INTRODUCTION

Inflammation is a type of defense response activated to protect organisms from detrimental factors, such as pathogens and tissue damage. Imbalance in inflammatory mechanisms can lead to chronic disorders, such as inflammatory bowel disease (IBD). The adaptive immune system is mainly involved in the pathogenesis of IBD, but some innate immune cells are also implicated (de Mattos et al., 2015). While many small-molecule drugs, such as aminosalicylates and corticosteroids, and biological molecules, for instance, infliximab and adalimumab, induce clinical remission in around 50% of the patients, novel therapies are still needed.

Abstract

GPR43 (also known as FFAR2), a metabolite-sensing G-protein-coupled receptor stimulated by short-chain fatty acid (SCFA) ligands is involved in innate immunity and metabolism. GPR43 couples with Gαi/o and Gαq/11 heterotrimeric proteins and is capable of decreasing cyclic AMP and inducing Ca2+ flux. The GPR43 receptor has additionally been shown to bind β-arrestin 2 and inhibit inflammatory pathways, such as NF-κB. However, GPR43 shares the same ligands as GPR41, including acetate, propionate, and butyrate, and determination of its precise functions in association with endogenous ligands, such as SCFAs alone, therefore remains a considerable challenge. In this study, we generated novel synthetic agonists that display allosteric modulatory effects on GPR43 and downregulate NF-κB activity. In particular, the potency of compound 187 was significantly superior to that of pre-existing compounds in vitro. However, in the colitis model in vivo, compound 110 induced more potent attenuation of inflammation. These novel allosteric agonists of GPR43 clearly display anti-inflammatory potential, supporting their clinical utility as therapeutic drugs.

Key Words: GPR43, Allosteric agonists, Anti-inflammation, NF-κB
patients, their toxicity, anti-drug antibody production, and ef-
cfficacy properties remain to be established.

Short-chain fatty acids (SCFAs) including acetate, propio-
nate, and butyrate produced from gut microbiota elicit several
effects on host metabolism and the immune system (Tedelind
et al., 2007; Khan et al., 2014). SCFAs play important roles
in the colonic mucosa (Parada Venegas et al., 2019). These
compounds are used as a fuel source by intestinal epithelial
cells and have the ability to reduce pro-inflammatory mediators
as well as increase anti-inflammatory factors. Following deor-
phanization of GPR43 as a SCFA-specific receptor (Brown
et al., 2003; Trompette et al., 2014), several efforts have been
made to elucidate the mechanisms by which GPR43 mediates
SCFA activity in IBD. In an acute and chronic dextran sulfu-
rate sodium (DSS)-induced colitis animal model, higher disease
activity and inflammation were detected in GPR43 KO mice
compared to their wild-type siblings. Furthermore, mice ad-
ministered weak GPR43 agonists appeared less susceptible
to colitis than vehicle control, suggesting that GPR43 medi-
ates the anti-inflammatory effects of SCFAs in IBD (Maslowski
et al., 2009; Masui et al., 2015; Agus et al., 2010). Subsequently, AZ1729 was established as a posi-
tive allosteric agonist of Gαi signaling and negative alloster-
ic antagonists of GPR43 against IBD and other inflam-
matory diseases.

Potential and selective modulators of GPR43 other than SC-
FAs may serve as an effective tool to address this discrepancy.
To date, several research groups have attempted to develop synthetic selective GPR43 modulators. The first syn-
thetic set of compounds generated were phenylacetamide der-
ivatives, which acted as allosteric agonists with significantly
improved potency relative to SCFAs (Lee et al., 2008; Wang et
al., 2010). Subsequently, AZ1729 was established as a posi-
tive allosteric agonist of Gαi signaling and negative allosteric
agonist of Gαq signaling for human GPR43 (Bolognini et al.,
2016). While these modulators are selective for GPR43, there
remains an urgent need for agonists with greater potency in
the clinic.

