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Immunopharmacology and inflammation

KAG-308, a newly-identified EP_4-selective agonist shows efficacy for treating ulcerative colitis and can bring about lower risk of colorectal carcinogenesis by oral administration

Yusuke Watanabe [a,*], Takahiko Murata [b], Masahiro Amakawa [a], Yoshihide Miyake [a], Tango Handa [a], Katsuhiko Konishi [c], Yasushi Matsumura [d], Takuji Tanaka [e], Koji Takeuchi [f]

[a] Pharmacology Department, Drug Research Center, Kaken Pharmaceutical Co., Ltd, 14 Shinomiya, Minamigawara-cho, Yamashina-ku, Kyoto 607-8042, Japan
[b] Drug Research Center, Kaken Pharmaceutical Co., Ltd, 14 Shinomiya, Minamigawara-cho, Yamashina-ku, Kyoto 607-8042, Japan
[c] Life Science Division, AGC Chemicals, Asahi Glass Co., Ltd., 1-5-1 Marunouchi, Chiyoda-ku, Tokyo 100-8405, Japan
[d] Research and Development Division, AGC Chemicals, Asahi Glass Co., Ltd, 1150 Hazawa-cho, Kanagawa-ku, Yokohama 221-8755, Japan
[e] Department of Diagnostic Pathology (DDP) & Research Center of Diagnostic Pathology (RC-DiP), Gifu Municipal Hospital, 7-1 Kashima-Cho, Gifu 500-8513, Japan
[f] Kyoto Pharmaceutical University, 5 Misasagi-Nakauchicho, Yamashina-ku, Kyoto 607-8414, Japan

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ABSTRACT

Agonists for EP_4 receptor, a prostaglandin E_2 receptor subtype, appear to be a promising therapeutic strategy for ulcerative colitis (UC) due to their anti-inflammatory and epithelial regeneration activities. However, the clinical development of orally-available EP_4 agonists for mild to moderate UC has not yet been reported. Furthermore, the possibility of an increased risk of colitis-associated cancer (CAC) through direct proliferative effects on epithelial cells via EP_4 signaling has not been ruled out. Recently, we identified KAG-308 as an orally-available EP_4-selective agonist. Here, we investigated the pharmacological and pharmacokinetic profiles of KAG-308. Then, we compared KAG-308 and sulfasalazine (SASP) for their abilities to prevent colitis and promote mucosal healing in a mouse model of dextran sulfate sodium (DSS)-induced colitis. Finally, the effect of KAG-308 treatment on CAC was evaluated in an azoxymethane (AOM)/DSS-induced CAC mouse model. KAG-308 selectively activated EP_4 and potently inhibited tumor necrosis factor-α production in peripheral whole blood and T cells. Oral administration of KAG-308, which showed relatively high bioavailability, suppressed the onset of DSS-induced colitis and promoted histological mucosal healing, while SASP did not. KAG-308 also prevented colorectal carcinogenesis by inhibiting colitis development and consequently decreasing mortality in a CAC model, whereas SASP had marginal effects. In contrast, MF-482, an EP_4 antagonist, increased mortality. These results indicated that orally-administered KAG-308 suppressed colitis development and promoted mucosal healing. Moreover, it exhibited preventive effects on colorectal carcinogenesis, and thus may be a new therapeutic strategy for the management of UC that confers a reduced risk of colorectal carcinogenesis.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with which patients experience intermittent remission and relapse over many years (Ghosh and Mitchell, 2007). The chronic inflammation increases the risk of colitis-associated cancer (CAC) and leads to CAC-related death; thus, CAC is regarded as a serious complication of IBD (Munkholm, 2003; Harpaz and Talbot, 1996). 5-Aminosalicylic acid (5-ASA) is currently used as a first-line therapy for mild-to-moderate UC (Tindall et al., 2007). In the clinic, treatment with 5-ASA therapy in active UC patients is useful; however, the ratio of refractory or relapsed cases is also comparatively high (Nielsen and Munck, 2007; Azad et al., 1977). Furthermore, the chemoprophylactic effects of 5-ASA on CAC remains controversial in UC patients (Bernstein et al., 2011; Van Staa et al., 2005).

The prostaglandin E_2 (PGE_2) and EP_4 receptor, a PGE_2 receptor subtype, plays a critical role in the suppression of inflammation and in the maintenance and repair of intestinal mucosa (Tanaka et al., 2009; Hatazawa et al., 2006; Kabashima et al., 2002). Several

* Corresponding author. Tel.: +81 75 594 0787; fax: +81 75 594 0790.
E-mail address: watanabe_yusuke@kaken.co.jp (Y. Watanabe).

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studies revealed that EP_4 agonists block induction of colitis and ameliorate established colitis in experimental murine models (Jiang et al., 2010; Jiang et al., 2007; Nitta et al., 2002). In a clinical trial, intravenous administration of EP_4 agonist was reported to be effective in treating patients with mild-to-moderate UC who were refractory to 5-ASA (Nakase et al., 2010). Thus, EP_4 agonist treatment is expected to be an alternative approach to UC.

For the clinical use of EP_4 agonist as a new therapeutic strategy for UC, the development of compounds with orally-available properties is hugely desirable to improve the convenience of dosing. However, an orally efficacious EP_4 agonist for UC has not yet been reported, aside from one previous study using microspheres formulation of orally-unstable EP_4 agonist (Okamoto et al., 2012). This may be due to the metabolic instability of several conventional EP_4 agonists. Moreover, there is a possibility of an EP_4 agonist increasing the risk of CAC. EP_4 is highly expressed in colorectal cancer cells, and it is suggested that it plays roles in their proliferation and motility (Chell et al., 2006). However, there is no report that reveals the effect of EP_4 agonist in CAC development.

We recently identified KAG-308, a chemically and metabolically stable 7,7-difluoroprostacyclin derivative that exhibits EP_4 agonist activity (Fig. 1) (Ishibashi et al., 2014). In the current study, we attempted to clarify the following questions: first, whether orally-administered KAG-308 is well absorbed and prevents colitis; second, whether KAG-308 has mucosal healing (MH) effects not shared by conventional anti-colitic agents; and third, the effect of KAG-308 on CAC development risk. We initially investigated the in vitro pharmacological and pharmacokinetic profiles of KAG-308. Then, we compared the effect of KAG-308 and sulfasalazine (SASP), a prodrug of 5-ASA, in a dextran sulfate sodium (DSS)-induced colitis model and in an azoxymethane (AOM)/DSS-induced CAC model. Finally, to clarify the involvement of the endogenous PGE_2–EP_4 axis in CAC development, we evaluated MF-482, an EP_4 antagonist.

