JPET Fast Forward. Published on September 28, 2006 as DOI: 10.1124/jpet.106.111146

Title Page

The prevention of colitis by EP4 agonist through enhancement of epithelium survival and regeneration

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JPET Fast Forward. Published on September 28, 2006 as DOI: 10.1124/jpet.106.111146
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Running Title Page

a) **Running title:** EP4 agonist and epithelial integrity in colitis

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c) **Number of text pages: 27**

Number of figures: 6

Number of references: 40

Words in the abstract: 249

Words in introduction: 637

Words in discussion: 1088

d) **ABBREVIATIONS:** AGN205203, {7-[2-(3-Hydroxy-4-phenyl-but-1-enyl)-6-oxo-piperidin-1-yl]-heptanoic acid methyl ester, $C_{23}H_{33}NO_4$}; AGN205204, {7-[2-(3-Hydroxy-4-phenyl-but-1-enyl)-6-oxo-piperidin-1-yl]-heptanoic acid, $C_{22}H_{31}NO_4$}; DP, D prostanoid receptor; EP, E prostanoid receptor; ERK, extracellular signal-related kinase; FP, F prostanoid receptor; HEK293, human embryonic kidney 293; IP, prostacyclin receptor; NSAID, nonsteroidal anti-inflammatory drug; ONO-AE1-329, {[3-[[(1R,2S,3R)-3-hydroxy-2-[(1E,3S)-3-hydroxy-4-[3-(methoxymethyl)phenyl]-1-butenyl]-5-oxocyclopentyl]thio]propyl]thio]-acetic acid, $C_{22}H_{30}O_6S_2$}; PGE_2, prostaglandin E2; PKA, protein kinase A; TP, thromboxane receptor.

e) **Recommended section:** Gastrointestinal, Hepatic, Pulmonary, and Renal

ABSTRACT:

Inflammatory bowel diseases (IBD) are often triggered and/or exacerbated by non-steroidal anti-inflammatory drugs (NSAIDs). Among various prostanoids affected by NASIDs, prostaglandin E_2 (PGE_2), in particular, seems to play critical roles in IBD via the EP4 receptor, one of the four PGE_2 receptor subtypes (EP1-4). An EP4 agonist, ONO-AE1-329, for example, when topically applied, has been reported to ameliorate typical colitis symptoms by suppressing the production of cytotoxic cytokines in the Dextran Sodium Sulfate (DSS)-induced colitis model. EP4 agonists are also known, however, for their ability to protect epithelial cells from apoptosis in vitro, which may contribute to the protection of mucosal barrier functions. To investigate this potential application, we have tested another EP4-selective agonist in the DSS-indomethacin mouse colitis model. AGN205203, an analog from the 8-azapiperidinone series of EP4 agonists, is metabolically and chemically more stable than the ONO agonist, due to its lack of oxidizable sulfur atoms in the α-chain and of 11-OH group, a potential source of β-elimination reaction. Treatment of mice subcutaneously with AGN205203 at 3 mg/kg/day minimized colitis symptoms such as weight loss, diarrhea and colonic bleeding. Further histological examination of colons revealed healthy surface columnar epithelial cells free of erosion and ulceration as compared to those without the drug treatment. At cellular level, the drug treatment decreased colon epithelial apoptosis, prevented goblet cell depletion and promoted epithelial regeneration. AGN205203 may be unique among known EP4 agonists for its metabolic and chemical stability, and amenable to systemic applications for the prevention and recovery of IBD.

Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), affect approximately 1.4 million U.S. patients with 15,000-30,000 new cases annually and the mean age of 30s and early 40s. IBD patients suffer from body weight loss, diarrhea, fecal blood, and pain. Such symptoms could last for 15 to 25 years, frequently alternating between exacerbation and remission, thus severely affecting the quality of patient life, and retarding the growth of young patients (Loftus, 2004; Isaacs et al., 2005). General consensus in the field is that IBD may arise from compromised colonic mucosal barrier functions which allow colonic antigens access to submucosal monocytes, which, upon activation, initiate innate immune responses and trigger cytotoxic cytokine production. Prevention and recovery of IBD thus largely depend on the integrity and maintenance of colonic mucosal barrier functions, which are compromised by inflammations along the entire bowel wall in CD, and at the mucosal surface in UC.

