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Novel GPR43 Agonists Exert an Anti-Inflammatory Effect in a Colitis Model

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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 $G\alpha_{i/o}$ and $G\alpha_{q/11}$ heterotrimeric proteins and is capable of decreasing cyclic AMP and inducing Ca^{2+} 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

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

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patients, their toxicity, anti-drug antibody production, and efficacy properties remain to be established.

Short-chain fatty acids (SCFAs) including acetate, propionate, 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 deorphanization 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 sulfate 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 administered weak GPR43 agonists appeared less susceptible to colitis than vehicle control, suggesting that GPR43 mediates the anti-inflammatory effects of SCFAs in IBD (Maslowski *et al*., 2009; Masui *et al*., 2013; Macia *et al*., 2015; Agus *et al*., 2016). However, conflicting reports have demonstrated that GPR43 KO mice with chemical and pathogen-induced colitis show less colonic inflammation compared to their wild-type littermates (Sina *et al*., 2009; Kim *et al*., 2013). They suggested that GPR43 induces neutrophil migration toward damaged tissue in the gut, resulting in elevation of inflammatory responses to defense the infection with residential bacteria. This contradiction need to be resolved.

Potent and selective modulators of GPR43 other than SCFAs 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 synthetic set of compounds generated were phenylacetamide derivatives, 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 positive 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 agonists 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 associated 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 inhibition of the NF-κB pathway than acetate and phenylacetyl aminothiazole (PAAT). Moreover, one of the allosteric modulators, 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 inflammatory diseases.

MATERIALS AND METHODS

Reagents

Compound 110 (2-methyl-4-(3-methylpiperidin-1-yl)-7-(phenylsulfonyl)-5H-pyrrolo[3,2-*d*]pyrimidine-6-amine), compound 187 (4-[(2R,6S)-2,6-dimethylmorpholin-4-yl]-7-(2-fluorobenzenesulfonyl)-2-methyl-5H-pyrrolo[3,2-d]pyrimidin-6-amine; Barker *et al*., 2015), and phenylacetyl aminothiazole (PAAT) ((S)-2-(4-chlorophenyl)-N-(5-fluorothizol-2-yl)-3-methylbutanamide; 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 (Rocky 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) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 1X GlutaMAX (Invitrogen), 1% Pen-Strep, and 2 μg/mL puromycin under 5% CO_2 at 37°C. pGloSensor-22F (E2301) and pGL4.32[luc2P/NF-κB-RE/Hygro] were purchased from Promega (Madison, WI, USA). The GPR43-SmBit-IRES-LgBit-βarr2 construct containing SmBit and LgBit derived from the Nano-Glo® Luciferase Assay System (Promega) was cloned into pB510B1 (System Biosciences). Cells were transfected with the relevant plasmids using X-tremeGENE™ HP DNA Transfection Reagent (Roche, Penzberg, Germany) according to the manufacturer's protocol. Cell lysis and western blot were performed as described previously (Park *et al*., 2016).

GloSensor, Ca^{2+} 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 CO_2-independent medium (Gibco, Carlsbad, CA, USA) including 2% GloSensor cAMP reagent (Promega) and cells incubated at 20°C for 2 h. After obtaining pre-read measurements for normalization 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 values 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 normalized to the 0.2% DMSO treatment group.

For measuring Ca^{2+} 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-βarr2 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% CO_2 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% CO_2 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

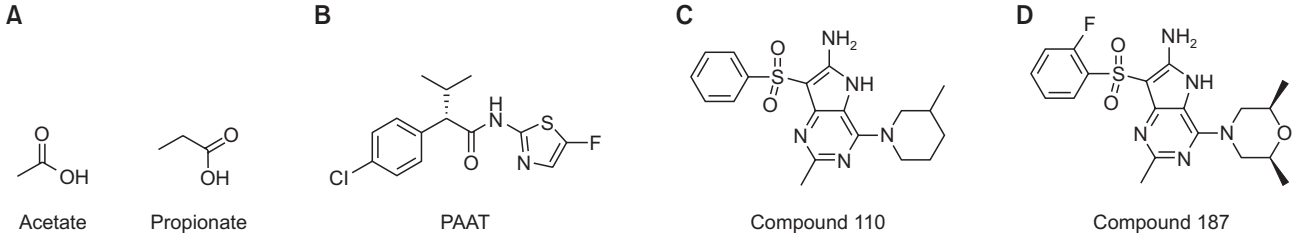

Fig. 1. Structures of compounds used in this study. (A) Endogenous ligands acetate and propionate. (B) Phenylacetyl aminothiazole, PAAT. (C) Compound 110; 2-methyl-4-(3-methylpiperidin-1-yl)-7-(phenylsulfonyl)-5*H*-pyrrolo[3,2-*d*]pyrimidine-6-amine. (D) Compound 187; 4-[(2R,6S)-2,6-dimethylmorpholin-4-yl]-7-(2-fluorobenzenesulfonyl)-2-methyl-5H-pyrrolo[3,2-d]pyrimidin-6-amine.