2. Materials and methods

2.1. Reagents

KAG-308, (2Z,3aR,4R,5R,6aS)-3,3-difluorohexahydro-4-[(1E,3R,4R)-3-hydroxyl-4-(3-methylphenyl)-1-penten-1-yl]-2-[4-(1H-tetrazol-5-yl)butylidene]-2H-cyclopenta[b]furan-5-ol (Fig. 1), was synthesized by Asahi Glass Co., Ltd. (Tokyo, Japan). MF-482, 4-{1-[({2,5-dimethyl-4-[4-(trifluoromethyl)benzyl]-3-thienyl}carbonyl)amino]cyclopropyl}benzoic acid, was synthesized by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). DSS (molecular weight 36,000–50,000) was purchased from MP Biomedicals Inc. (Solon, OH). AOM and SASP were obtained from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Receptor binding affinity and functional assays

Competitive binding experiments were performed using human recombinant EP_4-expressing cell membranes (ChemiSCREEN EP_4 Prostanoid Receptor Membrane Preparation from Millipore, Billerica, MA) and human recombinant prostaglandin I_2 receptor (IP)-expressing cell membranes (Kaken Pharmaceutical Co., Ltd.), according to conventional methods. Briefly, cell membranes and various concentrations of KAG-308 were incubated for 2 h at 25 °C in [^{3}H]–PGE_2 (PerkinElmer Inc., Waltham, MA) or [^{3}H]-Iloprost (PerkinElmer Inc.) -containing binding buffer, and the bound radioactivity was measured using a microplate scintillation luminescence counter (TopCount NXT, PerkinElmer Inc.). For comprehensive functional tests, KAG-308 was examined for agonistic/antagonistic activities against 158 human G protein-coupled receptors (GPCRs) including prostanoid receptors using GPCRProfiler Services provided by Millipore. To evaluate the functional agonistic activity of KAG-308 for human and mouse EP_4, activity of luciferase transcribed in response to intracellular cAMP production was examined using dual luciferase reporter assays in COS-7 cells transiently expressing human or mouse EP_4 receptor. COS-7 cells were incubated with various concentrations of KAG-308 or PGE_2 for 3 h at 37 °C. Reporter assays were performed using the Dual-Glo Luciferase Assay System (Promega Corp., Fitchburg, WI) according to the manufacturer's directions. Inhibition constant (Ki values) from receptor binding assays and 50% effective concentrations (EC_{50} values) from in vitro functional assays were calculated.

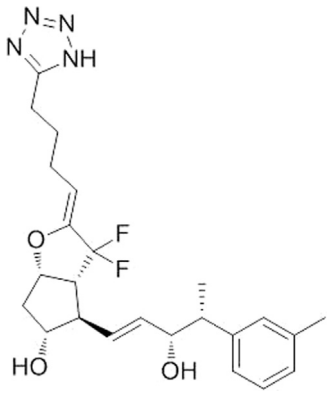

Fig. 1. Chemical structure of 7,7-difluoroprostacyclin derivative KAG-308.

2.3. Subjects

Ten ml peripheral whole blood samples were collected from 3 healthy volunteers for whole blood cell cultures. In addition, 50 ml peripheral whole blood samples were collected from 5 healthy volunteers for isolation of human peripheral blood mononuclear cells (PBMCs). These samples were withdrawn into blood collection tube containing sodium heparin. All experiments were approved by the local ethics committees. Informed consent was obtained from all healthy volunteers before obtaining samples.

2.4. Animals

Female 6-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan) or Japan Clea (Tokyo, Japan) and housed individually in plastic cages with free access to drinking water and a pelleted basal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (50 ± 20%), light (12/12 h light–dark cycle) and temperature (23 ± 3 °C). All experimental protocols were approved by the Animal Experimentation Ethics Committee of Kaken Pharmaceutical Co., Ltd.

2.5. Whole blood cell cultures and isolation of CD4+ T cells

Heparinized peripheral blood samples obtained from 3 healthy volunteers or 11 mice were diluted 4-fold with IMDM media (Life Technologies, Carlsbad, CA) supplemented with 1% fetal bovine serum (FBS), 100 U/ml Penicillin, and 100 μg/ml Streptomycin and were seeded on 96-well culture plates at 160 μl/well. Human PBMCs were prepared from heparinized peripheral blood samples of 5 healthy volunteers according to density-gradient centrifugation method. The harvested PBMCs were suspended in FACS flow (BD Biosciences, San Jose, CA) containing 0.5% bovine serum albumin (BSA). To prepare mouse splenic cells, spleens were

collected from 6 mice and mashed through cell strainer with 40 μm nylon mesh. Obtained splenic cells were suspended in FACS flow (BD Biosciences) containing 0.5% BSA. CD4+ T cells were isolated from mouse splenic cells or human PBMCs by magnetic-activated cell sorting system using autoMACS® Pro Separator (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions of CD4+ T cell Isolation Kit II (Miltenyi Biotech). Isolated CD4+ T cells were cultured in RPMI1640 medium (Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37 °C in 96-well plates at a density of 7.33×10^5 cells/ml.

2.6. Cytokine production from peripheral whole blood and CD4+ T cells

Cell cultures of whole blood and CD4+ T cells were pretreated with various concentrations of KAG-308 for 30 min at 37 °C. Following this, whole blood cell cultures were stimulated with lipopolysaccharide (LPS, 100 ng/ml, Sigma-Aldrich Co.), incubated for 24 h at 37 °C, and then supernatants were harvested. CD4+ T cells pretreated with KAG-308 were transferred to anti-CD3 pre-coated T cell activation plates (BD Biosciences), stimulated with anti-CD28 (Clone E18, 11 μg/ml, BioLegend, San Diego, CA), incubated for 48 h at 37 °C, and then supernatants were harvested. The concentration of tumor necrosis factor-α (TNF-α) in supernatants was measured by enzyme linked immunosorbent assay (ELISA) (Quantikine Mouse TNF-α Immunoassay, R&D Systems) according to the manufacturer's directions. 50% inhibitory concentrations (IC_{50} values) and inhibition rate for TNF-α production in maximum observed plasma concentration (C_{max}) when orally administered KAG-308 at 0.3 and 1 mg/kg were calculated.

2.7. Pharmacokinetic evaluation

In pharmacokinetic evaluation after single administration of KAG-308 to mice, KAG-308 was administered to non-fasted mice intravenously at 0.3 mg/kg and orally at 0.3 and 1 mg/kg. Then, blood was collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 10 h after intravenous administration or at 0.25, 0.5, 1, 2, 4, 8, 10, and 24 h after oral administration ($n=3$ at each time point for each treatment). Plasma samples were obtained from blood by centrifugation (3000 rpm, 15 min, 4 °C) and processed by solid-phase extraction. The concentration of KAG-308 in plasma was determined using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Areas under the plasma concentration-time curve covering different intervals ($AUC_{0-10\ h}$, $AUC_{0-24\ h}$, $AUC_{0-\infty}$) and bioavailability were calculated.

