pH Dependence of a GPR4 Selective Antagonist Hampers Its Therapeutic Potential

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Received December 8, 2022; accepted April 27, 2023

ABSTRACT

Inflammatory bowel disease (IBD) is characterized by chronic mucosal inflammation of the gastrointestinal tract and is associated with extracellular acidification of mucosal tissue. Several extracellular pH-sensing receptors, including G protein-coupled receptor 4 (GPR4), play an important role in the regulation of inflammatory and immune responses, and GPR4 deficiency has been shown to be protective in IBD animal models. To confirm the therapeutic potential of GPR4 antagonist in IBD, we tested Compound 13, a selective GPR4 antagonist, in the interleukin 10−/− mouse model of colitis. Despite good exposures and albute, there was a trend toward improvement for a few readouts, Compound 13 treatment did not improve colitis in this model, and there were no signs of target engagement. Interestingly, Compound 13 behaved as an “orthosteric” antagonist, i.e., its potency was pH dependent and mostly inactive at pH levels lower than 6.8 with preferential binding to the inactive conformation of GPR4. Mutagenesis studies confirmed Compound 13 likely binds to the conserved orthosteric binding site in G protein-coupled receptors, where a histidine sits in GPR4 likely preventing Compound 13 binding when protonated in acidic conditions. While the exact mucosal pH in the human disease and relevant IBD mice models is unknown, it is well established that the degree of acidosis is positively correlated with the degree of inflammation, suggesting Compound 13 is not an ideal tool to study the role of GPR4 in moderate to severe inflammatory conditions.

SIGNIFICANCE STATEMENT

Compound 13, a reported selective GPR4 antagonist, has been widely used to assess the therapeutic potential of GPR4, a pH-sensing receptor, for numerous indications. Its pH dependence and mechanism of inhibition identified in this study clearly highlights the limitations of this chemotype for target validation.

Introduction

Inflammatory bowel disease (IBD) is characterized by chronic, aberrant mucosal inflammation of the gastrointestinal tract. The exact etiology of IBD is unknown, but a complex interaction between immunologic, environmental, microbiome, and genetic constituents is believed to contribute to disease onset and continued progression. Acidosis has been shown to be associated with a variety of pathologic conditions including the inflammation observed in intestinal bowel disease due to hypoxia and build-up of glycolytic metabolites (Tsai et al., 2014; Cummins and Crean, 2017). In both active ulcerative colitis and Crohn’s disease, the intraluminal colonic pH is significantly lower than in normal subjects (Fallingborg et al., 1993; Nugent et al., 2001).

The proton-activated G protein–coupled receptors (GPCRs) GPR4, TDAG8 (or GPR65), and OGR1 (or GPR68) are pH sensors that can trigger intracellular signaling in response to alterations in extracellular pH around physiologic values, i.e., in the range between pH 7.5 and 6.5. These receptors have been linked to several well-studied pH-dependent physiologic processes, including the genesis and progression of several inflammatory diseases such as IBD (Imenez Silva and Wagner, 2022). GPR4 is predominantly expressed in endothelial cells, and GPR4 activation has been shown to increase the expression of numerous inflammatory and stress response genes in vascular endothelial cells, promoting leukocyte infiltration in the mucosa (Chen et al., 2011; Dong et al., 2013; Krewson et al., 2020). Absence of GPR4 has been shown to ameliorate colitis in several conditions.

This work received no external funding.
No author has an actual or perceived conflict of interest with the contents of this article.
dx.doi.org/10.1124/jpet.122.001554.
This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; DSS, dextran sulfate sodium; ECGS, Endothelial Cell Growth Supplement; GPCR, G protein-coupled receptor; GPR4, G protein-coupled receptor 4; HUVEC, human umbilical vein endothelial cells; IBD, inflammatory bowel disease; IL10, interleukin 10; PEI, polyethylenimine; pH50, pH of half-maximal activation; ROI, region of interest; SPR, surface plasmon resonance; SPRm, SPR microscopy; WT, wild type.
murine IBD models. GPR4−/− mice display an improved body weight loss and lower histopathology scores compared with wild type (WT) after induction of colitis with chronic dextran sulfate sodium (DSS) (Sanderlin et al., 2016; Wang et al., 2018). Compared with interleukin 10 (IL10)−/− mice, which develop spontaneous colitis, GPR4−/−/IL10−/− double knock-out mice have lower histopathology scores and reduced infiltration of immune cells associated with decreased expression of inflammatory genes (Wang et al., 2018). GPR4 inhibition has been proposed as a potential beneficial therapeutic approach for the treatment of IBD.

To explore the therapeutic potential of GPR4 antagonism in IBD, we tested Compound 13, a reported selective GPR4 antagonist, in IL10−/− mice. Compound 13 has been described as a selective, orally bioavailable GPR4 antagonist with a favorable safety and pharmacokinetic profile and showing efficacy in multiple models after oral administration at 30 mg/kg twice a day (Velicky et al., 2017; Sanderlin et al., 2019). In the present study, despite reasonable exposures consistent with previous reports, Compound 13 was ineffective at preventing colitis in IL10−/− mice, albeit there was a trend towards improvement for a few readouts. Those results led us to thoroughly characterize and identify the pharmacology and pH dependence of Compound 13 and its analogs at both human and mouse GPR4. All compounds tested behaved as orthosteric antagonists; their potency was dramatically reduced in low pH conditions, which is characteristic of inflammatory states. Metagenesis studies and surface plasmon resonance microscopy (SPRm) measurements confirmed Compound 13 likely binds to the conserved orthosteric binding site of GPCRs, preferentially binding to the inactive conformation of GPR4. Compound 13 and its analogs pH dependence strongly limits their utility in studying the role of GPR4 in acidosis-associated moderate to severe inflammatory conditions.

Materials and Methods

Compounds. Compound 13 was prepared as the hydrogen chloride according to Velicky et al. (2017). Compound 1 and Compound 2 were synthesized as described in Fukuda et al. (2016). Compound 39c was purchased from Dalton Pharma Services.

