of colon weight (colon weight included fecal content as previously described³¹), 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 5 ulcers). The score index represents the sum of all subscores and had a maximum of 16. In TNBS colitis, colonic damage was assessed by a semiquantitative scoring system 3 days after administration of TNBS and also adapted for mice in the present study.³² Macroscopic 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 of 6.99 ± 0.28 ; n=6); wall thickness measured in mm. The presence of hemorrhage, fecal blood, or diarrhea increased the score by 1 point for each additional feature.

Histology

Following macroscopic scoring, segments of the distal colon were stapled flat onto cardboard with the mucosal side up and fixed for 24 h in 10% neutral-buffered formalin. Tissue was then dehydrated, embedded in paraffin and standard hematoxylin/eosin staining was performed on 5 μ m thick sections. Five sections at least 50 μ m apart per colon were evaluated in a blinded manner and scored according to Kimball et al.³¹ Epithelial damage, cellular infiltration and muscle thickness were each subscored with 0–3 points where 0 depicts normal colon and 3 the maximally affected colon. The total score index for one colon was the sum of these three subscores and had a maximum of 9.

Determination of tissue myeloperoxidase activity

Myeloperoxidase (MPO) activity represents an index of neutrophil accumulation in the tissue.³³ Samples of colon were weighed, immediately frozen on dry ice, and stored at -80°C prior to further processing. For determination of MPO activity, tissue was placed in 0.5 % HTAB buffer (50 mg of tissue/mL; pH 6.0) and disrupted with a Polytron homogenizer (Brinkman Instruments, Mississauga, Ontario, Canada). HTAB (hexadecyltrimethyl-ammonium-bromide; Sigma-Aldrich, Oakville, Ontario, Canada) is a detergent that releases MPO from the primary granules of neutrophils and enhances enzyme activity through the presence of bromide. Afterwards, the homogenate was centrifuged for 15 min at maximum speed and 4°C . Before reading MPO activity, 7 μ L of supernatant was added to 200 μ L of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/mL of O-dianisidine hydrochloride and 0.5 μ L of 1% H_2O_2 /mL. The kinetics of MPO activity were measured at 460 nm (Thermo Fischer LabSystems Multiskan, Thermo Scientific, Ottawa, Ontario, Canada). A mean was calculated for the DSS+vehicle-treated group and set at

100%. Values of all other treatment groups are expressed as % of the DSS+vehicle-treated group.

Isolation of neutrophils and chemotactic assays

Neutrophils were collected from CD1 and CB1^{-/-}/CB2^{-/-} mice as previously described.³⁴ Briefly, bone marrow cells were flushed from the femurs and tibiae with a 5-mL syringe into 1x Hank's buffered salt solution (+ 1% fetal bovine serum; FBS), spun down, resuspended in 52% Percoll and added to a 72% and 64% Percoll gradient. After centrifugation at 1100g for 30 min, the neutrophils were collected from the Percoll, washed and counted. Neutrophils were then suspended in culture medium (RPMI, 10% FBS, 1% Pen/Strep) and preincubated for 30 min in 1, 10, 50 nM O-1602, and in RPMI without O-1602 to determine baseline migration. A suspension of 1×10^5 cells in 200 μ L RPMI was placed in the upper well of a 6.5 mm insert 24-well Transwell chemotaxis chamber (Corning Life Sciences, Lowell, MA, USA) with a 8- μ m polycarbonate membrane and 600 μ L of 10 nM of the synthetic neutrophil chemoattractant WKYMVM (Trp-Lys-Tyr-Met-Val-D-Met-NH₂; Phoenix Pharmaceuticals Inc. Burlingame, CA, USA) or keratinocyte-derived chemokine (KC; R&D Systems, Minneapolis, MN, USA) in the bottom wells of the plate. Plates were incubated at 37°C in a humidified incubator for 3 hrs. Cells that migrated to the lower chamber were counted in a hemocytometer in a blinded fashion.