2.8. Colitis and CAC induction and drug treatment

Effects of KAG-308 on colitis were investigated using two DSS-induced colitis models. In the first experiment (Experiment 1) to measure the inhibitory effects of KAG-308, acute colitis was induced in mice ($n=12$ / group) by allowing free access to 2.3% w/v DSS in drinking water for 8 days. Intact mice ($n=12$) were used as a normal group. KAG-308 was orally administered once daily at doses of 0.3 and 1 mg/kg (in distilled water) from 1 day before induction of colitis (day 1) until day 8. Likewise, 10 mg/kg SASP (in distilled water) was administered over the same time schedule (Fig. 2). In the second experiment (Experiment 2) to examine the MH effect of KAG-308, mice ($n=8$/group) were treated with 3% w/v DSS in drinking water for the first 9 days, and recovered after replacing DSS-containing drinking water with distilled water for 12 days. Intact mice ($n=6$) were used as a normal group. KAG-308 was orally administered once daily during day 9–21 at a dose of 1 mg/kg. SASP was administered at 30 mg/kg over the same time schedule (Fig. 2). In Experiments 1 and 2, mice were killed under anesthesia on the last day (day 8 and day 21, respectively). Excised colon lengths were measured, and the tissues were longitudinally cut for histological analysis. The effects of KAG-308 in the AOM/DSS-induced CAC (AOM/DSS-CAC) model were also investigated (Experiment 3). Mice ($n=10$/group) were intraperitoneally injected with 10 mg/kg AOM on day 0. One week later (day 7), mice were allowed free access to 2.3 w/v% DSS-containing drinking water for 7 days followed by free access to distilled water for 14 days. This DSS/distilled water treatment was repeated three times (distilled water-drinking was given for only 8 days in the third cycle). Intact mice ($n=8$) were used as a normal group. KAG-308 was orally administered to mice at 1 mg/kg once daily from one day before the first DSS treatment (day 6) until the day of autopsy (day 64). MF-482 (in 0.5% methylcellulose) and SASP (in distilled water) were administered at doses of 0.3 and 10 mg/kg, respectively, in the same time schedule as KAG-308 (Fig. 2). On day 64, mice were killed and blood samples were collected. Excised colons were measured and processed as described above.

2.9. Assessment of colitis symptoms

Body weight, stool consistency, and fecal occult blood were monitored daily. Colitis symptoms were evaluated according to the stool consistency score, fecal occult blood score and weight loss score as described in Table 1. Fecal occult blood scores were estimated using Shionogi II occult blood slides (Shionogi & Co., Ltd., Osaka, Japan). Disease activity index (DAI) score was calculated by sum of stool consistency score, fecal occult blood score and weight loss score. Area under the time curve (AUC) of DAI scores was also calculated using the trapezoidal rule.

2.10. Endoscopic procedures

Colitis activity and tumor development were monitored with AVS thin endoscope system (AVS Co., Ltd., Tokyo, Japan) in both Experiments 2 and 3. In Experiment 2, endoscopy was performed on days 9, 15, and 21 after initiation of DSS treatment (Fig. 2). The severity of mucosal injury was scored at colon region (2 cm) between 1 and 3 cm from the anus using the modified method of the murine endoscopic index of colitis severity (Becker et al., 2006). In Experiment 3, tumor development was evaluated on days 35, 56, and 63 after AOM injection (Fig. 2). The number of colon tumors observed at colon region (2 cm) between 1 and 3 cm from the anus were counted to determine overall tumor multiplicity. In addition, the size and status of every tumor observed was graded as described previously (Becker et al., 2005). The endoscopic tumor score was calculated as the sum of tumor grades observed in each mouse.

2.11. Macroscopic analysis of CAC

To evaluate tumor formation in entire colons of AOM/DSS-CAC mice, samples were fixed in 5% v/v neutral formalin and macroscopically observed to determine the presence of tumors. Tumor multiplicity was recorded, and individual tumors were measured to calculate volumes (length × width × width × 0.526) as described previously (Murray et al., 2009). Mean tumor volume was then determined for individual mice and the overall tumor burden was calculated by multiplying tumor volume with tumor multiplicity.

2.12. Histological analysis of colitis and CAC

Colonic tissues were fixed in 10% v/v formaldehyde solution, embedded in paraffin and sectioned (2 μm). To visualize goblet cell depletion and/or crypt loss, sections were stained with Periodic acid-Schiff (PAS) using a standard staining procedure. PAS-staining

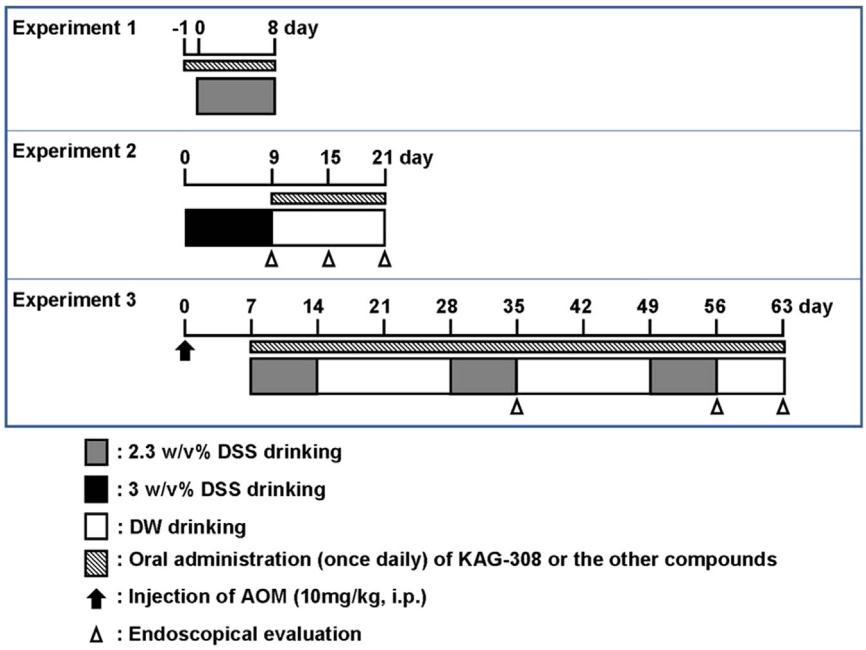

Fig. 2. Schematics of experimental designs 1, 2, and 3.

Table 1
Colitis symptoms score and PAS staining score.

Score	Colitis symptoms			PAS staining area
	Stool consistency	Fecal occult blood	Weight loss (g)	
0	Normal feces	Negative, no change in the slide color (yellow)	<1	Broad mucosal staining
1	Spherical feces comprising 50% of total feces	Slightly positive, faint blue	$1-<2$	No mucosal staining in the lower 1/3 of colons
2	Banana-shaped feces comprising 50% of total feces	Weakly positive, weak blue	$2-<3$	No mucosal staining in the lower 2/3 of colons
3	Banana-shaped feces comprising $\geq 50\%$ of total feces	Moderately positive, clear blue	$3-<4$	Dotted weak mucosal staining only in the upper 1/3 of colons or no mucosal staining
4	Muddy stool	Strongly positive, dark blue, color change occurs instantly with color developer	>4	–
5	Watery stool	Proctorrhagia	–	–

observations were made in sections at 4 cm distal from the anus using 32 High Power Fields (HPFs) for Experiment 1, and in sections at 1–3 cm distant from anus using 16 HPFs in Experiment 2, under 200 × magnification (Olympus BX5 microscope; Olympus, Tokyo, Japan). Changes in the observed PAS staining area, which indicated goblet cell depletion, were scored as described in Table 1. The severity of goblet cell damage was calculated as the sum of these scores obtained from 32 HPFs (score range, 0–96) for Experiment 1, and 16 HPFs (score range, 0–48) for Experiment 2. To evaluate neutrophil infiltration, paraffin-embedded sections were stained with hematoxylin and eosin according to a standard procedure. In the DSS-induced colitis model (Experiment 2), sections at 1–3 cm from the anus were observed under 400 × magnification to count neutrophils in the lamina propria. Total number of neutrophils observed in colon areas was recorded. Neutrophils were also counted in 32 randomly chosen visual fields from colon areas at 0–8 cm from the anus of AOM/DSS-CAC mice (Experiment 3) under 400 × magnification. Data were presented as the mean number of infiltrated neutrophils per HPF in lamina propria. For histological tumor analyses, paraffin-embedded sections were stained with hematoxylin and eosin. Colonic adenomas and adenocarcinomas were diagnosed as described previously (Ward, 1974). The number of adenomas and adenocarcinomas on longitudinal colon sections at 0–8 cm from the anus were counted.