Animal Studies. To confirm exposures and pharmacokinetics of Compound 13 reported in the literature, Compound 13 was formulated in 0.5% methylcellulose, 0.5% Tween 80, and 99% water. Thirty mg per kilogram body weight (mg/kg) was orally administered in BALB/c mice, and plasma samples were analyzed at time 0, 1, 2, 4, 8, and 24 hours after administration. The pharmacokinetic parameters such as C_{max}, half-life, CL/F, V_{ss}, and area under the curve were determined using noncompartmental analysis with Phoenix WinNonlin, version 8.3 (Pharsight Corporation, Sunnyvale, CA, USA).

Male IL10−/− (model #15660) and background control WT mice (BALB/c AnNTac strain) were purchased from Taconic Biosciences (Germantown, NY) and were 5 weeks old (18–22 g) at the time of delivery. Only male mice were included in this study as, compared with female mice, they display more consistent disease onset and progression in our vivarium. Importantly, a previous study assessing the function of GPR4−/− in IL10−/− mice showed that there were no sex differences in terms of efficacy (Wang et al., 2018). Four animals were housed per cage in a temperature- and humidity-controlled vivarium on a 12h light/12h dark schedule with ad libitum access to normal rodent chow (Envigo Teklad 18% protein non-autoclave pelleted diet #2018) and tap water. The experimental protocols were approved by the Institutional Animal Care & Use Committee of Ferring Pharmaceuticals and performed in accordance with the institutional and national guidelines for the use and care of animals for experiments.

Mice were monitored daily for overall health and at least weekly for qualitative assessment of stool consistency. Body weight measurements were taken at least three times a week. The IL10−/− model is a spontaneous model of colitis, thus the onset of disease is subject to environmental factors. Based on previous cohorts, disease onset (defined by a summed histopathology score of >0 for most mice in the cohort) was expected to occur at the first indication of body weight changes in 8–10 weeks old mice. In this cohort, body weights of IL10−/− mice started to deviate from WT control mice in 9-week-old mice (day 63), which is when treatments started.

Compound 13 was formulated in 0.5% methylcellulose, 0.5% Tween 80, and 99% water, which served as the vehicle control. Compound 13 was orally dosed at 10 mg/kg and 30 mg/kg twice a day via gavage. IgG control (catalog no. BE0089) and anti-IL12/23 (p40) (catalog no. BE0051) therapeutic control antibodies were purchased from Bioxcell (Lebanon, NH, USA). Both were dosed at 25 mg/kg, i.p. once a week. It was predetermined that treatments would last 6 weeks, which previous studies have shown is a sufficient timeframe to observe meaningful changes between IL10−/− mice treated with vehicle versus the therapeutic control anti-IL12/23 (Rennick et al., 1997; Koelink et al., 2020). Mice were euthanized using isoflurane followed by cervical dislocation. Distal colons were harvested and fixed using standard formalin/ethanol fixation procedures and submitted for histopathology. A small piece (1 cm) of mid distal colon was stored in RNAlater for transcriptomic analysis. Colon contents were collected for assessment of fecal lipocalin 2 levels using the mouse Lipocalin-2/NGL Quanti-kine ELISA kit (catalog no. MLCN20) from R&D Systems (Minneapolis, MN, USA).

Histopathology. Formalin-fixed distal colon segments, approximately 2.0 cm in length, from 69 mice were processed. Each colon sample was trimmed into three transverse pieces, which were paraffin embedded in a single block. Slides were sectioned and stained with H&E. Glass slides blinded to the treatment groups were evaluated using light microscopy by a board-certified veterinary pathologist (Ino-tive). Submucosal edema was quantified by measuring the distance from the muscularis mucosa to the internal border of the outer muscle layer in a nontangential area thought to most represent the severity of this change.

The extent of macrophage, lymphocyte, and polymorphonuclear leukocyte cell infiltration was assigned a severity score according to the following criteria:

0 = Normal
0.5 = Very Minimal, 1 or 2 small foci, mononuclear inflammatory cells, affects less than 1% of the mucosa
1 = Minimal, larger focal area with mononuclear inflammatory cells and neutrophils affecting 1% to 10% of the mucosa or minimal diffuse, may be mostly in areas of submucosal edema or mesentery
2 = Mild, diffuse mild, or multifocal affecting 11% to 25% of mucosa with minor focal or multifocal gland separation, no separation in most areas
3 = Moderate, 26% to 50% of mucosa affected with mild focal or multifocal separation of glands by inflammatory cell infiltrate, milder in remaining areas of mucosa with some areas having no gland separation by inflammation
4 = Marked, 51% to 75% of mucosa affected with mild to moderate separation of glands by inflammatory cell infiltrate, minimal to mild in remaining areas of mucosa but all glands have some separation by infiltrate
5 = Severe, 76% to 100% of mucosa affected with moderate to marked areas of gland separation by inflammatory cell infiltrate, mild to moderate in remaining areas of mucosa...

Crypt epithelial and remaining gland epithelial loss (gland loss score) were scored based on the approximate percent of the mucosa that was affected as follows: 0 = None; 0.5 = Very Minimal, 1 or 2 small focal areas of gland loss affecting less than 1% of the mucosa; 1 = Minimal, 1% to 10% of the mucosa affected; 2 = Mild, 11% to 25% of the mucosa...
affected; 3 = Moderate, 26% to 50% of the mucosa affected; 4 = Marked, 51% to 75% of the mucosa affected; and 5 = Severe, 76% to 100% of the mucosa affected.