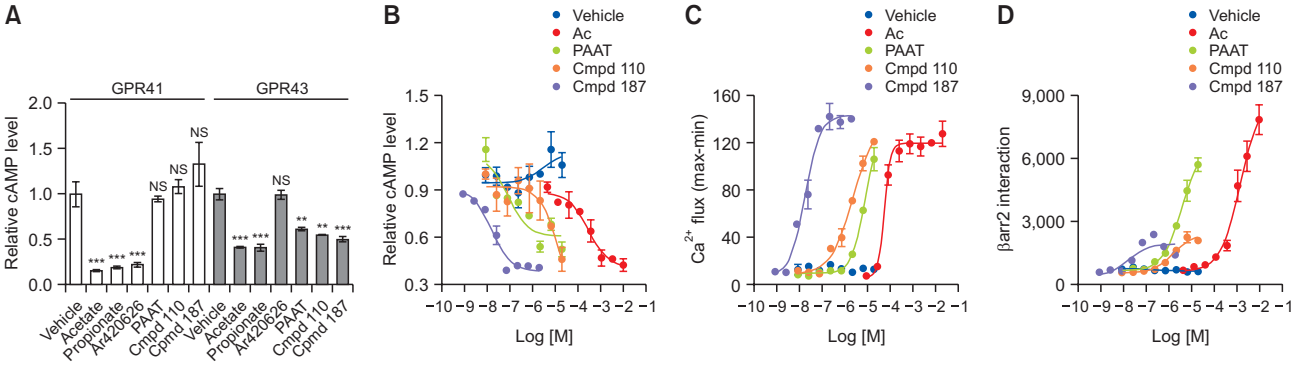

Fig. 2. Both compounds 110 and 187 are selective agonists for GPR43. (A) cAMP levels of GPR41 and GPR43-expressing cells treated with the indicated reagents. Acetate and propionate (10 mM) were added. AR420626, PAAT, compound 110, and compound 187 (10 μM) were added. Data are presented as mean values ± SEM from triplicate experiments. *p* values <0.05 were significant (**0.005<*p*<0.01, ***p<0.005). Measurement of (B) cAMP level, (C) Ca^{2+} flux, and (D) Nano Live Glo luminescence to evaluate the extent of interactions with GPR43 and β-arrestin 2 according to the concentrations of the indicated reagents.

Table 1. EC_{50} values of GPR43 agonists

	EC_{50} (μM)			
	cAMP	Ca^{2+}	β-Arrestin 2	NF-κB
Acetate	300.7	57.72	1,438	77.38
PAAT	0.089	7.673	4.15	12.06
Compound 110	18.65	2.410	1.609	3.406
Compound 187	0.016	0.019	0.018	0.019

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) while AR420626 exerted activity specifically on GPR41-expressing cells and PAAT on GPR43-expressing cells (Fig. 2A). Compounds 110 and 187 significantly suppressed cAMP levels in GPR43-expressing cell lines only (Fig. 2A), supporting their selectivity for GPR43. We further evaluated the potency of these modulators. In the cAMP assay, the EC_{50} value calculated for acetate was 300 μM, while that for PAAT was 89 nM with lower maximum efficacy (Fig. 2B, Table 1). Compound 110 showed micromolar potency, while compound 187 had significantly greater potency (EC_{50} of 16 nM; Fig. 2B, Table 1). Consistently, 187 displayed highest potency and efficacy in the calcium assay (Fig. 2C, Table 1). In contrast, 110 and 187 were determined as partial agonists in the β-arrestin assay than acetate and PAAT in terms of their maximum efficacy (Fig. 2D, Table 1). Our collective results indicate that compounds 110 and 187 are involved in G-protein signaling.