2.13. Immunohistochemistry

Paraffin-embedded sections were deparaffinized prior to staining. These sections were stained with anti-mucin 2, anti-CD3, or anti-F4/80 antibody. Detailed information on antibodies and description of the experimental procedures are provided in the Supplemental materials and methods. Mucin 2-immunopositive cells were serially observed at 1–3 cm from the anus in Experiment 2. Immunopositive cells were counted on five randomly chosen crypts from single HPF under 200 × magnification. Data were indicated as the mean number of immunopositive cells per crypt. In the AOM/DSS-CAC model (Experiment 3), F4/80-immunopositive macrophages and CD3-immunopositive T cells were counted on 32 randomly chosen visual fields from colon areas at 0–8 cm from the anus under 400 × magnification. Data were indicated as the mean number of immunopositive cells infiltrated into lamina propria per HPF.

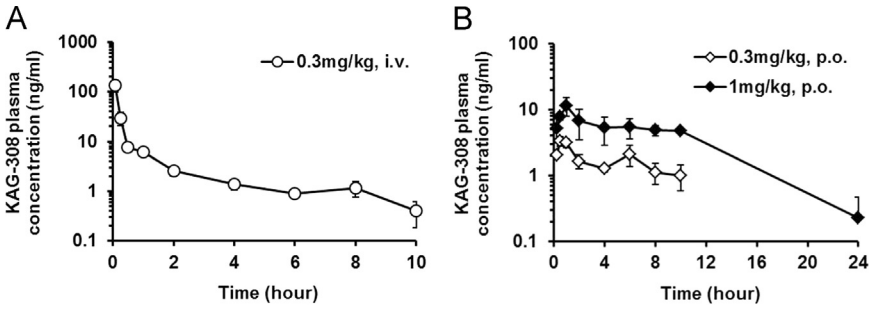

Fig. 3. Plasma concentrations of KAG-308 after single intravenous or oral administration. (A) KAG-308 at 0.3 mg/kg was intravenously administered and blood was collected at the indicated time. (B) KAG-308 at 0.3 and 1 mg/kg was orally administered and blood was collected at the indicated time. Data points and bars show the mean ± S.D. of data from 3 animals.

2.14. Measuring cytokine level in blood

Blood was collected, and the serum was isolated by centrifuging at 2200*g* for 20 min. Levels of TNF-α in serum samples were measured using ELISA (Quantikine Mouse TNF-α Immunoassay, R&D Systems) according to the manufacturer's directions.

2.15. Statistical analysis

The mean ± S.D. was calculated for all parameters determined. Statistical analysis was performed using Student's *t*-test, Dunnett's test, Mann–Whitney's *U*-test, and Steel's test and were considered significant when $P < 0.05$. Spearman's rank correlation test was used to calculate correlation coefficients.

3. Results

3.1. In vitro pharmacological profile of KAG-308

In the receptor binding assay, the Ki values of KAG-308 were 1410, 1540, 32.4, 2.57, and 52.9 nmol/l for human EP_1, EP_2, EP_3, EP_4, and IP, respectively. In comprehensive GPCR assays, KAG-308 exhibited agonistic activity only for human EP_1, EP_2, EP_3, EP_4, and IP toward 158 GPCRs tested, and their EC_{50} values were 1000, 1000, 160, 17, and > 10,000 nmol/l, respectively. KAG-308 had little agonist activity for the other receptors at a concentration of 1.25 μmol/l, and had no antagonist activity for the 158 GPCRs. Regarding Gαs signaling through EP_4, KAG-308 also displayed potent agonist activity for human and mouse EP_4 with an EC_{50} of 0.15 nmol/l and 1.0 nmol/l, respectively in the dual luciferase reporter assay. Likewise, PGE_2 showed an agonist activity for human and mouse EP_4 with an EC_{50} of 0.19 nmol/l and 0.22 nmol/l, respectively.

3.2. Effect of KAG-308 on TNF-α production from LPS-stimulated peripheral whole blood and anti-CD3/CD28 stimulated CD4+ T cells

To investigate the effect of KAG-308 on inflammatory cytokine production, an in vitro model producing TNF-α through activation of peripheral whole blood and CD4+ T cells in human and mouse was employed. KAG-308 dose-dependently inhibited TNF-α production from human and mouse peripheral whole blood stimulated by LPS. It also has similar effects on human and mouse CD4+ T cells stimulated by anti-CD3/CD28 antibody. The IC_{50} values of KAG-308 for TNF-α production in human and mouse peripheral whole blood were 4.53 nmol/l and 54.2 nmol/l, respectively. In human and mouse CD4+ T cells, the IC_{50} values of KAG-308 for TNF-α production were 0.831 and 92.6 nmol/l, respectively. Inhibitory effects of KAG-308 on TNF-α production from human and mouse CD4+ T cells were effectively antagonized by MF-482 (data not shown).

3.3. Pharmacokinetics of KAG-308

Plasma KAG-308 concentrations after intravenous administration at 0.3 mg/kg declined bi-phasically (Fig. 3A). The plasma concentration at 0 min (C_{0min}) and $AUC_{0-\infty}$, were 285.6 ng/ml and 55.5 ngh/ml, respectively. Plasma KAG-308 concentrations after oral administration at 0.3 mg/kg and 1 mg/kg reached time to peak plasma concentration (T_{max}) at 0.5 h and 1.0 h, respectively, and those C_{max} were 3.3 ng/ml and 11.5 ng/ml, respectively. After reached to T_{max}, those plasma concentrations gently declined to 10 h, and that at 1 mg/kg was detected even 24 h after administration (Fig. 3B). $AUC_{0-10\ h}$ at 0.3 mg/kg and 1 mg/kg were 16.6 ngh/ml and 59.0 ngh/ml, respectively, and $AUC_{0-24\ h}$ at 1 mg/kg was 94.0 ngh/ml. Bioavailability when orally administered at 1 mg/kg was 50.8%. Besides, inhibition rates for TNF-α production from mouse peripheral whole blood in C_{max} when administered at 0.3 and 1 mg/kg were estimated at 12.6% and 32.1%, respectively. In mouse CD4+ T cells, inhibition rates for TNF-α production in C_{max} when administered at 0.3 and 1 mg/kg were estimated at 19.4% and 33.4%, respectively.

3.4. Effect of KAG-308 on colonic mucosal injury development

The preventive effect of KAG-308 on colonic mucosal injury induced by 2.3 w/v% DSS was investigated in Experiment 1. Colon shortening observed in the DSS-treated control group was significant compared with that observed in the normal group (Fig. 4A). In histological examinations, crypt loss and goblet cell depletion were indicated by decreased PAS staining in colon areas (Fig. 4B). KAG-308 significantly suppressed colon shortening at doses of 0.3 and 1 mg/kg (Fig. 4A). KAG-308 at same doses also suppressed the increase in PAS staining scores, which indicates crypt loss associated with goblet cell depletion (Fig. 4B). SASP at 10 mg/kg also significantly inhibited colon shortening (Fig. 4A) and showed a weak but not significant inhibitory effect on the increase in PAS staining scores (Fig. 4B).