Whole blood from wildtype control or GPR55^{-/-} mice was withdrawn by intracardial puncture, collected in BD Vacutainers containing lithium heparin and layered over 5 mL of Polymorphprep (Axis-Shield, Norway) in 12-mL centrifuge tubes. The filled tubes were centrifuged at 550g for 35 min at 20°C, and the PMN layer was removed with a fine-tipped Pasteur pipette. To remove the residual Polymorphprep, the cells were suspended in a universal container with 20 mL of sterile serum-free DMEM medium at 37°C and centrifuged at 450g for 10 min at 20°C. The supernatant fluid was discarded, and the pellet was resuspended with 10 mL of PBS (containing CaCl₂ and MgCl₂) and 10 mL of 4°C distilled H₂O for further washing. The fluid was centrifuged again at 450g for 10 min at 20°C. The supernatant fluid was discarded, and the pellet resuspended with 210 μ L of serum-free DMEM. An estimation of the cell concentration was determined using 0.4% trypan blue solution and a hemocytometer. An appropriate amount of serum-free DMEM was used to resuspend the PMNs at a concentration of 1×10^6 cell mL⁻¹. *In vitro* cell migration assays were performed using a modified 96-well Boyden Chamber and PVP-free polycarbonate filters with 10 μ m diameter pores (Neuroprobe Inc., USA) coated with 25 μ g mL⁻¹ poly-D-lysine. The upper wells of the Boyden chamber were filled with 50 μ L of suspension of 1×10^6 cells mL⁻¹ in serum-free DMEM, before incubation with a 5% CO₂ atmosphere at 37°C for 3 h. 10 nM fMLP (Sigma, St. Louis, MO, USA) acted as positive controls. Following incubation, non-migrated cells were then removed before fixation and staining with Diff-Quik® stain set. Finally, the filter was sectioned and mounted onto microscope slides and the migrated cells counted in ten non-overlapping fields ($\times 40$ magnification) with a light microscope. Cells were pre-incubated with O-1602 (1, 10, and 50 nM) for 30 min at 37°C in a water bath before loading into the upper wells - the lower wells contained the equivalent concentrations of O-1602 and 10 nM of fMLP. This arrangement ensures that the only concentration gradient present is that generated by fMLP diffusing through the pores in the filter.

Reverse transcriptase (RT) and quantitative PCR

To determine mRNA expression of GPR55 in the colon of C57BL/6 mice, total RNA was extracted from the longitudinal muscle-myenteric plexus (LMMP) and the mucosal layer. Tissues were homogenized with a Polytron homogenizer (Kinematica) and RNA was isolated using QIAGEN RNeasy Plus Mini Kit (Qiagen), followed by DNase treatment for

15 min at room temperature (1U DNase I/ μ g RNA, Invitrogen, Karlsruhe, Germany). Total RNA was reverse transcribed in complementary DNA (cDNA) using random hexamere primers, essentially as described before.³⁵ Thirty five rounds of PCR amplification were carried out with intron-spanning primers GPR55-forward 5'-GGACTCATTGGTACTCCTAAGCTGT-3' and GPR55-reverse 5'-GCAGATCCCAAAGGTCTTCCT-3', 2.5 U Taq Polymerase (Sigma, Deisenhofen, Germany), 1 μ L of the RT-reaction mixture under the following conditions: "hot start" with an initial denaturation at 95°C for 3 min and amplification cycles consisting of denaturation at 94°C for 20 sec, annealing at 60°C for 20 sec and extension at 72°C for 45 sec. The last cycle was followed by an extension step at 72°C that lasted 7 min. As negative controls, we used isolated RNA amplified without reverse transcriptase or random hexamer primers. Amplification products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Bands were excised from the gel, purified by a Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into TOPO PCRII vector (Invitrogen). Nucleotide sequences were deduced by cycle sequencing of the isolated plasmids (Qiagen, QIAprep spin miniprep kit) with T7 sequencing primer (GATC, Konstanz, Germany). Sequences were analyzed by BLASTn homology search.