The loss of surface epithelium (erosion score) was scored based on the approximate percent of the mucosa that was affected, as indicated here. This is generally associated with mucosal hemorrhage (reflective of the bleeding seen clinically and at necropsy). 0 = None; 0.5 = Very Minimal, 1 or 2 small focal areas of mucosal erosion affecting less than 1% of the mucosa; 1 = Minimal, 1% to 10% of the mucosa affected; 2 = Mild, 11% to 25% of the mucosa affected; 3 = Moderate, 26% to 50% of the mucosa affected; 4 = Marked, 51% to 75% of the mucosa affected; and 5 = Severe, 76% to 100% of the mucosa affected.

Mucosal thickness was measured by optical micrometer in a nontangential area of the section that best represented the overall mucosal thickness. This parameter is indicative of gland elongation and mucosal hyperplasia. A hyperplasia score was derived from the measurement as follows: 0 = Normal, ≤ 200 μm; 0.5 = Very Minimal, 201–250 μm; 1 = Minimal, 251–350 μm; 2 = Mild, 351–450 μm; 3 = Moderate, 451–550 μm; 4 = Marked, 551–650 μm; 5 = Severe, >650 μm.

A sum of inflammation, gland loss, erosion, and hyperplasia scores was calculated to generate the overall histopathological score. Note that gland loss and erosion scores are not shown as the values were negligible in this model, thus inflammation and hyperplasia overwhelmingly contributed to the overall histopathology score.

**RNA Sequencing.** Terminal colon tissues were collected from IL-10−/− mice treated with vehicle, anti-IL12/IL23, and Compound 13. RNA was extracted using RNeasy kit from QiaGen (catalog no. 74106) according to the manufacturer’s guidelines and used for RNA sequencing. An mRNA library was prepared using a poly A enrichment method and sequencing using NovaSeq at pair-end 150 bp. Raw sequencing reads were processed using cutadapt v3.7 to remove adapter remnants. Trimmed reads were then aligned using STAR aligner version 2.7.8a and sequencing using NovaSeq at pair-end 150 bp. Raw sequencing reads were processed using cutadapt v3.7 to remove adapter remnants. Trimmed reads were then aligned using STAR aligner version 2.7.8a (Dobin et al., 2013) to human genome version 88. Quantification was performed based on RSEM annotation version 88. Quantification was performed based on RSEM v1.3.3 (Li and Dewey, 2011) for raw read counts and Transcript Per differentially expressed genes. Pathway enrichment analysis was performed using Gene Ontology Biologic Process using Fisher’s exact test. RNA sequencing raw and processed data were deposited to GEO: GEO accession GSE225694.

**Cell Culture and Mutagenesis.** HeLa cells (ATCC) were maintained at 37 °C in a humidified 5% CO2 incubator in culture medium [Eagle’s minimum essential medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FBS]. cDNA for human (NM_005282.3) and mouse GPR4 (NM_175668.4) were subcloned into pcDNA3.1(-)Hygro (Invitrogen) using EcoRV and XhoI restriction sites. Human GPR4 mutants (W73A, F97A, F97D, K172A) were subcloned into pcDNA3.1(−)Hygro (Invitrogen) using EcoRV and XhoI restriction sites. Human GPR4 mutants (W73A, F97A, F97D, K172A) were subcloned into pcDNA3.1(−)Hygro (Invitrogen) using EcoRV and XhoI restriction sites.

**cAMP Assays.** We first developed a cAMP assay in human GPR4-expressing cells. For agonist mode, WT or GPR4 mutant cells were incubated directly with assay buffer at the corresponding pHs in presence of 500 μM 3-isobutyl-1-methylxanthine at room temperature. For antagonist mode, cells were first incubated for 15 minutes in 10 μL assay buffer at pH 7.9 in presence of Compound 13 and 500 μM 3-isobutyl-1-methylxanthine. An equal volume (10 μL) of assay buffer at predetermined pH (between pH 3.0 and pH 4.0) was then added to reach desired final pH.

After 30 minutes incubation at room temperature, analytes were detected according to the manufacturer’s protocol (cAMP Dynamic kit; CisBio). Fluorescence was read with a PHERAstar plate reader (BMG Labtech, Ortenberg, Germany) using an excitation of 357 nm and emissions of 620 and 665 nm. Raw data were converted to nomocam cAMP values by interpolation from a cAMP standard curve. The IC_{50} and the pH of half-maximal activation (pH_{50}) determinations were made from an antagonist- or agonist-response curve analyzed with a curve-fitting program using a four-parameter logistic dose-response equation in GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data presented are representative of three independent experiments performed in quadruplicate for each compound. Data are represented as averages ± S.D. Data in Table 1 are averages from three independent experiments.

**Gene Expression.** HUVEC were first incubated overnight in complete media pH 8.0 in 6-well plates. The next day, the media was replaced by an optimized pH-stabilizing media in presence or absence of Compound 13 (DMEM, 0.5 g/L Sodium Bicarbonate, 20 mM HEPES, 10% FBS, 1X ECOS, 1X Pen/Strep, pH 8.0 or DMEM, 0.5 g/L sodium bicarbonate, 7.5 mM HEPES, 7.5 mM EPFS, 7.5 mM MES, 10% FBS, 1X ECOS, 1X Pen/Strep, pH 6.8 or 6.4). Cells were incubated for 5 hours in the cell culture incubator. RNA was extracted using Qagen kit (catalog no. 74104). Gene expression was assessed using 50 ng RNA per well and TagMan Fast Virus 1-Step Multiplex Master Mix (Thermo Fisher Scientific, catalog no. 5555532) according to the manufacturer’s protocol, on a QuantStudio 6 Flex Real-Time PCR Instrument. Data were normalized on housekeeping gene (HPRT), as well as on basal expression at pH 8.0 and represented as fold-increase over baseline (fold-over pH 8.0). Data presented are representative of two independent experiments performed in duplicate for each gene. Data are represented as averages ± S.D.