3.5. Healing-promoting effect of KAG-308 on colonic mucosal injury

The effects of KAG-308 on MH of inflamed mucosa in 3% w/v DSS-treated mice were examined in Experiment 2. Mice with established colitis showed a mild weight loss, and displayed severe diarrhea and occult bleeding. Colon length in the control group was significantly shortened on day 21 compared with that in the normal group. KAG-308 at 1 mg/kg and SASP at 30 mg/kg restored body weight to normal levels and recovered colon length, although these effects were not significant. In endoscopic evaluations, mucosal injuries were markedly increased (Fig. 4C). KAG-308 induced rapid recovery from mucosal injury in a time-dependent manner, as indicated by significant decreases in endoscopic mucosal injury scores compared with those in the control group on day 15 and day 21 (Fig. 4C). Likewise, SASP

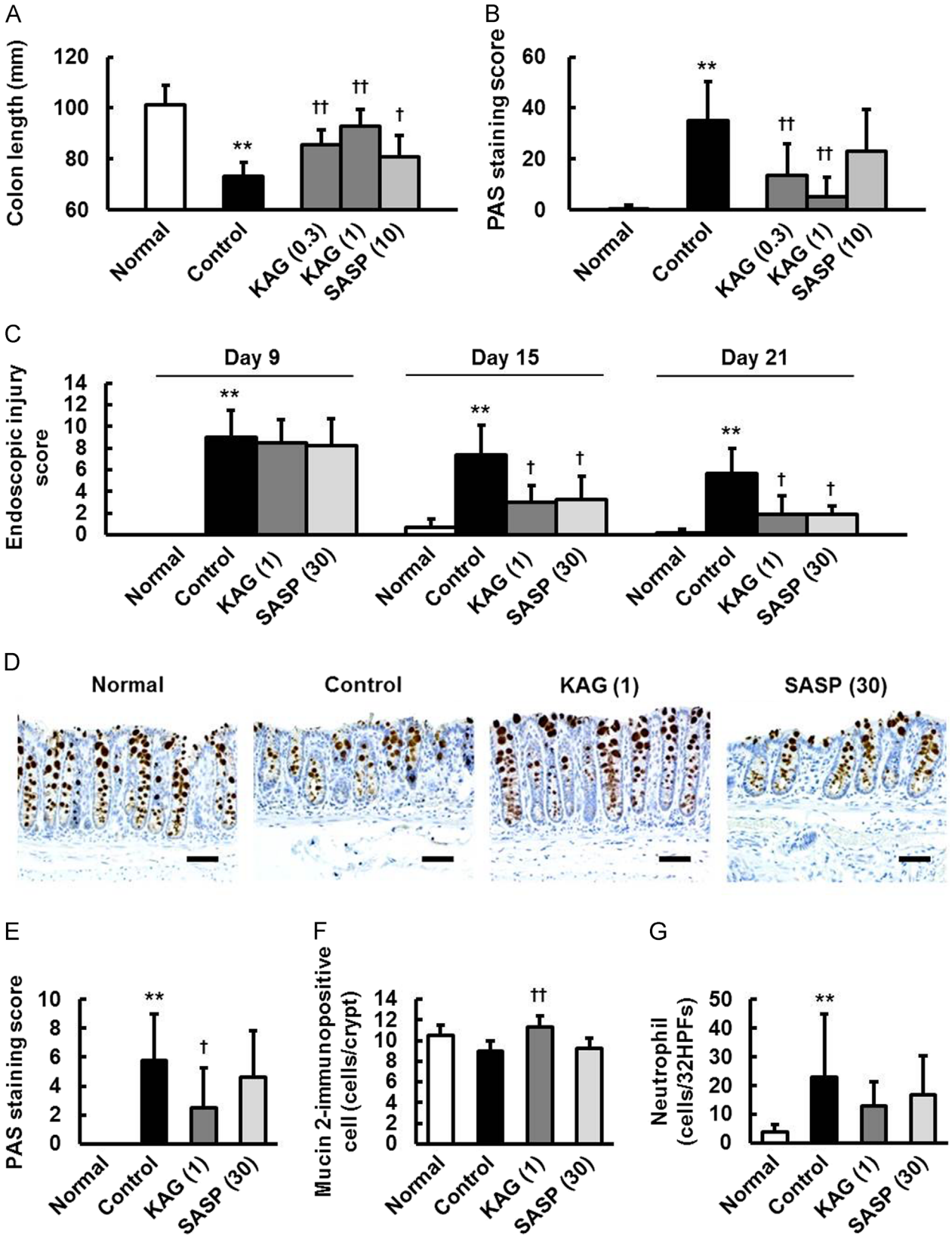

Fig. 4. Suppression of colitis development and promotion of mucosal healing in the DSS-induced colitis model. (A, B) KAG-308 (KAG) at 0.3 and 1 mg/kg and SASP at 10 mg/kg were administered once daily from 1 day before induction of colitis, as shown in Fig. 2 (Experiment 1). Mice were killed on day 8, and colon length was measured. Colons were sectioned for histological staining: (A) Colon length, (B) PAS staining score. Columns and bars show the mean ± S.D. from 11 or 12 mice. (C–G) Mice were treated with KAG-308 (KAG) at 1 mg/kg and SASP at 30 mg/kg as shown in Fig. 2 (Experiment 2). KAG-308 and SASP were administered once daily during day 9–21. (C) Chronological changes in the endoscopic injury score. Mice were killed on day 21, and colons were sectioned for histological staining and immunohistochemistry. (D) Representative microscopic images of immunohistochemical staining with anti-mucin 2 antibody on day 21; scale bar, 100 μm; (E) PAS staining score; (F) Number of mucin 2-immunopositive cells; (G) Number of infiltrated neutrophils in lamina propria. Columns and bars show the mean ± S.D. of data from 6 or 8 animals; $^{**}P < 0.01$ vs. the normal group; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ vs. the control group.