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^{2+} 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^{2+} flux assay, while maximum efficacy of PAAT was not altered (Fig. 3A). In the β-arrestin-2 assay, both basal activity and maximum efficacy

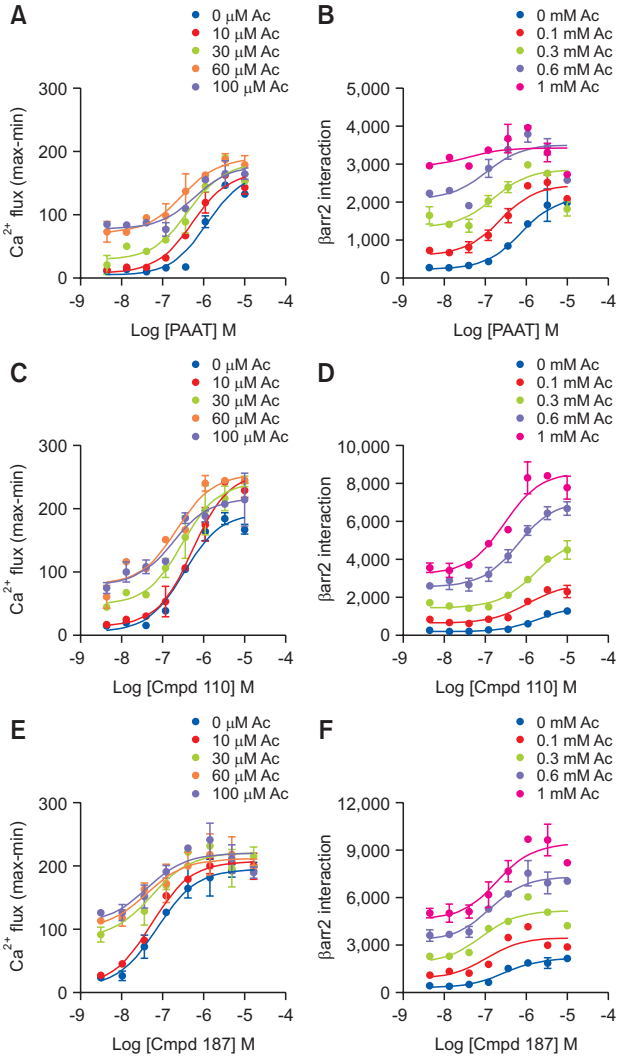

Fig. 3. Novel agonists for GPR43 with allosterism. Various fixed concentrations of acetate were added to Ca^{2+} and Nano Live Glo assay to examine interactions between GPR43 and β-arrestin 2 for (A, B) PAAT, (C, D) compound 110, and (E, F) compound 187. GPR43-expressing HEK293 cells were simultaneously treated with acetate and the indicated agonists.

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

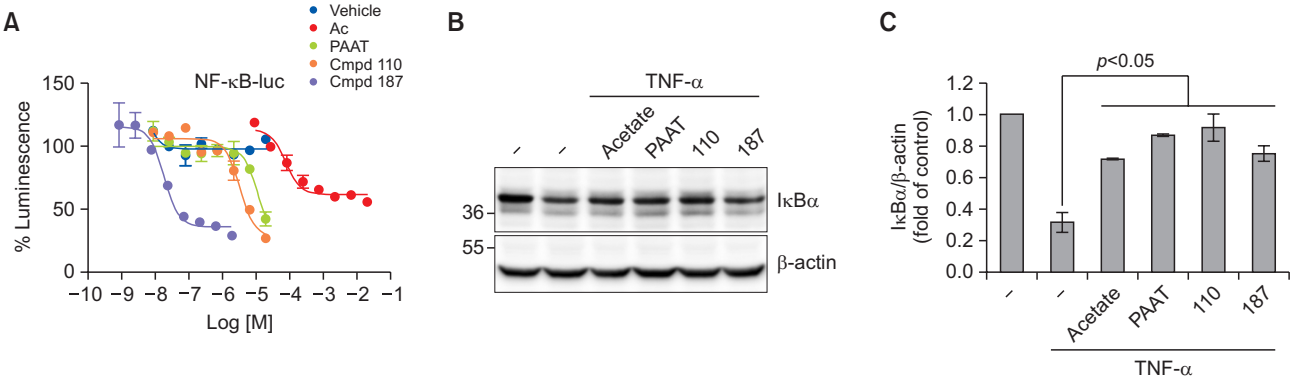

Fig. 4. Inhibition of NFκB activity by activated GPR43. (A) Concentration responses of the indicated agonists in downregulation of NF-κB activity induced by 10 ng/mL TNF-α from GPR43-expressing cells. (B) Reduced IκBα via stimulation of TNF-α was rescued by treatment with agonists for GPR43. (C) Quantification of total IκBα: β-actin ratio analyzed using Image J software. Data are expressed as means ± SEM from three independent experiments with Student's *t*-test. *p* values <0.05 were considered significant.