For quantitative RT-PCR, full thickness samples were taken from the colon and immediately stored in RNeasy lysis buffer at -20°C (RNA Stabilization Reagent; Qiagen, Mississauga, ON, Canada). Total RNA was then extracted using QIAzol Lysis Reagent (Qiagen) and determined by UV-spectrophotometry at 260 nm, as previously described.¹⁷ cDNA was generated from 1000ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. In some experiments, reverse transcriptase was withheld from the mix reaction to determine if there was any contamination with genomic DNA. TaqMan Gene Expression assay kits for tumor necrosis factor alpha (TNF- α) (Mm00443258_m1) and KC/chemokine (C-X-C motif) ligand 1 (Mm00433859_m1) were purchased from Applied Biosystems for this study. The rodent GAPDH probe (VIC) from Applied Biosystems was used as an endogenous control. Triplicate samples of 20 ng of each cDNA were amplified by real-time PCR in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The cycle parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The results were collected and analyzed using RQ Manager 1.2 (Applied Biosystems). A control sample from mice exposed to tap water was selected as calibrator and expression of TNF- α and KC/C-X-C motif ligand 1 was equaled to 1 for that sample. The relative quantification value (RQ) was calculated from each sample and compared to the control sample. mRNA expression was presented as the mean $\pm$ SEM of RQ values per treatment group.

Locomotor activity test

Ambulatory locomotor activity was measured 30 min after i.p. injection of O-1602 (5 mg/kg), WIN 55,212-2 (1 mg/kg) or vehicle using an infrared beam activity monitor (Columbus Instruments, Columbus, OH, USA). Mice were pre-exposed to the recording equipment in the morning and the experiments were done in the afternoon of the same day. Each individual mouse was placed in the apparatus and the ambulatory count was recorded over a 10 min period. Movement of the mice was recorded as the ambulatory activity count when the infrared beams were sequentially broken.

Statistical analysis

From every experimental group, a mean was calculated and differences of means between treatment (DSS+vehicle, DSS+O-1602) and control groups (tap water+vehicle) were analyzed either by Student's t-test or One-way ANOVA followed by Tukey's posthoc test

using GraphPad Prism (GraphPad Software, San Diego, CA). *P* values <0.05 were considered significant.

Results

O-1602 improves pathology of DSS colitis in C57BL/6 and CD1 mice (Figs.1–3)

In pilot studies, we initially determined the optimal dose for O-1602 by injecting 1, 3 and 5 mg/kg O-1602 i.p. once daily to separate groups of DSS-exposed C57BL/6 mice and evaluating macroscopic damage and myeloperoxidase (MPO) levels (Fig. 1). DSS-treated C57BL/6 mice started to lose weight on day 4–5 and lost about 9% of their body weight by day 7. No weight loss was seen in the control groups which had only access to tap water. A dose of 3 and 5 mg/kg O-1602 led to a significant decrease in macroscopic damage score while only 5 mg/kg of O-1602 significantly lowered MPO levels (Fig. 1B). The body weight curve of O-1602-treated mice did not differ from that of vehicle-treated mice, showing a drop in weight between day 4–5 and a total weight loss of 10.4% ($n=6$; n.s.; O-1602- vs vehicle-treated mice; One-way ANOVA). In accordance with a previous study,³⁶ a dose of 5 mg/kg of O-1602 was used for all following experiments. To investigate whether O-1602 efficacy was strain-specific, we chose CD1 mice and exposed them to DSS. CD1 mice that received DSS with their drinking water started to lose weight after 4 days and lost about 12% of body weight by day 7. After exposure to DSS they showed prominent signs of a strong colitis with increased macro- and microscopic damage scores (Figs. 2A and B), increased MPO levels (Fig. 2E), decreased colon weight (Fig. 2C) and a decrease in colon length (Fig. 2D). Treatment of DSS-exposed mice with O-1602 (5 mg/kg, twice daily) had no effect on the body weight ($n=5-12$; n.s.; O-1602- vs vehicle-treated mice; One-way ANOVA). However, colitis parameters in the DSS group showed significant improvement after O-1602 treatment with the twice daily treatment being slightly superior to the once daily treatment. The macroscopic and microscopic colitis scores were significantly lower in the DSS+O-1602 than in the DSS+vehicle group (Fig. 2A and B). Colon weight almost regained control values (Fig. 2C) and shrinkage of the colon was markedly improved (Fig. 2D). In histological sections of DSS+O-1602-treated mice, the colonic epithelium displayed partially preserved crypt architecture and the submucosa was less densely packed with infiltrated cells (Fig. 3A). Both daily and twice daily treatment significantly reduced MPO activity in O-1602-treated DSS-colitic mice (Fig. 2E). DSS exposure also led to a strong rise in keratinocyte-derived chemokine (KC) and tumor necrosis factor alpha (TNF- α) levels (Figs. 2F and G) which remained unaffected by twice daily O-1602 treatment. There was no effect of O-1602 alone (5 mg/kg once daily) on macroscopic scores and MPO activity in control mice (tap water only; data not shown).