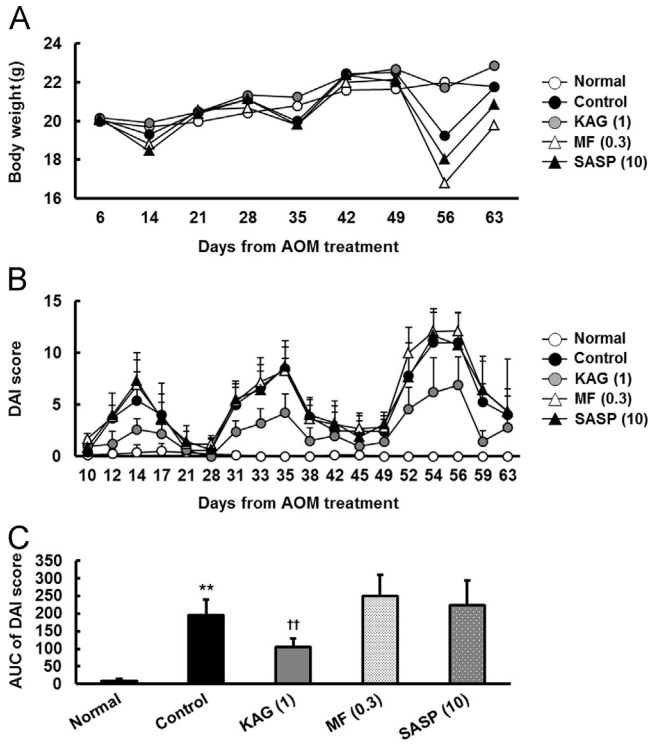

Fig. 5. Suppression of colitis in the AOM/DSS-CAC model. Mice were treated with KAG-308 (KAG) at 1 mg/kg, MF-482 (MF) at 0.3 mg/kg, or SASP at 10 mg/kg, as shown in Fig. 2 (Experiment 3). Test compounds were administered once daily during cyclic treatment with DSS/distilled water. (A) Chronological changes in body weight; data points show the mean of data from 5 to 10 animals. (B) Chronological changes in DAI scores; data points and bars show the mean ± S.D. of data from 5 to 10 animals. (C) The AUC of DAI scores from days 10–63; columns and bars show the mean ± S.D. of data from 4 to 9 animals; $^{**}P < 0.01$ vs. the normal group; $^{\dagger\dagger}P < 0.01$ vs. the control group.

Table 2
Effects of tested compounds on colon shortening and infiltration of inflammatory cells into colonic mucosa of surviving animals in AOM/DSS-CAC model. The values represent the mean ± S.D. of data from 4 to 9 animals.

Treatment (no. of mice examined)	Colon length (mm)	Infiltrated cells (cells/HPF)		
		Neutrophil	Macrophage	T cell
Normal (8)	103.8 ± 8.0	0.8 ± 0.7	8.1 ± 2.1	9.4 ± 2.0
Control (7)	91.8 ± 6.6[a]	13.8 ± 3.1[a]	41.1 ± 8.3 [a]	44.3 ± 9.0[a]
KAG-308 1 mg/kg (9)	98.6 ± 7.7	4.4 ± 2.2[b]	22.8 ± 5.9[b]	23.4 ± 7.0[b]
MF-482 0.3 mg/kg (4)	87.8 ± 3.0	10.8 ± 5.0	35.4 ± 11.0	38.7 ± 6.1
SASP 10 mg/kg (6)	90.9 ± 4.5	10.7 ± 3.8	45.8 ± 14.5	36.2 ± 9.8

[a] $P < 0.01$ vs. the normal group,
[b] $P < 0.01$ vs. the control group.

showed similar significant decreases in endoscopic scores (Fig. 4C). With regard to remission of colitis, which was defined by endoscopic scores of less than 2, endoscopic remission rates were 62.5% and 75.0% in mice treated with KAG-308 and SASP, respectively, compared with 12.5% for the control group. In histological evaluations, mucosal injury in the control group was denoted by significant increases in PAS staining scores, and mild decreases in mucin 2-immunopositive goblet cells, in comparison with those in the normal group (Fig. 4D, E and F). Marked neutrophil infiltration was observed even on day 21 (Fig. 4G). Treatment with KAG-308 slightly decreased this neutrophil infiltration, significantly decreasing PAS staining scores (Fig. 4E), and led to complete recovery of mucin 2-immunopositive goblet cell numbers (Fig. 4D and F). In contrast, SASP had no evident effects on these three histological parameters (Fig. 4D, E, F and G). With regarding to histological remission of colitis, which was defined by PAS staining scores of less than 2, histological remission rates in the control, KAG-308, and SASP groups were 12.5%, 75.0%, and 37.5%, respectively. Thus, KAG-308 was found to have superior histological MH effects than SASP.

3.6. Effect of KAG-308 on colitis development in the AOM/DSS-CAC model

The effects of KAG-308, MF-482, and SASP on chronic colitis and colorectal carcinogenesis were examined in the AOM/DSS-CAC model (Experiment 3). One animal of SASP group died during the first cycle and some animals died in all test groups during the third cycle of DSS/distilled water. The final survival rates of the control, KAG-308, MF-482, and SASP groups were 70%, 90%, 40%, and 60%, respectively. In the control group, loss in body weight and DAI scores increased depending on cycles of DSS/distilled water (Fig. 5A and B), and colon shortening was observed. KAG-308 prominently inhibited body weight loss (Fig. 5A) and significantly inhibited increases in DAI scores during the experimental period (Figs. 5B and C). In addition, KAG-308 weakly suppressed the colon shortening, although this effect was not significant (Table 2). In contrast, the body weight in the MF-482 group was markedly decreased by the third cycle of DSS/distilled water (Fig. 5A). MF-482 and SASP slightly enhanced increases in DAI score in the first cycle of DSS/distilled water, while the DAI scores remained at the same level as that in the control group during the second and third cycles (Fig. 5B). In consequence, AUC of DAI scores obtained in the MF-482 group was highest of all AUC values for other test groups (Fig. 5C). On day 64, serum TNF-α level remained slightly high in the control group (48.7 ± 4.9 ng/ml) compared to the normal group (40.7 ± 2.5 ng/ml) with a significant difference, even though colitis symptoms already improved. In contrast, it was not significantly increased in the KAG-308 group (43.6 ± 2.8 ng/ml). Histological examinations conducted in the control group showed that neutrophils, macrophages, and T cells markedly infiltrated into tumor sites and only moderately into non-tumor sites. KAG-308 significantly prevented the infiltration of these three types of cells into both tumor and non-tumor sites (Table 2).

3.7. Effect of KAG-308 on colorectal carcinogenesis development in the AOM/DSS-CAC model

In the control group, colorectal tumors were endoscopically observed during the second cycle of DSS/distilled water treatment and developed in a time-dependent manner (Fig. 6A and B). On day 64, extensive tumors of various sizes were macroscopically observed in colorectal areas, and almost all of these were adenocarcinomas (Fig. 6C, Table 3). KAG-308 significantly inhibited the formation of colorectal tumors in endoscopic observations (Fig. 6A and B) and significantly inhibited the increase in tumor burden with decreasing tumor size and multiplicity (Fig. 6C, Table 3). Histological examinations showed that KAG-308 significantly decreased multiplicity of adenocarcinomas compared with that observed in the control group (Table 3). In contrast, MF-482 and SASP slightly promoted tumor formation at day 35 and 56 in endoscopic examinations, although this was not significant (Fig. 6B and C). There were no significant changes in macroscopic and histological parameters obtained in these groups, possibly because of small number of surviving mice, except that SASP increased the multiplicity of adenomas (Table 3).The multiplicity of adenocarcinomas and the AUC of DAI scores were significantly correlated ($r = 0.7918$, $p < 0.01$).