A newly published patent provided a series of GPR43 ago-
nists that require further characterization (Barker et al., 2015).
Data from the current study showed that two of the above
novel agonists are selective for GPR43, but not GPR41, and
act as positive allosteric modulators. In addition, the associ-
ated mechanisms appear to be G-protein signaling-biased,
since β-arrestin 2 agonism was markedly weaker than that of
acetate. Moreover, the two agonists induced more potent in-
hibition of the NF-κB pathway than acetate and phenylacetate
aminothiazole (PAAT). Moreover, one of the allosteric modula-
tors, compound 110, attenuated disease activity and elicited
an increase in colon length in a DSS-induced colitis mouse
model. Our collective data support the therapeutic potential
of allosteric agonists for GPR43 against IBD and other inflam-
matory diseases.

MATERIALS AND METHODS

Reagents

Compound 110 (2-methyl-4-(3-methylpiperidin-1-yl)-7-(phe-
nylsulfonyl)-8H-pyrido[3,2-d]pyrimidine-6-amine), compound
187 (4-[(2RS,6S)-2,6-dimethylmorpholin-4-yl]-7-(2-fluorobenzo-
nesulfonyl)-2-methyl-8H-pyrido[3,2-d]pyrimidine-6-amine;
Barker et al., 2015), and phenylacetyl aminothiazole (PAAT)
((S)-2-(4-chlorophenyl)-N-(5-fluorothiazol-2-yl)-3-methylbutan-
amide; Wang et al., 2010) were synthesized according to the
patent guidelines. Compounds 110 and 187 were completely
soluble in dimethyl sulfoxide (DMSO) at a concentration of 20
mM. sodium acetate and forskolin were obtained from Sigma
Aldrich (St. Louis, MO, USA), AR420606 (Cat No. 17531)
from Cayman Chemical (Ann Arbor, MI, USA), recombinant human
TNF-α (300-01A) from PeproTech (Rockey Hill, NJ, USA), and
cumate stock (10,000X) from System Biosciences (Palo Alto,
CA, USA).

Cell culture and plasmid transfection

Cumate-inducible human GPR43-expressing HEK293 cells
(HEK293-hGPR43) were maintained in Dulbecco’s Modified
Eagle’s Medium (DMEM; Welgene, Gyeongsan, Korea) sup-
plemented with 10% FBS (Invitrogen, Carlsbad, CA, USA),
1X GlutaMAX (Invitrogen), 1% Pen-Strep, and 2 µg/mL puro-
mycin under 5% CO2 at 37°C. pGloSensor-22F (E2301) and
pGL4.32luc2P/NF-κB-RE/Hygro were purchased from Pro-
mega (Madison, WI, USA). The GPR43-SmBit-IRE5-LgBit-
Jarr2 construct containing SmBit and LgBit derived from the
Nano-Glo® Luciferase Assay System (Promega) was cloned
into pBS101B1 (System Biosciences). Cells were transfected
with the relevant plasmids using X-tremeGENE™ HP DNA
Transfection Reagent (Roche, Penzberg, Germany) accord-
ing to the manufacturer’s protocol. Cell lysis and western blot
were performed as described previously (Park et al., 2016).

GloSensor, Ca2+ flux, and Nano-Glo live cell assays

At 24 h after transfection of HEK293-hGPR43 cells with
pGloSensor-22F, cells were re-plated in 96-well white plates
for 16 h. The medium was replaced with CO2-independent
medium (Gibco, Carlsbad, CA, USA) including 2% GloSen-
sor cAMP reagent (Promega) and cells incubated at 20°C for
2 h. After obtaining pre-read measurements for normaliza-
tion of data, cells were treated with the test compounds for 5
min. Forskolin (1 µM) was added to cells for a further 15 min
and post-read measurements obtained. Luminescence val-
ues were measured using FlexStation 3 ( Molecular Devices,
Sunnyvale, CA, USA). The cyclic AMP level was normalized
by dividing post-read by pre-read values and further normal-
ized to the 0.2% DMSO treatment group.

For measuring Ca2+ flux, HEK293-hGPR43 cells were
seeded into a 96-well black clear-bottomed plate overnight.
Calcium 6 Assay reagent (R8190, Molecular Devices) with 2.5
mM probenecid (Sigma Aldrich) was added to cells for 2 h.
The fluorescence of the sample was automatically read at an
excitation wavelength of 485 nm and emission wavelength of
525 nm in FlexStation 3 every 2.1 s for 50 s.