The protective effect of O-1602 is retained in $CB_1^{-/-}/CB_2^{-/-}$ mice (Fig. 4)

O-1602 was next tested in $CB_1^{-/-}/CB_2^{-/-}$ mice to investigate whether CB_1 and CB_2 could be involved in the protection against DSS colitis. $CB_1^{-/-}/CB_2^{-/-}$ mice started to lose weight 3 days after the beginning of DSS treatment. At the end of the DSS period, these mice lost 7.8% of their body weight. Since twice daily treatment with O-1602 produced a slightly increased improvement of colitis in DSS-exposed CD1 mice over once daily treatment, we used the twice daily treatment for the $CB_1^{-/-}/CB_2^{-/-}$ mice and in all following experiments. Body weights of DSS-exposed $CB_1^{-/-}/CB_2^{-/-}$ mice treated with O-1602 did not differ from those of DSS+vehicle-treated $CB_1^{-/-}/CB_2^{-/-}$ mice (8.6% vs. 7.8%, resp., $n=5-6$; n.s. One-way ANOVA). However, DSS+O-1602-treated $CB_1^{-/-}/CB_2^{-/-}$ mice showed a large reduction in macroscopic and microscopic damage scores (Figs. 4A and B) and a significantly lower MPO activity as compared to DSS+vehicle treated $CB_1^{-/-}/CB_2^{-/-}$ mice (Figs. 4D). Shortening of colon length was also significantly less prominent in DSS

+O-1602-treated $CB_1^{-/-}/CB_2^{-/-}$ mice (Fig. 4C). Histological sections displayed areas with preserved epithelium and reduced cell infiltration into the colon submucosa (Fig. 3B).

Protection against colitis by O-1602 is also retained in $GPR55^{-/-}$ mice (Fig. 5)

Because O-1602 is a known agonist of the GPR55 receptor,⁵ we wanted to test whether this receptor played a role in the protection by O-1602 against DSS colitis. In figure 5E, RT-PCR of GPR55 transcripts in the mucosa and the muscle layer of the colon from wild type mice is shown. As seen in the macroscopic score index, DSS treatment only induced a mild colitis in $GPR55^{-/-}$ mice (Fig. 5A). The macroscopic score index was slightly lower in the $GPR55^{-/-}$ mice (6.3 ± 0.5) than in age-matched C57BL/6 wild type mice (8.8 ± 0.5 ; $p < 0.05$; Student's t-test; $n = 7$). DSS-vehicle-treated $GPR55^{-/-}$ mice lost around 6% of their body weight while O-1602-treated DSS-exposed mice lost 3.8% of their weight ($n = 9$; n.s. One-way ANOVA). After O-1602 treatment (5 mg/kg twice daily), macroscopic and microscopic damage scores (Figs. 5A and B), and MPO activity (Fig. 5D) were decreased while colon length was increased (Fig. 5C) indicating reduced inflammation. Compared with DSS + vehicle mice, reduced infiltration of inflammatory cells was also seen in histological sections of colons from DSS + O-1602-treated mice (Fig. 3C). Thus, O-1602 is effective in ameliorating colitis even in mice lacking GPR55.