NF-κB luciferase reporter assay and compared the potencies of compounds 110 and 187 along with PAAT. Compound 187 had an EC_{50} 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

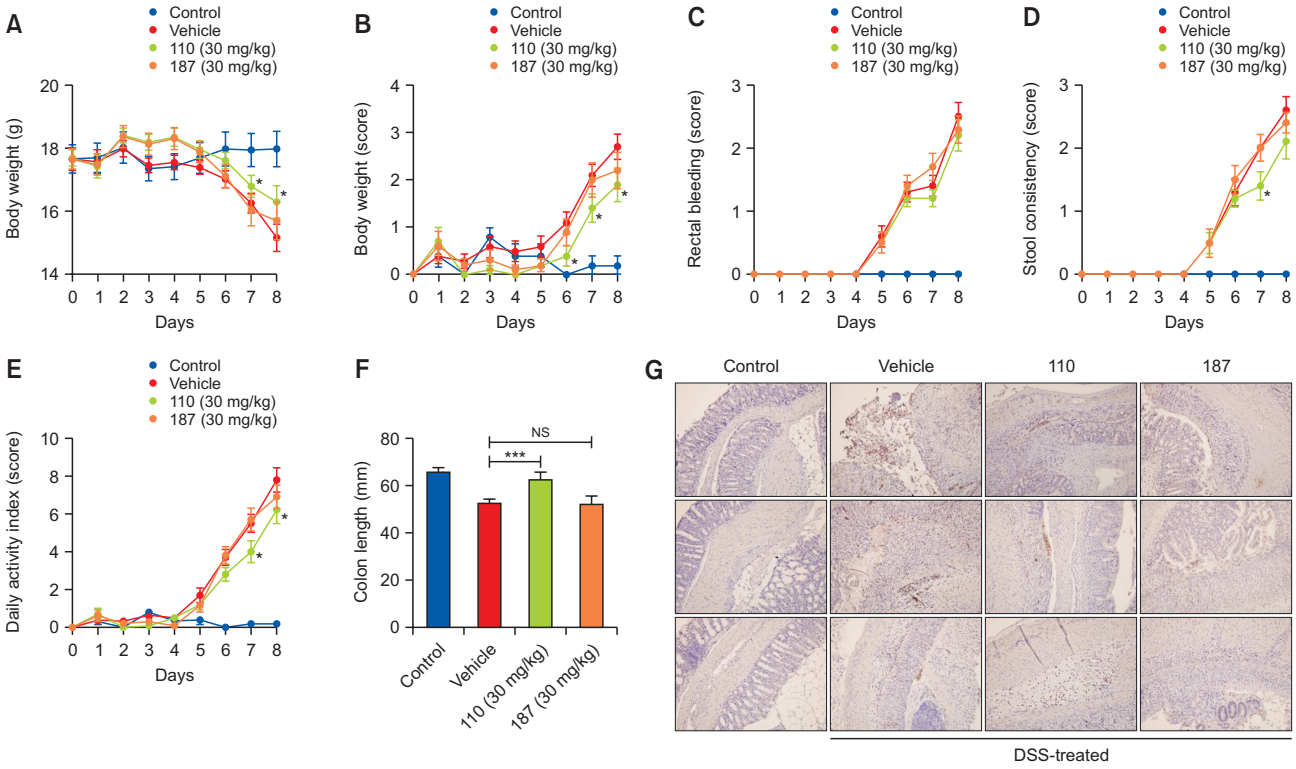

Fig. 5. Attenuation of DSS-induced colitis by the novel GPR43 agonists. Colitis was induced as described in Materials and Methods. The phenotypes were examined every day. Daily activity index (E) was calculated by summation of clinical scores for body weight (A, B), rectal bleeding (C) and stool consistency (D). On day 8, the entire colon was excised and colon length measured (F). The excised colon was subject to MPO staining (G). The representative samples from three mice were shown. n=5 in Control and n=10 in other groups. Data are expressed as means ± SEM from three independent experiments with Student's *t*-test. **p*<0.05, ****p*<0.005 were considered significant.

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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