For measuring interactions between GPR43 and β-arrestin
2, cells transfected with GPR43-SmBit and LgBit-Jarr2 were
seeded into 96-well white plates overnight. Cells were treated
with test compounds for 25 min after replacing the medium
with FBS-free DMEM and incubated under 5% CO2 at 37°C.
for 1 h. The Nano-Glo Live cell assay (N2011, Promega) was performed according to the manufacturer’s protocol.

**NF-κB luciferase reporter assay**

HEK293-hGPR43 cells were transfected with NF-κB luciferase reporter plasmid (Promega), incubated under 5% CO2 and 37°C for 24 h, and plated in 96-well white plates overnight. The next day, cells were treated with the relevant compounds for 20 min, followed by 10 ng/mL TNF-α, and incubated at 37°C for a further 6 h. Luminescence was measured using FlexStation 3 at the 15 min time-point after adding One-Glo assay reagent (Promega) to each well.

**Animals**

Female C57BL/6 mice (5 weeks old) supplied by Laboratory Animal Resource Center at the Korea Research Institute of Bioscience and Biotechnology (Cheongju, Korea) were housed under specific pathogen-free conditions. Rooms were maintained under a 12 h light-dark cycle at 21 ± 2°C. Animals were allowed to acclimate to the local environment for one week before experimental use.

**Animal model of colitis**

Colitis was induced as described previously (Wirtz et al., 2007). To induce acute colitis, mice were provided 2% dextran sodium sulfate (DSS; 36-50 KDa, MP Biomedicals, CA, USA) dissolved in sterile distilled water ad libitum. DSS solutions were freshly made every 3 days. Compounds 110 (30 mg/kg, p.o.) and 187 (30 mg/kg, p.o.) were administered daily. Fecal occult blood was assessed using a Tri-Slide stool blood test kit (Cenogenics Corporation, NJ, USA). On day 8, the entire colon was excised and colon length measured. All animal experiments were approved by the Institutional Animal Care and Use Committee of Korea Research Institute of Bioscience and Biotechnology (Approval #: KRIBB-AEC-17119).

**Determination of clinical scores**

Body weight, stool consistency and rectal bleeding were examined daily. The baseline clinical score was determined on day 0. In terms of clinical score assessment, no weight loss was registered as 0 points, weight loss of 1-5% from baseline as 1 point, 6-10% as 2 points, 10-20% as 3 points and >20% as 4 points. For stool consistency, well-formed pellets were assigned 0 points, slightly loose pellets 1 point, very soft pellets 2 points and diarrhea 3 points. For rectal bleeding, 0 points were assigned for negative hemoccult, 1 point for positive hemoccult, 2 points for bloody stool and 3 points for gross bleeding. Daily activity index was calculated by summation of clinical scores for body weight, stool consistency and rectal bleeding.

**Immunohistochemistry**

Harvested colons were washed with cold PBS, cut longitudinally, swiss-rolled and fixed with 4% paraformaldehyde (Junsei Chemical Co. Ltd., Tokyo, Japan). Paraffin sections were deparaffinized, rehydrated and stained with primary antibody.
Table 1. EC<sub>50</sub> values of GPR43 agonists

<table>
<thead>
<tr>
<th></th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>β-Arrestin 2</th>
<th>NF-κB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>300.7</td>
<td>57.72</td>
<td>1,438</td>
<td>77.38</td>
</tr>
<tr>
<td>PAAT</td>
<td>0.089</td>
<td>7.673</td>
<td>4.15</td>
<td>12.06</td>
</tr>
<tr>
<td>Compound 110</td>
<td>18.65</td>
<td>2.410</td>
<td>1.609</td>
<td>3.406</td>
</tr>
<tr>
<td>Compound 187</td>
<td>0.016</td>
<td>0.019</td>
<td>0.018</td>
<td>0.019</td>
</tr>
</tbody>
</table>

against myeloperoxidase (Abcam, Cambridge, UK). After incubation with biotinylated secondary antibody, sections were developed using VECTASTATIN® ABC kit (VECTOR Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin.

**Statistical analysis**

Assays and immunoblot experiments were performed in duplicate or triplicate and data presented as means ± SEM. All statistical analyses were performed using Microsoft Excel program, p-values < 0.05 were considered significant with Student’s t-test.