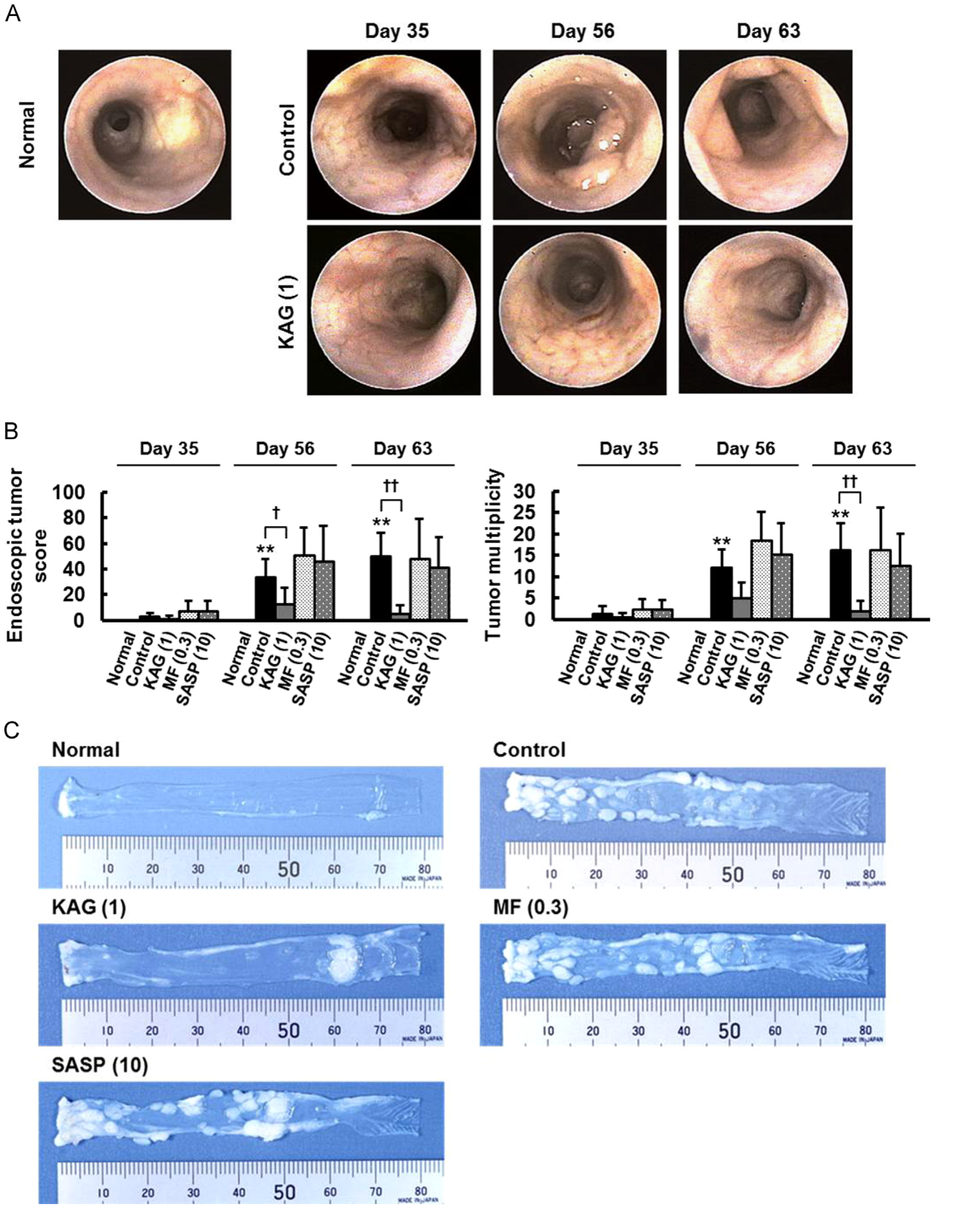

Fig. 6. Suppression of colorectal tumor formation in the AOM/DSS-CAC model. Mice were administered once daily during cyclic treatment with DSS/DW with KAG-308 (KAG) at 1 mg/kg, MF-482 (MF) at 0.3 mg/kg, or SASP at 10 mg/kg, as shown in Fig. 2 (Experiment 3). (A) Representative endoscopic images of colorectal tumors; (B) Chronological changes in endoscopic tumor scores and tumor multiplicity. Columns and bars show the mean ± S.D. of data from 5 to 10 animals; $^{**}P < 0.01$ vs. the normal group; $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$ vs. the control group at corresponding time points. (C) Representative macroscopic images from the anus (left side of the images) to 8 cm of the colon.

Table 3
Macroscopic and histological evaluation of colorectal tumor formation isolated from surviving mice in AOM/DSS-CAC model. The values represent the mean ± S.D. of data from 4 to 9 animals.

Treatment (no. of mice examined)	Macroscopic analyses			Histological analyses	
	Multiplicity (number/mouse)	Tumor size (mm^3/tumor)	Tumor burden (mm^3/mouse)	Adenoma (number/section)	Adenocarcinoma (number/section)
Normal (8)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control (7)	16.7 ± 4.8[b]	5.2 ± 4.9[b]	70.6 ± 30.4[b]	0.7 ± 0.8[a]	6.4 ± 2.6[a]
KAG-308 1 mg/kg (9)	6.8 ± 4.1	2.1 ± 1.2	15.7 ± 11.2[d]	1.1 ± 1.1	1.1 ± 1.1[d]
MF-482 0.3 mg/kg (4)	13.8 ± 10.6	1.5 ± 0.3	22.5 ± 20.2	1.0 ± 0.8	2.8 ± 1.5
SASP 10 mg/kg (6)	24.2 ± 12.8	3.2 ± 0.7	79.5 ± 48.2	2.7 ± 1.6[c]	4.5 ± 2.9

[a] $P < 0.05$.
[b] $P < 0.01$ vs. the normal group.
[c] $P < 0.05$.
[d] $P < 0.01$ vs. the control group.

4. Discussion

In the present study, we confirmed that KAG-308, a newly discovered 7,7-difluoroprostacyclin derivative, is an orally-available EP_4-selective agonist with potent in vitro efficacy. KAG-308 was shown to be stable to β-oxidation since the carboxy group of α-chain of prostaglandin skeleton, which is susceptible to β-oxidation, is converted to the tetrazole group; however other EP_4 agonists such as compounds produced by Ono Pharmaceutical Co., Ltd and Allergan, Inc. have the carboxy group. (Murata et al., 2013; Kambe et al., 2011; Jiang et al., 2007). Therefore, it had a prolonged plasma half-life and can maintain an effective plasma concentration for a long time. Furthermore, it showed high permeability in a study of epithelial cell monolayers (data not shown). Since the metabolic stability in the body and the intestinal permeability were improved as seen above, the bioavailability of KAG-308 could be improved. It was estimated that the bioavailability of KAG-308 in mice is superior to the only previously-reported orally-available EP_4 agonist (Kambe et al., 2011).

Previous research indicated the effectiveness of EP_4 agonist treatment in animal colitis models. In this study, KAG-308 effectively suppressed the onset of colitis and also inhibited goblet cell depletion, and its effect was equal to or better than that of SASP. KAG-308 suppressed colitis when administered orally or subcutaneously but not intrarectally at comparable doses in our preliminary examination. Based on this result, we presumed that orally-administered KAG-308 showed efficacy against colitis diseases mainly via systemic circulation. In addition, we first demonstrated that KAG-308 repaired colonic mucosa from established colitis induced by DSS, while SASP lacked such activity. Specifically, therapeutic administration of KAG-308 promoted endoscopic healing of injured mucosa and increased the number of mucin 2-positive goblet cells observed by histological methods. Interestingly, SASP also improved endoscopic MH but had no increasing effect on the number of goblet cells. Apparent differences were observed between the histological characteristics of mucosa observed in mice treated with KAG-308 and SASP.