**Bioluminescence Resonance Energy Transfer-Based Biosensor Assays.** BioSensAll biosensor assays were conducted at Domain Therapeutics NA Inc. (Montreal, QC, Canada). Assays were performed in HEK-293T cells, cultured in DMEM (Wiset, Quebec, Canada) supplemented with 1% penicillin-streptomycin (Wiset) and 10% FBS (Wiset), and then maintained at 37 °C with 5% CO2. All biosensor coding plasmids and related information are the property of Domain Therapeutics NA Inc.; GAPL-G6, GAPL-G7, GAPL-G11, GAPL-G12, GAPL-G2, GAPL-Gb, GAPL-Gz, GAPL-G15, GAPL-G12, and GAPL-G13. Transfections were performed using 25-kDa linear polyethyleneimine (PEI) (Polysciences, Warrington, PA, USA) at a 3:1 μl of PEI/microgram of DNA ratio. Briefly, DNA and PEI were diluted separately in 150 mM NaCl, mixed, and then incubated for at least 20 minutes at room temperature (note: the total amount of DNA transfected was adjusted to a final quantity of 2 μg with salmon sperm DNA; Thermo Fisher Scientific). During the 20-minute incubation, HEK-293T cells were detached, counted, and resuspended into cell culture medium to a final density of 350,000 cells/ml. At the end of the 20-minute incubation, DNA/PEI complexes were added to the cells followed by gentle mixing. Cells were subsequently distributed in cell culture-treated 96-well plates (White Opaque 96-well microplates;
Greiner Kremsmünster, Austria) at a density of 35,000 cells per well (i.e., 100 μl of cell suspension per well) and incubated at 37°C for 48 hours. Then, 48 hours after transfection, culture medium was aspirated and replaced with 100 μl Hank’s balanced salt solution buffer (Wisent, catalog no. 319-067CL with sodium bicarbonate, with calcium and magnesium, with HEPES) per well. For agonist testing, different pH conditions were added manually. For antagonist testing, Compound 13 was added to each well using the HP D300 digital dispenser (Tecan) in Hank’s balanced salt solution buffer at different pHs (pH equivalent to pH5–pH 7.9 or pH80–pH6.5; Supplemental Fig. 3) and cells were then incubated 10 minutes at room temperature. Ten μl of 10μM e-Coelenterazine Prolume Purple (Methoxy e-CTZ) (Nanolight, catalog no. 369) was added to each well for a final concentration of 1μM. Bioluminescence resonance energy transfer (BRET) readings were subsequently collected with a 0.4-second integration time on a Synergy NEO plate reader (BioTek Instruments, Inc., Winooski, VT, USA; filters: 400/70 and 515/20 nm, donor and acceptor filters, respectively). The BRET signal was calculated as the ratio of acceptor emission to donor emission. Data from two independent experiments for each compound and performed in duplicates were combined and the symbols presented are the mean ± S.D.

SPRm. Surface plasmon resonance (SPR) experiments were carried out on the SPRm 200AP system from Biosensing Instrument, Inc., which combines bright field optical imaging and high-resolution imaging-based SPR measurement. SPR has been the go-to technique for measuring binding affinity and kinetics in a label-free manner. However, measuring membrane proteins binding with SPR has been a challenge because of the need to immobilize purified receptors on the sensor chip surface. SPRm is a label-free technology used to study binding interactions of membrane proteins without extracting the proteins from the cell, ensuring intact native conformations of the membrane proteins (Wang et al., 2012). The SPR uses a 690 nm LED as the light source with a sensing area of ~600 x 450 μm. SPR sensorgrams can be generated and analyzed for each spot inside the sensing area with a lateral resolution of ~1μm. The Au sensor chip has a silicon chamber placed on top for cell culture.

Chips were poly-D-lysine-coated before human GPR4-expressing HeLa cells were plated at a density of 100,000 cells per chip in complete medium, pH 7.9. The cells were maintained at 37°C in a humidified 5% CO2 incubator in culture medium for ~48 hours until they reached a suitable confluence. To lock GPR4 in either its active or inactive conformation, human GPR4-expressing cells were stimulated and then adjusted to either pH 7.9, pH 6.8, or pH 6.4 and cells were incubated at room temperature for 15 minutes. Cells were then fixed right away for 10 minutes by addition of formaldehyde 4% final to fix GPR4 in either its active or inactive conformation. Cells were washed with PBS several times and chips were stored at 4°C until the SPRm experiment could be performed.

The chip was then mounted on to the system and the growth medium was replaced with running buffer (Hank’s balanced salt solution, 2mM CaCl2, 1 mM MgSO4, 20 mM HEPES, 0.1% fatty-acid free BSA, 0.1% DMSO, and pH 7.4) for measurement. A dose-response of Compound 13 was prepared in the same running buffer. A microfluidic flow-cell was equipped to ensure the flow dynamics of the running buffer during the experiment, for maintaining the biologic condition, and for accurate SPR measurement. All measurements were performed at room temperature.

SPRm data were analyzed statistically using the provided ImageSPR analysis software. The measured SPRm images were divided into about 600 small regions of interest (ROIs), about 20 x 20 μm each. SPR sensorgrams were generated for each ROI for all concentrations injected. Kinetic analysis has not been possible in this study due to a relatively low signal, a characteristic of low-expressing systems (Zhang et al., 2015), leading to low confidence in the kinetic KD values measured. The thermodynamic binding parameters from the valid ROIs are presented in the form of a histogram distribution and the corresponding mean values are reported. Figure 5 shows the optical image (left) and the corresponding SPR image (right) of the sensing area on the chip.