**RESULTS**

**Compounds 110 and 187 are specific agonists of GPR43**

To ascertain whether both compounds 110 and 187 act specifically on GPR43 (Fig. 1), the cAMP assay was performed using GPR41- and GPR43-expressing HEK293 cell lines. The SCFAs acetate and propionate induced a decrease in cAMP level in both cell lines (Fig. 2A), supporting their selectivity for GPR43. We further evaluated the potency of these modulators. In the cAMP assay, the EC<sub>50</sub> value calculated for acetate was 300 µM, while that for PAAT was 89 nM with lower maximum efficacy (Fig. 2B, Table 1). Consistently, 187 showed micromolar potency, while compound 187 had significantly greater potency (EC<sub>50</sub> of 16 nM; Fig. 2B, Table 1). SCFAs acetate and the indicated agonists.

**Compounds 110 and 187 are positive allosteric agonists for GPR43**

To elucidate the mechanisms of action of these compounds, cells were treated with various concentrations of agonists and acetate for measurement of Ca<sup>2+</sup> flux and β-arrestin-2 using the NanoBiT assay. Cells were subjected to serum starvation, and activity of PAAT, a known allosteric agonist of GPR43, was initially assessed (Wang et al., 2010). It was observed that EC50 values of PAAT gradually decreased as the increasing concentrations of acetate in Ca<sup>2+</sup> flux assay, while maximum efficacy of PAAT was not altered (Fig. 3A). In the β-arrestin-2 assay, both basal activity and maximum efficacy were elevated and the potency of PAAT was increased by acetate (Fig. 3B), supporting allosteric activity of PAAT on GPR43. Compounds 110 and 187 showed a similar pattern in the calcium assay (Fig. 3C, 3E). Interestingly, addition of acetate to 110 and 187 treatment groups induced stiffer slopes in the β-arrestin-2 NanoBiT assay (Fig. 3D, 3F). Our results strongly suggest that compounds 110 and 187 act as positive allosteric agonists for GPR43.

**Both GPR43 agonists inhibit NF-κB activity**

SCFAs have been shown to exert anti-inflammatory effects in a variety of disease models (Di Sabatino et al., 2005; Cox et al., 2009; Maslowski et al., 2009). Previously, our group demonstrated that β-arrestin-2 mediates inhibition of the NF-κB pathway by GPR43 (Lee et al., 2013). To elucidate the activities of our novel compounds in inflammation, we performed a...
NF-κB luciferase reporter assay and compared the potencies of compounds 110 and 187 along with PAAT. Compound 187 had an EC50 of 19 nM (Fig. 4A, Table 1), similar to its potency in the cAMP, calcium flux and β-arrestin-2 assays (Fig. 2B-2D, Table 1), while PAAT and 110 displayed markedly lower potencies (12 μM and 3.4 μM, respectively; Fig. 4A, Table 1). Notably, acetate inhibited NF-κB luciferase reporter activity with half the efficacy of these allosteric agonists without a significant change in potency relative to cAMP and calcium assays. In addition, compounds 110 and 187 inhibited NF-κB luciferase reporter activity with similar efficacy as PAAT, implying that β-arrestin-2 is not the sole mediator of anti-inflammatory signaling. To validate results from the reporter assay, we examined the extent of IκBα degradation induced by TNF-α via immunoblot analysis. IκBα destabilization by TNF-α was significantly rescued following the addition of orthosteric and allosteric agonists (Fig. 4B, 4C).

Dextran sodium sulfate (DSS)-induced colitis is attenuated by an allosteric agonist

