Cannabinoid Receptor 2 (CB_2) Localization in Colonic Tissue and Primary Sensory Dorsal Root Ganglia (DRG) Neurons Isolated From Rodents With Colitis and Chronic Visceral Hypersensitivity

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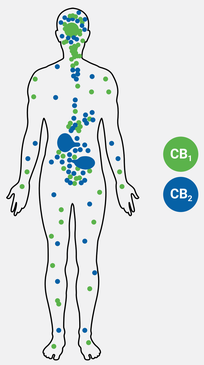
INTRODUCTION

- Abdominal pain is a key symptom of irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)[1,2]
  - More than 90% of patients with IBS have abdominal pain weekly[2]
  - Abdominal pain occurs in up to 70% of patients with IBD[1]
- Treatment options for abdominal pain in IBS and IBD are limited for many patients,[3,4] and current options do not fully alleviate the pain
- Modulation of somatic and visceral pain via the endocannabinoid system and its receptors, cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2), is emerging as a potential management approach[5]
  - CB_1 is widely distributed and highly expressed in the brain and mediates the psychoactive effects of cannabis (**Figure 1**)[5]
  - CB_2 is mainly expressed in immune cells and peripheral tissue, including the gastrointestinal tract (**Figure 1**), and is upregulated in disease states, such as inflammation[6-8]
- CB_2 may be an attractive target for the treatment of abdominal pain
  - Increased expression in the colon of patients with IBS and IBD[9-10]
  - Modulated visceral hypersensitivity in animal models[11-14]
- Olorinab (APD371) is a highly selective, peripherally acting, full agonist of the CB_2 receptor[15,16]
  - Exhibited >1000-fold functional selectivity for CB_2 over CB_1[15,16]
  - Demonstrated low brain penetration in rats,[16] reducing the potential for CNS effects
  - Sustained efficacy in several nonclinical models of chronic pain, including osteoarthritis and neuropathic pain[15,17]
  - Generally safe and well tolerated in healthy volunteers in a single-dose study of up to 400 mg and in multiple doses up to 200 mg 3 times a day[18,19]

Figure 1. Expression of CB_1 and CB_2 in the Body[5-8]



CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2.
*Graphic is illustrative of cumulative CB2 expression data from Arena and External Sources.

OBJECTIVES

- To investigate the potential antinociceptive effects and mechanism of action of olorinab in an animal models of IBS and IBD
- To identify potential sites of olorinab activity by determining the expression of CB_1 and CB_2 in the colon and dorsal root ganglia (DRG) in animal models of IBS and IBD and in human DRG from donors with IBD and heathy donors

METHODS

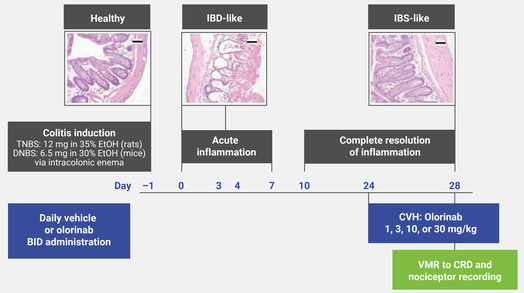
ANIMAL MODELS OF CHRONIC VISCERAL HYPERSENSITIVITY (CVH; IBS-LIKE) AND COLITIS (IBD-LIKE)

- Colitis was induced in mice and rats as previously described in Hughes et al[20] (**Figure 2**)
  - Male 6- to 7-week-old Sprague Dawley rats were administered an intracolonic enema of 2,4,6-trinitrobenzene sulfonic acid (TNBS) 12 mg in 35% ethanol (0.3 mL)
  - Male 13-week-old C57BL/6 mice were administered an intracolonic enema of 2,4-dinitrobenzene sulfonic acid (DNBS) 6.5 mg in 30% ethanol (0.1 mL)
- CVH was induced in male 10- to 11-week-old C57BL/6 mice using an intracolonic enema of DNBS 6.5 mg in 30% ethanol (0.1 mL) (**Figure 2**)