## Results

### Compound 13 Treatment Does Not Improve Colitis in the IL10−/− Mouse Model with No Sign of Target Engagement.

Compound 13 potency was confirmed at mouse GPR4 to be 197.6 ± 55.2 nM at pH 6.8 (Table 2). Those data are discussed later in more detail. Oral exposures of Compound 13 in male BalbC mice (Table 1 and Supplemental Fig. 1) were very similar to oral exposures reported previously in female

### Table 2

<table>
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<tr>
<th>Compound</th>
<th>Activity at GPR4</th>
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N.D., non determined.
Sprague-Dawley rats (area under the curve 6.44 \( \mu \)M*h in mouse versus 6.7 \( \mu \)m*h in rat, both values dose-normalized to 1 mg/kg). Taken together, these data indicated that oral administration of Compound 13 at 30 mpk (twice a day) would provide sufficient exposures and target engagement (C\(_{\text{min}}\) of 0.86 \( \mu \)M with the uninterrupted coverage above fourfold the IC\(_{50}\) (Supplemental Fig. 1). Actual plasma concentration of Compound 13 in terminal samples taken 16 to 18 hours after the last dose were at or above the projected levels (Supplemental Fig. 1).

IL10\({}^{-/-}\) mice developed mild colitis over a period of 15 weeks from birth. Compound 13 treatment was initiated when signs of disease (decreased weight gain) were evident in this cohort at approximately 9-weeks (Day 63) (Supplemental Fig. 2A). After another 6 weeks, there were no signs of disease progression in the IL10\({}^{-/-}\) control mice, and the study was terminated on day 106. Over the treatment period (day 63–106), healthy control (WT) mice gained on average \sim 15% of their bodyweight from baseline (day 63), whereas IL10\({}^{-/-}\) control mice and Compound 13-treated mice failed to gain weight (Fig. 1A). In contrast, mice treated with anti-IL12/23 (therapeutic positive control) gained weight comparable to the healthy controls. As a gross readout of colon inflammation, colon weight to length ratio increased twofold in IL10\({}^{-/-}\) mice compared with healthy controls (Fig. 1B). Mice treated with anti-IL12/23 had comparable colon weight to length ratios as the healthy controls. Those treated with Compound 13 had elevated ratios suggesting a lack of improvement in inflammation. Other markers of gut inflammation (Supplemental Fig. 2B) and general inflammation (Supplemental Fig. 2C) were consistent with a lack of significant efficacy in Compound 13-treated mice.

Histopathological analysis showed that colitis in this model, although mild, was reversed in mice treated with anti-IL12/23 but not with Compound 13 (Fig. 1C and Supplemental Supplemental Fig. 1, D–E). There was a trend toward improvement in the high-dose group (30 mg/kg), but the reduction was not statistically significant.

The lack of efficacy prompted us to investigate whether there were any pharmacodynamic effects in Compound 13-treated mice. Terminal colon tissues were collected from IL10\({}^{-/-}\) mice treated with vehicle and Compound 13 for transcriptomic analysis. Compared with WT mice, IL10\({}^{-/-}\) mice exhibited significant alterations in gene expression related to defense and immune response (Supplemental Fig. 3A). While those effects were reversed following anti-IL12/23 treatment (adjusted Fisher’s exact \( P < 2e-49 \)) (Supplemental Fig. 3, C–E), consistent with previous reports (Kiesler et al., 2015), there were no significant changes in gene expression induced by Compound 13, suggesting a lack of or minimal target engagement and a more complex mechanism of inhibition of Compound 13.

**Compound 13’s Potency Is pH-Dependent.** The lack of detectable target engagement of Compound 13 in IL10\({}^{-/-}\) mice despite good exposures led us to more thoroughly investigate the pharmacology and mechanism of inhibition of Compound 13. We evaluated the potency of Compound 13 at inhibiting the activation of GPR4-mediated pathways at various pHs in both human and mouse GPR4-expressing cells. As GPR4 has been shown to couple to Gs/cAMP (Chen et al., 2011; Velcicky et al., 2017), we first developed a cAMP assay in human GPR4-expressing HeLa cells and confirmed the pH50 to be at \( \sim \) pH 7.1 and pH80 to be \( \sim \) pH 6.8 in that assay (Supplemental Fig. 5). We then measured Compound 13’s activity at various pHs. Compound 13’s potency was highly dependent on pH (Fig. 2A), with an IC\(_{50}\) of 111.8/+/− 91.5 nM at pH 6.8 and 7.2/+/− 3.4 nM at pH 7.0 (Table 2). Compound 13 was mostly inactive at pH lower than 6.8 (Fig. 2A; Table 2).

Additionally, GPR4 had been shown to couple to other G proteins, such as the G12/13 pathway (Krewson et al., 2020). Using BRET-based biosensors (BioSensAll), we confirmed that GPR4 could also couple strongly to Gq/11 and G12/13 with a pH50 of \( \sim \) 7.0 for both pathways (Supplemental Fig. 4). In antagonist mode at pH 7.0, the potency of Compound 13 at inhibiting GPR4-mediated Gq and G13 recruitment was 28.5+/− 7.0 nM and 14.0+/− 5.4 nM, respectively. At both Gq and G13, the potency of Compound 13 was also significantly reduced at pH 6.5, similarly to results observed in the cAMP assay (Fig. 2B, Table 2).

Additionally, we confirmed the pH dependence of Compound 13 observed in overexpressing systems in HUVEC cells that endogenously express GPR4. As previously shown in the literature, in endothelial cells, GPR4 activation by acidosis stimulates the expression of a wide range of inflammatory genes, such as SELE and CSP2 (Fig. 2C) (Dong et al., 2013). Compound 13 (1 \( \mu \)M) only significantly blocked those effects at pH 6.8 but was mostly inactive at lower pH (pH 6.4), confirming the pH dependence seen in recombinant systems.

In mouse GPR4-expressing cells, the pH50 was similar to that observed in human GPR4 expressing cells. Compound 13 had an IC\(_{50}\) of 197.6+/− 55.2 nM at pH 6.8 and was inactive at pH lower than 6.8 (Fig. 2D; Table 2). Compound 13 mostly behaved as an orthosteric antagonist, reducing the potency of the agonist (H+/+). The presence of increasing concentrations of Compound 13 dramatically affected the pH50 of mouse GPR4;
i.e., the apparent affinity of protons (H+) for the receptor was reduced, right-shifting the agonist curve (Fig. 2E).