Several reports suggest that GPR43 and its ligand effectively attenuate inflammatory disease pathogenesis (Maslowski et al., 2009; Masui et al., 2013; Smith et al., 2013) while other studies have described the opposite phenotype in animal disease models (Sina et al., 2009; Kim et al., 2013). To establish whether these novel allosteric agonists exert beneficial effects on immune responses, we induced colitis chemically by adding 2% dextran sulfate sodium (DSS) to drinking water as described in Materials and Methods. Surprisingly, compound 110 displaying markedly lower in vitro activity than 187 induced a small but significant decrease in the inflammatory response compared to vehicle-treated mice. Mice treated with DSS and compound 110 (30 mg/kg) showed a decrease in stool consistency score and daily activity index (DAI; combined measure of weight loss, rectal bleeding and stool consistency; Fig. 5A-5E). Moreover, decreased colon length in DSS-treated mice was significantly restored in the group treated with compound 110 (Fig. 5F). To examine the status of inflammation in colon, expression of myeloperoxidase (MPO) was measured. Increased MPO staining was observed in DSS-treated mice and the level of MPO was reduced by compound 110 and 187 (Fig. 5G). Our collective results support an anti-inflammatory effect of agonists for GPR43 in inflammatory bowel disease.

DISCUSSION

Short-chain fatty acids are primarily produced via gut microbiota metabolism of indigestible dietary fiber (den Besten et al., 2013). These compounds serve as energy sources in most tissues and exert multiple physiological effects through stimulation of relevant receptors. Although slight differences may be observed depending on the ingested diet, SCFAs generated from the gut commensal flora are present in cecum and colon at millimolar (mM) levels (Topping and Clifton, 2001).

GPR43 was initially identified in the intestinal epithelium and L cells that secrete incretin hormones, such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Tolhurst et al., 2012). GPR43 is additionally expressed not only in adipocytes and pancreatic β-cells but also innate immune-related cells, such as macrophages and neutrophils (Kimura et al., 2013; McNelis et al., 2015; Nakajima et al., 2017). A recent study demonstrated that GPR43 activated by acetate in pulmonary epithelial cells prevents respiratory syncytial virus (RSV) infection and exerts antiviral effects via induction of type 1 interferon response (Antunes et al., 2019). Interestingly, acetate treatment promotes NF-κB activation and protects against RSV infection through initiating the interferon-β response in a GPR43-dependent manner. Our group previously demonstrated that NF-κB signaling is negatively modulated via the GPR43-β-arrestin 2 pathway using acetate and PAAT in HeLa cells (Lee et al., 2013). Interestingly, compounds 110 and 187, G protein-biased allosteric agonists of GPR43, also potently inhibited NF-κB signaling in HEK293 cells in the current study (Fig. 4). One explanation for this finding is that the weak activity of 110 and 187 on the β-arrestin 2 pathway may be sufficient to inhibit NF-κB signaling. Another possibility is that G protein signaling is required for attenuation of NF-κB signaling along with β-arrestin 2. Furthermore, we cannot exclude the likelihood that differences in the cell lines used may be accountable. In addition, our unpublished data recently showed that GPR43 is specifically expressed in M1 macrophage-like cells derived from THP-1 cell line and might mitigate the transition to M2-like status (under review), suggesting that GPR43
has pleiotropic functions in inflammatory processes.

Numerous investigations on the involvement of GPR43 in IBD have employed acetate or butyrate as an active ligand. However, SCFAs can activate GPR41 and GPR109A in addition to GPR43 and, in the case of butyrate, inhibit histone deacetylases (Parada Venegas et al., 2019). Accordingly, care should be exercised while evaluating results from studies using SCFAs. Potent and selective modulators of GPR43 are necessary to assess its physiological and therapeutic functions. In this study, novel compounds 110 and 187 selectively stimulated GPR43, with no effects on GPR41 (Fig. 2A). Moreover, compound 187 was markedly more potent than 110 and PAAT in vitro (Fig. 2B-2D, 4A). However, only compound 110 induced significant restoration of Daily Activity Index and colon length in the DSS-induced colitis animal model (Fig. 5). One possible reason is that 187 is not active on mouse GPR43. Indeed, physiologic ligands, such as SCFAs, are reported to activate human and mouse GPR43 with altered potencies and even synthetic compounds, such as CATPB and BTI-A-404, modulate human orthologs only (Hudson et al., 2012; Park et al., 2016). The pharmacokinetic properties of 187 may thus not be favorable in the murine system, highlighting the necessity of further characterization of these compounds.

In summary, we have identified compounds 110 and 187 as novel allosteric agonists for GPR43 that are capable of attenuating inflammation. Including this finding, further structure-activity relationship analyses and mechanism of action study should reveal more potent anti-inflammatory candidates with improved therapeutic efficacy in vivo.

CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

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