IN VIVO PAIN ASSESSMENT BY VISCEROMOTOR RESPONSE (VMR) TO COLORECTAL DISTENSION (CRD)

- Visceral hypersensitivity was assessed in vivo by quantifying VMR to CRD (0 to 80 mm Hg) on Day 28 (CVH) post-TNBS/DNBS administration (**Figure 2**)
  - Noxious distension of the colorectum triggers the VMR, a nociceptive brainstem reflex consisting of the contraction of the abdominal muscles,[21] and was used as an indicator of pain
- After TNBS/DNBS administration in rodents, CRD was induced using a barostat, and VMR was measured using an amplifier connected to an analog-to-digital converter
- Olorinab or vehicle (0.5% methylcellulose) was administered to
  - CVH or healthy control rodents at 1, 3, 10, or 30 mg/kg twice a day (BID) on days 24 to 28 after induction of colitis

Figure 2. Induction of Colitis and CVH for Assessing VMR to CRD



BID, twice daily; CRD, colorectal distension; CVH, chronic visceral hypersensitivity; DNBS, 2,4-dinitrobenzene sulfonic acid; EtOH, ethanol; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; VMR, visceromotor response. Histology images adapted with permission from BMJ Publishing Group Limited (Post-inflammatory afferent sensitization: different subtypes, different pathways, and different time courses. Hughes et al. Volume 58, Issue 10, Pages 1333-1341, Copyright 2009). [20] Scale bar, 20 µm.

IN VITRO MECHANOSENSORY RESPONSE ASSESSMENT OF COLONIC NOCICEPTORS

- Single-unit extracellular recordings from splanchnic colonic nociceptors were performed as previously described[20]
- Olorinab and/or a CB_2 antagonist (SR144528) were applied to the surface of the mucosal epithelium of splanchic colonic nociceptors from CVH and healthy control animals
- After baseline firing rate was recorded in response to mechanical stimulation with von Frey filaments (2g), compounds were applied for 10 minutes at each concentration:
  - Baseline (0), 0.01, 0.1, 1.0, 10 µM olorinab
  - Baseline, 1.0 µM SR144528, 1.0 µM SR144528 + 1.0 µM olorinab

MEASUREMENT OF CB_1 AND CB_2 EXPRESSION BY QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (qRT-PCR)

- RNA was isolated for qRT-PCR from the following sources:
  - Colonic tissue (mucosa and muscle + enteric nervous system [ENS]) and DRG (thoracolumbar [T10-L1] and lumbosacral [L6-S1]) from healthy, colitis, and CVH mice
  - Human DRG from healthy donors (T10-L1; AnaBios) and from donors with IBD (T11)
- The mouse and human CB_2 genes have 2 distinct promoter regions, resulting in differential tissue expression (CB_{2A} and CB_{2B}).[22] Therefore, expression of each CB_2 isoform was assessed individually (where possible) and combined
- qRT-PCR was performed using TaqMan® probes for genes coding for the CB_1 and CB_2 proteins in mice and humans (*CNR1*, *CNR2*, $CNR2_A$ [only mouse probe available], and $CNR2_{1\alpha\beta}$) and reference genes for β-actin, peptidylprolyl isomerase A, and glyceraldehyde-3-phosphate dehydrogenase
- Results were analyzed using the delta cycle threshold (Ct) method to calculate relative expression levels: $N(0) = 2^{(Ct_{[geometric\ mean\ of\ reference\ genes]} - Ct_{[target]})}$

RNA IN SITU HYBRIDIZATION (ISH)