To determine if other known GPR4 antagonists were pH dependent, we prepared three additional molecules that had been well characterized in the literature, Compounds 1 and 2 (Fukuda et al., 2016), as well as Compound 39c (Miltz et al., 2017). We measured their activity in the cAMP assay at pH 6.8 and at pH 6.4 alongside Compound 13 (Table 2). The potency of all compounds dropped dramatically at pH 6.4 (Fig. 3; Table 2).

**Compound 13 Binds to Conserved Orthosteric Binding Site of GPR4 in Its Inactive Conformation.**

To determine whether Compound 13 binds to the conserved orthosteric binding site of GPCRs, we used a model structure of GPR4 that was released from AlphaFold (Jumper et al., 2021; Varadi et al., 2022) to select residues for mutagenesis. Given that Compound 13 originated from a lead found to be active at H3R (Velcicky et al., 2017), we hypothesized that GPR4 and histamine receptors shared a common binding site. To probe this idea, we decided to use available crystal structures of the only available histamine family receptors at the time, H1 receptors (PDB 3RZE and 7DFL) (Shimamura et al., 2011; Xia et al., 2021) to define a binding region. The H1 receptors were structurally aligned with the AlphaFold model of GPR4, and residues within the H1 receptors binding pocket region were manually selected for mutagenesis. The GPR4 residues selected (W73, F97, Y98, R185, Y240, Y268, H269, and L272) all fall into regions in which AlphaFold suggests a
very high model confidence. Subsequent publications of H2R (PDB 7UL3) (Robertson et al., 2022) and H3R (PDB 7F61) (Peng et al., 2022) confirmed that the selected GPR4 residues for mutagenesis fall within their respective binding sites as well.

We performed mutagenesis of the main residues in the proposed “orthosteric” binding pocket (W73, F97, Y98, R185, Y240, Y268, H269, and L272) and assessed the expression and functionality of those mutants (Supplemental Fig. 5). Several of those mutations, such as F97A, F97D, H269A, L262A, Y240A, and R185A, led to either a completely inactive receptor or a dramatic loss-of-function (Supplemental Fig. 5), preventing us from assessing the functionality of Compound 13 in those mutants. The key role of H269 in the molecular mechanisms of GPR4 activation had already been reported in the literature (Rowe et al., 2021). W73A and Y268A mutants (Fig. 4A) were expressed and had a similar pH50 to human GPR4 WT (Supplemental Fig. 5).

We tested Compound 13 activity in the cAMP assay at the W73A and Y268A GPR4 mutants at pH 6.8. The potency of Compound 13, 111.8 ± 91.5 nM at the WT receptor, was significantly reduced at the W73A mutant (>10 μM) and slightly reduced at the Y268A mutant (477.6 ± 313.0 nM) (Fig. 4B), suggesting Compound 13 interacts with these residues and binds in this conserved orthosteric pocket.

A histidine, H269, sits in the middle of the putative orthosteric binding pocket proximal to both W73 and Y268 and is critical for GPR4 coupling to multiple pathways (Rowe et al., 2021). As many histidines have been suggested to be protonated leading to GPR4 activation, we hypothesized that Compound 13 binding is likely affected in low pH conditions, possibly due to protonation of H269 (Rowe et al., 2021). To confirm Compound 13 binding properties, we performed SPRm experiments to measure Compound 13 binding affinities to GPR4 in either its inactive or active state.

SPRm thermodynamic analysis in hGPR4-expressing cells revealed that Compound 13 could bind to hGPR4 at both pH 7.9 and 6.8 with equilibrium dissociation constants of 3.9 and 5.5 nM, respectively (Fig. 5, A–B), consistent with the IC50 values obtained in the different functional assays at pH below pH50 (Table 2). No binding was detected at pH 6.4 (Fig. 5C). No binding was detected either at pH 7.9 and pH 6.8 in mock cells that did not express hGPR4 (not shown). An interesting advantage of SPRm is its ability to measure binding events to multiple regions of the cells, capturing the diversity in receptor conformations. We observed a number of high-affinity subnanomolar binding events at pH 7.9 that were absent at pH 6.8 and 6.4, consistent with the affinity of Compound 13 decreasing at lower pH. A small number of binding events were detected at pH 6.4, but they were not sufficient to generate a

![Fig. 3. The potency of Compound 13 analogs, Compound 1, Compound 2, and Compound 39c is pH-dependent at both human (A) and mouse (B) GPR4 in a cAMP assay.](image-url)

![Fig. 4. Compound 13 potency is significantly reduced by GPR4 mutations to conserved orthosteric site comprising H269. (A) AlphaFold model of GPR4’s conserved orthosteric site with residues selected for mutagenesis shown as sticks. (B) W73A in red and Y268A in green were determined to decrease the potency of Compound 13 in a cAMP assay in cells expressing either WT or mutants human GPR4, while H269 in yellow has previously been shown to be critical to GPR4 function (Rowe et al., 2021). GPCR transmembrane helices are labeled in the gray boxes.](image-url)
Wang et al., 2018), suggesting that IL10 mice showed protection in the development of severe colitis.

IL10 in intestinal inflammation would be an appropriate model for studying the role of GPR4.

human IBD, genetic polymorphism at the IL10 locus confers resistance to enterocolitis and could confound the potential therapeutic efficacy of Compound 13. GPR4 deficiency has been shown to very mildly improve body weight loss and histopathology scores in the DSS colitis model (Sanderlin et al., 2016; Wang et al., 2018). It is worth noting Compound 13 only showed minimal activity in this model (Sanderlin et al., 2019). Moreover, given the known limitations of the DSS model at recapitulating fundamental aspects of human IBD (Kiesler et al., 2015) and the proposed mechanism of action of GPR4 at promoting leukocytes infiltration, GPR4 inhibition could also be evaluated in the T-cell transfer model (Ostanin et al., 2009). Additionally, it has been suggested GPR4 antagonists could have therapeutic potential in several other diseases, such as angiogenesis and Parkinson's disease (Wyder et al., 2011; Mitzi et al., 2017; Velicky et al., 2017; Haque et al., 2021; Weder et al., 2022), and we cannot exclude Compound 13 could show efficacy in those models.