O-1602 improves TNBS colitis in C57BL/6 mice (Fig. 6)

To investigate whether O-1602 would be effective in a different model of colitis we administered TNBS to a group of C57BL/6 mice and evaluated the development of colitis after 3 days. TNBS + vehicle-treated mice lost 16.4% of body weight while TNBS + O-1602-treated mice (5 mg/kg twice daily) lost 10.8% ($n = 6$; $p < 0.05$; One-way ANOVA). TNBS-colitic mice showed extensive macro- and microscopic mucosal damage (Fig. 6A and B), elevation of MPO activity, increase in TNF- α levels (Figs. 6C and E), and colon shrinkage (Fig. 6D). As in the DSS model, mice treated with twice daily O-1602 had significantly less macro- and microscopic damage, a decrease in MPO levels, and diminution of colon shrinkage. TNF- α levels also tended to decrease after twice daily O-1602 treatment, although this was not statistically significant (Fig. 6E).

O-1602 inhibits neutrophil migration (Fig. 7)

To define a mechanism for the protective effect of O-1602 we performed chemotactic assays with mouse neutrophils using N-formyl-methionyl-leucyl-phenylalanine (fMLP), N-formyl-peptide receptor ligand WKYMVM and keratinocyte-derived chemokine (KC; CXCL1), the mouse orthologue of human interleukin-8,³⁷ as chemoattractants. In all chemoattractants, O-1602 (1, 10, 50 nM) concentration-dependently not only inhibited migration of neutrophils from control mice (CD1 and C57BL/6), but also from mice lacking CB_1/CB_2 (Figs. 7A and B) or GPR55 receptors (Fig. 7C), indicating O-1602 was mediating this effect via targets other than CB_1 , CB_2 or GPR55. There were no significant differences between CD1 or wild type mice and knockout mice in the inhibition of neutrophil migration by O-1602 except between CD1 and $CB_1^{-/-}/CB_2^{-/-}$ mice at 50 nM O-1602 with KC as a chemoattractant ($n = 4-9$; $p < 0.05$; One-way ANOVA). O-1602 alone (10 and 50 nM) had no effect on neutrophil migration (data not shown).

O-1602 does not alter locomotor activity

To test whether O-1602 exhibits centrally-mediated behavioural activity we submitted CD1 mice to a locomotor test. A dose of 5 mg/kg O-1602 (i.p.) did not alter locomotor activity in O-1602-injected mice as compared to mice that received vehicle only. In contrast, i.p. injection of the CB_1 and CB_2 agonist WIN 55,212-2 (1 mg/kg) significantly reduced locomotor activity ($n = 6-9$; One-way ANOVA, Tukey's multiple comparison).

Discussion

Major advances have been made in the therapy of IBD,^{38,39} nevertheless there are many limitations with currently established medications largely due to side-effects and high costs. In the search for alternative treatment options, cannabinoids have come to be considered, because some IBD patients take marijuana and anecdotal reports suggest it confers a degree of therapeutic benefit. Animal studies with CB₁ and CB₂ receptor ligands and endocannabinoid degradation blockers have shown improvement in the pathology of experimental colitis,¹¹ supporting the anecdotal human evidence. Unfortunately, central effects that occur with CB₁ receptor active drugs limit their usefulness or translation into humans. Thus, cannabinoid compounds that bypass central CB₁ receptor pathways are of growing interest for the development of alternative drugs. Given this line of reasoning, phytocannabinoids or synthetic cannabinoids that are known to be devoid of psychotropic actions, such as the neurobehaviorally inactive abnormal cannabidiol (abn-CBD),⁴ are of substantial interest. However, the effect of abn-CBD in experimental colitis has not yet been evaluated. In the present study, we have therefore tested an analogue of abn-CBD, O-1602 (Fig. 8), in models of DSS and TNBS colitis. Like abn-CBD, it has no binding affinity to CB₁ and CB₂ receptors,^{4,5} and did not change locomotor behavior in our experiments.