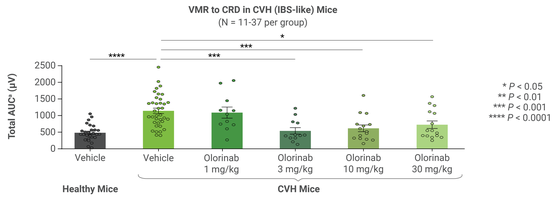
- Tissue sections were cut at 10-µm thickness
- Sections were mounted in duplicate or triplicate for each sample of colon and DRG, respectively, with a randomly selected sample from each group per slide; some slides contained a section of spleen (positive control)
- In situ labeling, for the singleplex CB_2 staining, was performed with the RNAscope® 2.5 HD Manual Assay–BROWN Assay
  - RNAscope® Probes for CB_2 (NM_009924.3), a negative control (dihydrodipicolinate reductase gene [dapB]; EF191515), and a positive control (peptidylprolyl isomerase B gene [PPIB]; NM_011149.2) were used
- CD45 and CB_2 double staining was performed with the RNAscope® 2.5 HD DUPLEX Assay using RNAscope® Probes for CB_2 (NM_009924.3) and PTPRC/CD45 (NM_001111316.2)
  - A duplex negative control (dapB; EF191515), positive control (PPIB; NM_011149.2), and RNA Polymerase II Subunit A (POLR2A; NM009089.2) were used
- Sections were imaged with a Nanozoomer Digital Slide Scanner (Hamamatsu Photonics) using 5× to 40× objectives, with no modifications made to the images

RESULTS

IN VIVO VISCERAL HYPERSENSITIVITY

- VMR to CRD was significantly increased in CVH mice compared with healthy mice (**Figure 3**)
- Olorinab significantly attenuated the increased visceral sensitivity in CVH mice at doses ≥3 mg/kg, reducing the VMR to CRD in CVH mice to levels similar to those in vehicle-treated healthy mice (**Figure 3**)
  - Healthy animals treated with olorinab (30 mg/kg) did not show altered VMR to CRD (data not shown)

Figure 3. VMR to CRD in CVH Mice With and Without Olorinab Treatment



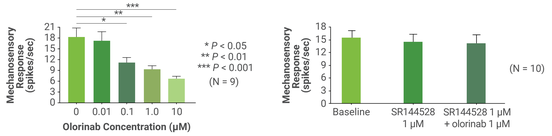
AUC, area under the curve; CRD, colorectal distension; CVH, chronic visceral hypersensitivity; IBS, irritable bowel syndrome; VMR, visceromotor response.
*Sum of all distension pressures.
All comparisons were made with a post hoc generalized estimating equation using least squares difference. AUC was calculated as the difference of area values obtained before distension (20 seconds) minus those obtained during distension (20 seconds).
All data are presented as mean ± standard error of the mean.

- No changes in colonic compliance were observed in healthy or CVH animals treated with olorinab at any dose (data not shown)[17]

IN VITRO COLONIC NOCICEPTION

- Colonic nociceptors displayed enhanced responses to mechanical stimuli in CVH animals when compared with healthy controls (data not shown)
- Olorinab application to colonic nociceptors from CVH mice caused a concentration-dependent reduction in mechanosensory responses at doses ≥0.1 µM (**Figure 4**)
  - The effect of olorinab on colonic nociceptors was inhibited by the CB_2 antagonist SR144528,[17] confirming the activity of olorinab is mediated by CB_2

Figure 4. Colonic Nociceptor Mechanosensory Response in CVH Rodents With and Without Olorinab Treatment



CVH, chronic visceral hypersensitivity.
All data are presented as mean ± standard error of the mean.