EP_4 is strongly expressed in the goblet cells of the rat gastrointestinal tract, and its expression is increased in colonic mucosa during colitis development (Nitta et al., 2002; Northey et al., 2000). PGE_2 is suggested to induce the proliferation of goblet cells (Phillips et al., 1993). Signaling via EP_4 is also shown to stimulate colonic mucin exocytosis in human colonic epithelial cells and rat colon (Belley and Chadee, 1999). Thus, our results indicated that KAG-308 may promote wound healing through the robust secretion of mucus and the stimulation of goblet cell proliferation. Moreover, it has been reported that PGE_2 regulates murine hematopoietic stem/progenitor cells through EP_4 activation, which cells recruit to injured sites to aid in tissue repair, implying that KAG-308 elicits epithelial regenerative repair through mobilization of stem cells (Ikushima et al., 2013; Kavanagh et al., 2013). However, 5-ASA inhibits production of PGE_2 in colonic cells (Serra et al., 2013). Furthermore, it suppresses the proliferation and induces the apoptosis of colonic epithelial cells (Reinacher-Schick et al., 2000). These findings indicate that SASP has little ability to heal colonic mucosa when therapeutically administered in the presence of mucosal injury, although its prophylactic administration prevented the onset of mucosal damage mediated via the anti-inflammatory effect. Thus, the difference in efficacy for mucosal injury between KAG-308 and SASP is thought to be based on the exertion of opposite actions in the regulation of intestinal epithelial cell function. The histological MH is essential for true remission in patients with UC (Neurath and Travis, 2012; Iacucci and Ghosh, 2011). KAG-308 treatment for UC patients is expected to show more prominent healing effects on injured mucosa than 5-ASA treatment.

It is known that patients with UC who undergo a long-lasting cycle of flare/remission are at increased risk of CAC; that is, suppression of colonic inflammation and achievement of MH is essential for a decreased risk of CAC. In a mouse model of AOM/DSS-CAC, it was reported that colonic inflammation characterized by production of proinflammatory cytokines such as TNF-α is related to colorectal cancer development and its development was suppressed by anti-TNF-α therapy (Onizawa et al., 2009; Popivanova et al. 2008). In addition, hepatocyte growth factor, which facilitates the repair of injured mucosa, inhibits the development of colorectal cancer (Yamaji et al., 2011). Thus, it is expected that EP_4-selective agonists such as KAG-308 may be able to decrease CAC risk through suppressing colonic inflammation and promoting MH. On the other hand, intestinal epithelial proliferation observed in patients with UC in a recovery phase has the potential to cause an increased incidence of carcinoma (Serafini et al., 1981). Hence, the promotion of MH during the recovery phase of UC patients may influence the induction of colorectal cancer. The AOM/DSS-CAC model is characterized by colorectal carcinogenesis with the recurrence of colitis induction and following recovery from injured mucosa by repeated DSS treatment; therefore, we considered that the impact of promoted MH by treatment with EP_4 agonist in CAC development can be evaluated with this model. In this study, we demonstrated that KAG-308 treatment suppressed repeated incidences of colitis, reduced damage of mucosal injury and strongly prevented colorectal tumor formation. This result suggests the possibility that the enhanced MH in addition to the anti-inflammatory action by KAG-308 treatment provided beneficial effects for UC patient without enhancing the incidence of carcinoma.

Endogenous PGE_2/EP_4 axis is important in diverse physiological functions. EP_4 deficiency or EP_4 antagonist treatment enhances cytokine production from inflammatory cells and aggravates the colonic inflammation based on impaired mucosal integrity and intestinal immune response (Kuroda and Yamashita, 2003; Kabashima et al., 2002), suggesting that endogenous PGE_2-EP_4 axis is essential for intrinsic anti-colitogenic action. In AOM/DSS-

CAC model which showed a colorectal carcinogenesis associated with colitis, COX-2 deficiency exhibit increased number of tumors and higher mortality (Ishikawa and Herschman, 2010). This indicates that the activation of endogenous COX-2-PGE_2 axis acts cancer-protectively under the colitis condition. Therefore, MF-482 might promote cancer development through the enhancement of inflammatory response and mucosal damage in the presence of colitis in this study. However, such results seem to conflict with the theory that locally-enhanced production of PGE_2 and activated EP_4 signaling in colonic cancer cells promotes tumor growth. Colorectal cancer development induced by AOM alone which is not accompanied by inflammation was suppressed by COX-2 or EP_4 deficiency (Ishikawa and Herschman, 2010; Mutoh et al., 2002), suggesting that endogenous PGE_2–EP_4 axis serves as one of the pro-tumorigenic factors. Reportedly, other EP receptors also play a role in colorectal carcinogenesis. Different EP receptors participate in the regulation at different stages (initiation versus progression) of carcinogenesis (Hull et al., 2004); that is, more than one EP receptor subtype is involved in carcinogenesis. Therefore, we consider that the exogenous activation of EP_4 alone may have insignificant impact on colorectal carcinogenesis. Indeed, cancer-promoting effects by EP_4 agonist treatment has not been reported in a mouse model of colorectal cancer. In the future, more research is considered to be needed to clarify the impact of EP4 agonist on colorectal carcinogenesis in in vivo model. Collectively, our results indicate that blockade of the PGE_2–EP_4 axis impairs intestinal homeostasis and leads to exacerbation of CAC but not suppression, implying that the mucosal PGE_2–EP_4 axis plays a critical role in CAC prevention through colitis suppression.

In this study, SASP failed to prevent tumorigenesis in the AOM/DSS-CAC model. The optimal dosage of SASP is reportedly variable according to experimental conditions in animal studies (Reinecke et al., 2012; Petersen et al., 2009, Xu et al., 2009). Thus, the anti-inflammatory actions of SASP at 10 mg/kg may be insufficient in this model and higher doses, having more potent anti-inflammatory action, would have changed these results. Clapper et al. (2008) reported that 5-ASA at 75 mg/kg but not higher doses reduced tumor burden in the AOM/DSS-CAC model. We also demonstrated that the dose-response curve of SASP was bell-shaped, and SASP at 10 and 30 mg/kg were effective, but not at 100 mg/kg in the colitis model in preliminary experiments. 5-ASA is reported to suppress PGE_2 production from colonic cells and epithelial cell proliferation (Serra et al., 2013; Reinacher-Schick et al., 2000). Intestinal mucosal protection/healing and inhibition of cancer cell proliferation in CAC are considered to be in a reciprocal relationship. That is, SASP at high doses likely deteriorates the spontaneous intestinal MH, although it may suppress cancer cell proliferation in CAC. Therefore, selection of the optimal dose for treating colitis and CAC is difficult and may result in patients who are refractory to 5-ASA therapy in the clinic.

In conclusion, KAG-308 as an orally-available EP_4-selective agonist should improve the ease of dosing in clinical therapy, a weak point for the conventional EP_4 selective agonist. The oral administration of KAG-308 is more efficacious than 5-ASA for the prevention and recovery of DSS-induced colitis and also in prevention of CAC development in AOM/DSS-CAC model. Therefore, KAG-308 may be a new therapeutic strategy to treat UC and appears to confer a lower risk of colorectal carcinogenesis.

Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.02.021.

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