We demonstrated that O-1602 has anti-inflammatory properties and ameliorates the extent of colitis. Macroscopic scores, histological scores and MPO activity improved significantly after daily or twice daily treatment with O-1602. Twice daily treatment was slightly more efficacious than the single treatment as illustrated by the decrease in the macroscopic score index. Experiments were also performed in CB₁^{-/-}/CB₂^{-/-} mice to investigate a possible involvement of CB receptors in the effects of O-1602. Double knockouts were used because both CB receptors have been shown to contribute to the protection against experimental colitis.^{15,17} The protection of O-1602 against DSS colitis was maintained in the double knockouts indicating that CB₁ and CB₂ receptor activity was not mediating these effects. Since O-1602 is also known to bind and activate GPR55, a novel putative cannabinoid receptor,^{5,24,25} and since we found GPR55 mRNA in colon tissue, we performed experiments in GPR55^{-/-} mice. In GTPγS binding assays of hGPR55 transfected HEK293s cells, O-1602 was a potent agonist, with an EC₅₀ of 13 nM.⁵ GPR55 shares little sequence identity with the classical CB receptors,^{5,40} and couples to a G_{α12/13} G protein and not to G_{i/o}, as do CB₁ and CB₂.^{5,41} Its signaling pathways are therefore distinct from the downstream signalling of CB₁ and CB₂.⁴² So far, not much is known about the *in vivo* role of GPR55 although one study in GPR55^{-/-} mice suggests that it might play a role in mechanical hyperalgesia.⁴³ Like in the CB₁^{-/-}/CB₂^{-/-} mice, the protective effect of O-1602 was retained in GPR55^{-/-} mice indicating the involvement of an unknown, novel atypical cannabinoid receptor in protection against colitis. These observations are similar to a study by Johns et al. in which O-1602 was investigated for its vasodilator actions in GPR55^{-/-} mice.¹⁰ O-1602 was originally found to cause non-CB₁/CB₂-mediated vasodilatation in rat mesenteric vascular bed, an effect that was diminished after endothelial denudation,⁴ and it was suggested that GPR55 may be the potential endothelial target. Vasodilation, however, was still present in GPR55^{-/-} mice.¹⁰

To investigate a mechanism of action of O-1602, we performed migration assays with neutrophils. Neutrophil accumulation, activation and release of neutrophil granule proteins are crucial pathological features in IBD,^{44–47} and they significantly contribute to the development and progression of the disease.^{27,28} Recently, McHugh et al. reported that O-1602 inhibits human neutrophil migration via a non-CB₁/CB₂ receptor.²⁶ Since our experiments showed that MPO activity in the colon (and therefore the presence of neutrophils,³³) decreased in DSS and TNBS colitis after O-1602 treatment, we examined whether O-1602 might affect neutrophil migration. Although there are reports demonstrating

that neutrophil depletion with neutralizing antibodies may worsen DSS colitis,⁴⁸ others observed an improved protection against DSS colitis after depletion of neutrophils,⁴⁹ and after pharmacological interference of neutrophil recruitment and activity of neutrophil granule proteins.^{50–52} Chemotactic assays with the bacterial peptide fMLP (or the N-formyl-peptide receptor ligand WKYMVm) and KC (CXCL1), which was found upregulated in our experiments during DSS colitis, revealed that O-1602 concentration-dependently inhibited chemotaxis of neutrophils in wild type and CB₁^{-/-}/CB₂^{-/-} mice confirming the findings of McHugh et al and illustrating the involvement of a non-CB₁/non-CB₂ target for O-1602. We also performed chemotactic assays with neutrophils isolated from GPR55^{-/-} mice. GPR55 has been previously shown to activate small GTPases, such as rhoA, cdc42 and rac1,⁵ and to facilitate calcium release from internal stores,⁴² implicating a role in cytoskeleton arrangement and cell locomotion. In accordance with the preserved protection against DSS colitis in GPR55^{-/-} mice, the dose-response dependent inhibition of neutrophil chemotaxis by O-1602 was not abolished in GPR55^{-/-} mice either, refining the novel cannabinoid receptor hypothesis such that a non-CB₁/non-CB₂/non-GPR55 target transduces the protective signaling effects of O-1602 against colitis. In this regard, a new study from McHugh et al demonstrates that N-arachidonoyl glycine (NAGly), abn-CBD and O-1602 potently drive cellular migration in microglial cells via GPR18 receptors, and provides multiple lines of evidence that GPR18 is the unidentified ‘abn-CBD’ receptor.⁵³ Finally, we did not see a significant influence of O-1602 on TNF-α and KC levels in the DSS mice indicating that the protective effect of O-1602 did not involve these two cytokines. Some studies suggest that TNF-α not only functions as a proinflammatory cytokine but may also have anti-inflammatory effects, in particular during acute DSS colitis.^{54–56} In light of these findings, it is possible that the unchanged level of TNF-α following O-1602 treatment in DSS mice indicates an involvement of TNF-α in the protection against colitis.