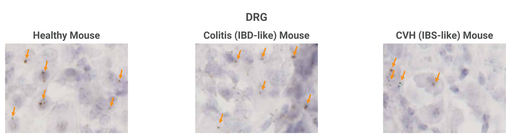
RELATIVE mRNA EXPRESSION OF CB_1 AND CB_2 IN COLONIC TISSUE AND DRG

- CB_2 was observed at low levels in all tissue examined and was more predominant than CB_1 in the colonic mucosa, whereas CB_1 was predominant on DRG and on colonic muscle + ENS (data not shown)
  - Expression levels of CB_2 or CB_1 were not different among healthy, colitis, or CVH mice (data not shown)
- CB_2 was more predominantly expressed than CB_2 in human DRG (data not shown)

CB_2 LOCALIZATION FROM HEALTHY, IBD-LIKE, AND IBS-LIKE MICE

- CB_2 was localized within DRG neurons across healthy, colitis, and CVH mice (**Figure 5**)

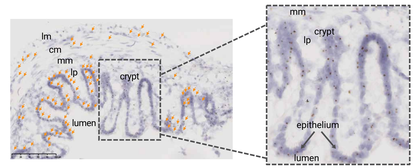
Figure 5. CB_2 Localization in DRG Neurons From Healthy, IBD-Like, and IBS-Like Mice



CB_2, cannabinoid receptor 2; CVH, chronic visceral hypersensitivity; DRG, dorsal root ganglia; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.
*Orange arrows pointing at CB2 expression. Brown dots represent CB2 expression

- In healthy control colonic tissue, CB_2 expression was localized to the colonic mucosa with prominent staining within the region containing epithelial cells lining the lumen edge and crypts (**Figure 6**)
- Similar staining of CB_2 was observed in colitis and CVH mice (data not shown)

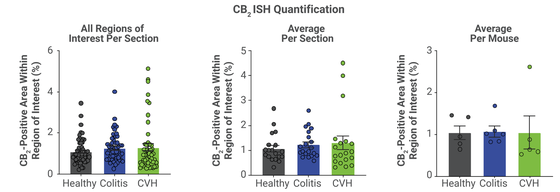
Figure 6. CB_2 Localization in Mouse Colonic Tissue



CB_2, cannabinoid receptor 2; cm, circular muscle; lm, longitudinal muscle; lp, lamina propria; mm, muscularis mucosae.
Representative image of CB_2 localization shown in healthy mouse.
*Orange arrows pointing at CB2 expression outside of magnified section. Brown dots represent CB2 expression.

- No significant difference in CB_2 mRNA expression by ISH was found in healthy, colitis, or CVH mice (**Figure 7**)

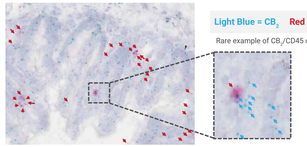
Figure 7. Quantification of CB_2 Expression in Colonic Tissue From Healthy, IBD-Like, and IBS-Like Mice



CB_2, cannabinoid receptor 2; CVH, chronic visceral hypersensitivity; ISH, in situ hybridization.

- In colonic mucosa, CB_2 is rarely colocalized with CD45, a broad marker for immune cells (**Figure 8**)

Figure 8. CB_2 and CD45 Colocalization in Mouse Colonic Tissue*



CB_2, cannabinoid receptor 2.
*Image is derived from a mouse with CVH.

CONCLUSIONS

- Olorinab reduced visceral hypersensitivity in a dose- and CB_2- dependent manner in an animal model of IBS but not in healthy controls, suggesting that activation of CB_2 causes antinociceptive effects in visceral sensory pathways in disease states
- CB_2 was observed at low levels in all tissue examined and was more predominant than CB_1 in the colonic mucosa, supporting the hypothesis that olorinab activates CB_2 receptors located in the colon to decrease visceral hypersensitivity
- Expression of CB_2 in the colonic mucosa and DRG neurons was confirmed through ISH
- Duplex staining in healthy, colitis, and CVH colons with CD45 confirmed the presence of immune cells, but with little colocalization with CB_2 receptors, suggesting that CB_2 receptors are predominantly expressed on nonimmune cells in the colon
- These data support further clinical development of olorinab for use as a novel therapeutic approach for the management of chronic visceral pain in gastrointestinal disorders

Poster presented at Digestive Disease Week (DDW);
May 21-23, 2021; Virtual

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