Despite adequate exposure levels in this study, Compound 13 treatment in IL10−/− mice did not yield significant target engagement (Supplemental Fig. 3), suggesting that the pharmacology of Compound 13 was more complex than previously elucidated. Compound 13 showed decreased potency with decreasing pH with both human and mouse GPR4 and was inactive at pH lower than 6.8. The pH dependence of Compound 13 led us to investigate if other known GPR4 antagonists shared the same property. The molecules disclosed in the literature can broadly be viewed as belonging to the same chemotype, characterized by a trisubstituted bicyclic heterocycle connected to a basic amine (piperazine or piperidine) via a linker. Only the linker part was significantly different among the various series. Of the three additional compounds investigated, the most interesting of them was Compound 2, lacking the basic amine part. We speculated that this feature of the general chemotype might be responsible for the pH dependence via a different interaction with the receptor in its protonated versus unprotonated state. However, we found that the potency of Compound 2 dropped dramatically at pH 6.4, similarly to the other compounds. Our results indicate that the basic amine part of the pharmacophore, while important for activity, was not in itself a determinant of pH dependence. Interestingly, these findings seem consistent with previous observations that those compounds were much more effective at weak acidic pH or neutral pH but less effective at pH lower than 6.8 (Tobo et al., 2015; Hosford et al., 2018). However, those compounds were described as negative allosteric modulators, which is not consistent with the observed pH dependence. The pH dependence observed for all compounds tested here appears to be general for the entire chemotype and is probably related to the mechanism of action and the binding mode of GPR4.

Fig. 5. Compound 13 only binds to GPR4 in its inactive conformation. Binding affinities of Compound 13 to human GPR4 were assessed by SPR at various pHs. To lock GPR4 in either its active or inactive conformation, human GPR4-expressing cells were stimulated by pH 7.9 (A), pH 6.8 (B), or 6.4 (C) adjusted buffers and then fixed right away before flowing Compound 13 (in pH 7.4 buffer) for binding determination. The measured SPR images were divided into about 600 small ROIs, about 20 × 20 μm each. SPR sensorgrams were generated for each ROI for all concentrations injected. The histograms represent the distribution of measured thermodynamic KD from all isothermal curves. The corresponding statistically significant histogram distribution (Supplemental Fig. 6).

Discussion

IBD is a complex, multifactorial inflammatory disease of the gastrointestinal tract with unknown etiology. Acidosis is associated with inflammation, and it has been suggested that pH-sensing receptors, including GPR4, could contribute to the onset and/or worsening of the disease (Imenez Silva and Wagner, 2022). GPR4 is predominantly expressed in endothelial cells and thought to promote leukocyte infiltration and inflammation in the mucosa through the upregulation of inflammatory and stress response genes (Chen et al., 2011; Dong et al., 2013; Kreweski et al., 2020).

While no single mouse model captures the complexity of human IBD, genetic polymorphism at the IL10 locus confers increased risk of both ulcerative colitis and Crohn’s disease (Andersen et al., 2010; Moran et al., 2013). Mice with targeted deletion of (IL10−/−) develop spontaneous inflammation of the colon, which are characterized by the presence of lymphocytes, macrophages, and neutrophils (inflammatory infiltrates). Several Food and Drug Administration-approved therapies for the treatment of IBD, such as anti-IL-12/IL-23, were moderately effective in this model (Kiesler et al., 2015; Mizoguchi et al., 2020). Moreover, GPR4−/− IL10−/− double knockout mice showed protection in the development of severe colitis (Wang et al., 2018), suggesting that IL10−/− colitis mice would be an appropriate model for studying the role of GPR4 in intestinal inflammation. However, Compound 13, a selective GPR4 antagonist, failed to improve colitis in the present study despite having good oral bioavailability. Although the lack of target engagement suggested the pharmacology and mechanism of inhibition of Compound 13 was more complex than initially suggested, we cannot exclude that GPR4 inhibition would show efficacy in other models. One limitation of the IL10−/− colitis mice model is the generation of interferon-γ-producing CD4+ T cells (Th1 type) (Rennick and Fort, 2000), which have been shown to play a critical role in the development of enterocolitis and could confound the potential therapeutic efficacy of Compound 13. GPR4 deficiency has been shown to very mildly improve body weight loss and histopathology scores in the DSS colitis model (Sanderlin et al., 2016; Wang et al., 2018). It is worth noting Compound 13 only showed minimal activity in this model (Sanderlin et al., 2019). Moreover, given the known limitations of the DSS model at recapitulating fundamental aspects of human IBD (Kiesler et al., 2015) and the proposed mechanism of action of GPR4 at promoting leukocytes infiltration, GPR4 inhibition could also be evaluated in the T-cell transfer model (Ostanin et al., 2009). Additionally, it has been suggested GPR4 antagonists could have therapeutic potential in several other diseases, such as angiogenesis and Parkinson's disease (Wyder et al., 2011; Mitzi et al., 2017; Velicky et al., 2017; Haque et al., 2021; Weder et al., 2022), and we cannot exclude Compound 13 could show efficacy in those models.