In summary, we have demonstrated that the atypical cannabinoid O-1602 offers significant protection against experimental colitis and shows no change in locomotor behaviour, implying that it does not have central effects at the doses used in this study. Its protective effects involve inhibition of neutrophil recruitment independent of CB₁, CB₂ and GPR55 receptors, indicating that another target is responsible for the protection against colitis. O-1602 represents a new class of non-CB₁/non-CB₂ cannabinoid drugs with anti-inflammatory actions in the colon and with no central sedative activity and therefore warrants further investigation and development.

Acknowledgments

We thank Winnie Ho for performing the genotyping of the CB₁ and CB₂ receptor gene-deficient mouse colony and Scott Hastings for excellent technical assistance with the GPR55^{-/-} mice. This work was supported by a grant from the Crohn’s and Colitis Foundation of Canada (to MS and KAS), the Canadian Institutes of Health Research (to KAS) and by the NIH (DA021285 and DA021696, to KM). Keith Sharkey is an Alberta Heritage Foundation for Medical Research (AHFMR) Medical Scientist and holds the Crohn’s and Colitis Foundation of Canada Chair in IBD Research at the University of Calgary. Donna-Marie McCafferty is an AHFMR Senior Scholar.

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Effect of O-1602 on DSS-induced colitis in C57BL/6 mice

Figure 1.

In pilot experiments, 1, 3 and 5 mg/kg O-1602 were given once daily for 6 days (i.p.) to C57BL/6 mice suffering from dextran sulfate sodium (DSS)-induced colitis to evaluate an effective dose for O-1602. **(A)** Macroscopic scoring revealed significant decreases in tissue damage for 3 and 5 mg/kg O-1602 while a decrease in **(B)** myeloperoxidase (MPO) activity was significant for 5 mg only. A dose of 5 mg/kg was subsequently used in all experiments (Students t-test; n=5–7).

Effect of O-1602 on DSS-induced colitis in CD1 mice

Figure 2.

The figure shows the main parameters used for evaluation of dextran sulfate sodium (DSS)-induced colitis. (A) macroscopic score, (B) microscopic score, (C) colon weight, (D) colon length, (E) myeloperoxidase (MPO) activity. O-1602 at 5 mg/kg (once or twice daily) improved parameters of colitis in CD1 mice but did not significantly effect (F) keratinocyte-derived chemokine (KC) and (G) tumor necrosis factor alpha (TNF- α) mRNA levels. Colon weight and colon length are subscored to show the dose-dependent changes but are also part of the macroscopic score index (One-Way ANOVA; Tukey's multiple comparison; n=5–12).

Figure 3.

Hematoxylin/eosin staining of colon wall sections from mice exposed to dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS) and treated with 5 mg/kg O-1602 twice daily. DSS-colitis and O-1602 treatment in (A) CD1 mice, (B) Cannabinoid receptor 1 and 2 (CB₁/CB₂) double knockouts and (C) GPR55 knockouts. (D) TNBS colitis and O-1602 treatment in C57BL/6 mice. Note the destruction of the mucosal architecture and the massive infiltration of immunocytes in all DSS+vehicle- and TNBS+vehicle-treated mice. Mucosal damage, cell infiltration and colon thickness decreased noticeably in the O-1602-treated groups. Calibration bars = 100 μ m.

Effect of O-1602 on DSS colitis in CB₁/CB₂ double knockout mice **Figure 4.**

Parameters used for evaluation of dextran sulfate sodium (DSS)-induced colitis in cannabinoid receptor 1 and 2 (CB₁/CB₂) double knockout mice: **(A)** macroscopic score, **(B)** microscopic score, **(C)** colon length and **(D)** myeloperoxidase (MPO) activity. O-1602 (5 mg/kg; twice daily) kept its colitis-protective effect in CB₁/CB₂ double knockout mice. Colon length is subscored here but is also part of the macroscopic score index (Student's t-test; n=5–6).

Effect of O-1602 on DSS colitis in GPR55 knockout mice

Figure 5.

Parameters used for evaluation of dextran sulfate sodium (DSS)-induced colitis in GPR55 knockout mice: **(A)** macroscopic score, **(B)** microscopic score, **(C)** colon length and **(D)** myeloperoxidase (MPO) activity. As in the in cannabinoid receptor 1 and 2 double knockout mice, protection against DSS colitis by O-1602 (5 mg/kg; twice daily) is also maintained in the GPR55 knockout mice (One-way ANOVA; Tukey's multiple comparison; $n=9$). **(E)** Reverse transcriptase polymerase chain reaction (RT-PCR) shows GPR55 mRNA expression in the mucosa and the muscle layer of the colon.

Effect of O-1602 on TNBS colitis in C57BL/6 mice

Figure 6. Parameters used for evaluation of trinitrobenzene sulfonic acid (TNBS)-induced colitis in C57BL/6 mice: (A) macroscopic score, (B) microscopic score, (C) myeloperoxidase (MPO) activity, (D) colon length and (E) tumor necrosis factor alpha (TNF- α) mRNA levels. Like in the DSS colitis model, O-1602 (5 mg/kg; twice daily) was also protective in the TNBS colitis model. Colon length is subscored here but is also part of the macroscopic score index (One-way ANOVA; Tukey's multiple comparison; n=5–8).

Chemotaxis of neutrophils

Figure 7.

(A) Chemotactic assays with neutrophils that were isolated from control (CD1) and cannabinoid receptor 1 and 2 double knockout mice (CB₁^{-/-}/CB₂^{-/-}) mice displayed concentration-dependent inhibition of O-1602 to 10 nM of the N-formyl-peptide receptor ligand WKYMVm. There are no differences in neutrophil migration between CD1 and CB₁^{-/-}/CB₂^{-/-} mice at any concentration of O-1602.

(CD1: *p<0.05; 1 and 10 nM O-1602 vs WKYMVm only; **p<0.001; 50 nM O-1602 vs 1 and 10 nM O-1602 and WKYMVm only. CB₁^{-/-}/CB₂^{-/-}: # p<0.05; 50 nM O-1602 vs 1 and

10 nM O-1602; ## $p < 0.01$; 1 nM O-1602 vs WKYMVm only; ### $p < 0.001$; 10 and 50 nM O-1602 vs. WKYMVm only; $n = 9$; One-way ANOVA, Tukey's multiple comparison).

(B) Chemotactic assays with neutrophils that were isolated from control (CD1) and cannabinoid receptor 1 and 2 double knockout ($CB_1^{-/-}/CB_2^{-/-}$) mice displayed concentration-dependent inhibition of O-1602 also to 10 nM keratinocyte-derived chemokine (KC). There are no differences in neutrophil migration between CD1 and $CB_1^{-/-}/CB_2^{-/-}$ mice at any concentration of O-1602 except for at 50 nM O-1602 (CD1: * $p < 0.01$; 1 nM O-1602 vs KC only; ** $p < 0.001$, 10 and 50 nM O-1602 vs KC only, 50 nM O-1602 vs 1 and 10 nM O-1602. $CB_1^{-/-}/CB_2^{-/-}$: # $p < 0.001$; 1, 10 and 50 nM O-1602 vs KC only; ## $p < 0.01$; 10 nM vs 1 nM O-1602; $n = 9$; One-way ANOVA, Tukey's multiple comparison).

(C) Chemotactic assays with neutrophils that were isolated from control mice (C57BL/6) and GPR55 knockout mice ($GPR55^{-/-}$) display concentration-dependent inhibition of O-1602 to 10 nM N-formyl-methionyl-leucyl-phenylalanine (fMLP). There are no differences in neutrophil migration between wildtype and knockout mice at any concentration of O-1602

(C57BL/6: ** $p < 0.001$ between all concentrations; $GPR55^{-/-}$ mice: ### $p < 0.001$ between all groups; $n = 4$; One-way ANOVA, Tukey's multiple comparison).

O-1602

Figure 8.
Chemical structure of O-1602 (5-Methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl)-1,3-benzenediol])