Despite adequate exposure levels in this study, Compound 13 treatment in IL10−/− mice did not yield significant target engagement (Supplemental Fig. 3), suggesting that the pharmacology of Compound 13 was more complex than previously elucidated. Compound 13 showed decreased potency with decreasing pH with both human and mouse GPR4 and was inactive at pH lower than 6.8. The pH dependence of Compound 13 led us to investigate if other known GPR4 antagonists shared the same property. The molecules disclosed in the literature can broadly be viewed as belonging to the same chemotype, characterized by a trisubstituted bicyclic heterocycle connected to a basic amine (piperazine or piperidine) via a linker. Only the linker part was significantly different among the various series. Of the three additional compounds investigated, the most interesting of them was Compound 2, lacking the basic amine part. We speculated that this feature of the general chemotype might be responsible for the pH dependence via a different interaction with the receptor in its protonated versus unprotonated state. However, we found that the potency of Compound 2 dropped dramatically at pH 6.4, similarly to the other compounds. Our results indicate that the basic amine part of the pharmacophore, while important for activity, was not in itself a determinant of pH dependence. Interestingly, these findings seem consistent with previous observations that those compounds were much more effective at weak acidic pH or neutral pH but less effective at pH lower than 6.8 (Tobo et al., 2015; Hosford et al., 2018). However, those compounds were described as negative allosteric modulators, which is not consistent with the observed pH dependence. The pH dependence observed for all compounds tested here appears to be general for the entire chemotype and is probably related to the mechanism of action and the binding mode of GPR4.
these molecules. Interestingly, the original lead compound in the series, Compound 39c, coded Compound 1a in the original report, was reported to be also active at the histamine H3 receptor (Velevicky et al., 2017), displacing the binding of [3H]-
\( R^- \)-\( \alpha \)-Methylimidazole-2.5(n)/histamine and suggesting a competitive mode of action. Those results combined with the observed pH dependence (Fig. 2) suggested Compound 13 could bind to the conserved orthosteric binding site of GPCRs. Orthosteric antagonists bind to the same site as and compete with the binding of endogenous agonists, so their potency is highly dependent on the concentration of the present agonist (Kenakin and Strachan, 2018). The orthosteric binding site of class A GPCRs is widely conserved (Yanamala and Klein-Seetharaman, 2010; Hedderich et al., 2022). On the other hand, allosteric antagonists bind to a distinct site and modulate the activity of the receptor. While negative allosteric modulators can negatively affect the affinity of the endogenous agonist, this effect is saturable, and the loss of potency of Compound 13 with decreasing pH was more consistent with a competitive orthosteric mode of inhibition (Christopoulos and Kenakin, 2002). Those observations were confirmed through mutagenesis of the main residues in the proposed orthosteric binding pocket (Fig. 4). Those mutations, namely W73A and Y268A, significantly abrogated Compound 13 potency. While we cannot exclude those mutations may indirectly destabilize Compound 13-induced GPR4 inactive conformation, combined with the pH-dependent orthosteric-like behavior of Compound 13 (Fig. 2), those data strongly suggest Compound 13 binds to GPR4 putative orthosteric binding site, making key interactions with W73 and Y268.

Although Compound 13 behaved as an orthosteric antagonist, displaying pH dependence, i.e., loss of potency at low pH (high concentration of agonist present), the concept of orthosteric antagonism for pH sensing receptors remains poorly characterized. It has been proposed that multiple residues in GPR4 are protonated in low pH conditions, resulting in conformation changes necessary for activation (Rowe et al., 2021). Interestingly, H269, a histidine situated in the presently described orthosteric binding site of GPR4, is pivotal for GPR4 function (Rowe et al., 2021). To further confirm the orthosteric nature of Compound 13, we postulated Compound 13 would not be able to bind to GPR4 in presence of its agonist, hence its protonated state in low pH conditions. SPRm measurements largely confirmed Compound 13 preferentially binds to the inactive conformation of GPR4. Thermodynamic analysis was consistent with what was previously observed in that Compound 13 could not bind to GPR4 at pH 6.4.

In light of the present results, we believe Compound 13 is not an ideal tool to study the role of GPR4 in inflammatory conditions. It is well established that the degree of inflammation in IBD is associated with increased acidosis (Taafe et al., 2014; Anderson et al., 2016), and reports indicate that the pH in the colon of IBD patients ranges between 5.5 and 7.0 (Krarup HB, and Vogel U (2010) The polymorphism rs3024505 proximal to IL-10 is

**Conclusion**

Our results indicate that Compound 13 failed to improve colitis in the IL10−/− mouse model of IBD despite good exposures. The lack of apparent and significant target engagement led us to investigate the mechanism of inhibition of Compound 13. Compound 13 binds to the conserved GPCRs orthosteric binding site, so it behaves as an orthosteric antagonist, only binding to GPR4 in its inactive, unprotonated state. As the degree of inflammation is generally correlated with increased acidity, Compound 13 is unlikely the appropriate compound for studying GPR4 and its role in inflammatory bowel conditions.

**Acknowledgments**

The authors would like to thank Adanze Onwuliri and Chantelle Rein-Smith of Whitsell Innovations, Inc. (Chapel Hill, NC, USA) for editorial assistance during manuscript preparation, funded by Ferrin Pharmaceuticals.

**Data Availability**

The data that support the findings of the RNAseq study are openly available at https://www.ncbi.nlm.nih.gov/geo/: GEO accession GSE225694. All other data presented are contained within the manuscript/supplemental data.

**Authorship Contributions**

*Participated in research design:* Stalewski, Papazyan, Fuchs, Rives.

*Conducted experiments:* Stalewski, Shih, Papazyan, Ramirez, Ibanez, Yin, Badger, Wu, Ueki, Fuchs, Rives.

*Contributed new reagents or analytic tools:* Stalewski.

*Performed data analysis:* Stalewski, Shih, Papazyan, Ibanez, Hsiao, Yue, Yin, Badger, Wu, Ueki, Fuchs, Rives.

*Wrote or contributed to the writing of the manuscript:* Stalewski, Shih, Papazyan, Yin, Rives.

**References**

*Andersen V, Errot A, Christensen J, Østergaard M, Jacobsen BA, Tjønneland A, Kragstrup HB, and Vogel U (2010) The polymorphism rs3024505 proximal to IL-10 is
associated with risk of ulcerative colitis and Crohn's disease in a Danish case-control study. *BMJ Genet* 11:82.


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