Current therapies primarily aim at the symptomatic remission by anti-inflammatory agents such as aminosalicylates, and/or immunosuppressive agents such as steroids, purine analogs, and monoclonal antibodies against cytotoxins (Colombel et al., 2004; Isaacs et al., 2005). These therapies have been successful in alleviating symptoms during outbreaks, but have not been effective in maintaining remission and preventing relapses under various regimens including combination therapies (Colombel et al., 2004; Loftus et al., 2004; Isaacs et al., 2005). Moreover, severe side effects of immunosuppressants limit their long-term usage (Colombel et al., 2004; Loftus et al.,

2004; Isaacs et al., 2005). Eventually, 25% of UC and 60 % of CD patients have to undergo colectomy in 10 to 15 years following the onset of IBD (Bernstein and Nabalamba, 2006).

Such inadequacies in current therapies could arise from their failure to mend compromised colonic mucosal barrier functions involving epithelial cell layers, mucus and goblet cells in IBD patients. This hypothesis is supported by several papers reporting that the impaired integrity of colonic mucosal epithelial barrier is central in the course of IBD (Stein et al., 1998; Lim and Hanauer, 2004). Therefore, strengthening of epithelial resistance to noxious stimuli and enhancement of intestinal repair would provide novel and effective approaches for the treatment of IBD (Dignass, 2001; Yu et al., 2004).

NSAIDs and COX-2 inhibitors often trigger and worsen IBD in humans (Thiefin and Beaugerie, 2005; Meyer et al., 2006). Among various prostanoids affected by COX inhibitors, PGE_2 has received much attention because of its important roles in GI physiology. Functionally, PGE_2 interacts with the four receptor subtypes (EP1-4). EP1 and EP3 primarily contribute to inflammatory responses, while EP4 promotes both cell survival and growth by activating anti-apoptotic and proliferative cellular signaling pathways (Fujino et al., 2003; Hase et al., 2003; Hoshino et al., 2003; Goulet et al., 2004; Joseph et al., 2005). Moreover, EP4 is constitutively expressed in the colonic epithelium, and further induced during IBD, along with PGE_2 (Wiercinska-Drapalo et al., 1999; Northey et al., 2000; Takafuji et al., 2000; Nitta et al., 2002). Recently, EP4 antagonists have been reported to impair epithelial proliferation in the colon (Kabashima et al., 2002). Few studies, however, have delineated effects of EP4-selective agonists on the survival of epithelia and Goblet cells during IBD. Earlier studies primarily concerned

immunosuppressive effects of EP4 agonists (Nitta et al., 2002). For instance, an EP4 agonist, ONO-AE1-329, {[[3-[[(1R,2S,3R)-3-hydroxy-2-[(1E,3S)-3-hydroxy-4-[3-(methoxymethyl)phenyl]-1-butenyl]-5-oxocyclopentyl]thio]propyl]thio]-acetic acid, $C_{22}H_{30}O_6S_2$}, inhibited the production of cytotoxic cytokines, when topically applied in the Dextran Sodium Sulfate (DSS)-induced colitis model.

In this study, we examined the effect of an EP4-selective agonist, AGN205203, {7-[2-(3-Hydroxy-4-phenyl-but-1-enyl)-6-oxo-piperidin-1-yl]-heptanoic acid methyl ester, $C_{23}H_{33}NO_4$}, on colonic epithelial and Goblet cells in the mouse DSS-Indo colitis model. The drug is metabolically and chemically more stable than the ONO agonist, because it has no oxidiazable sulfur atoms in the α-chain and no 11-OH group, a potential source of β-elimination reaction which abolishes its interaction with EP4 (Fig. 1).

Materials and methods

Ligand binding and functional tests.

Competition binding experiments were performed in a medium containing Hank's balanced salt solution, Hepes 20 mM, pH 7.3, $2x10^5$ cells or membranes (~60 µg protein) from HEK 293 cells stably expressing the human EP4 receptor, [^{3}H] PGE_2 (10 nM) and various concentrations of test compounds in a total volume of 300 µl, as described in detail elsewhere (Alberts et al., 2003). cAMP assay was carried out using AlphaScreen cAMP assay kits (PerkinElmer, Boston, MA) following manufacturer instructions. Intracellular Ca^{2+} was monitored using a FLIPR Tetra system and assay kits from Molecular Devices following manufacturer instructions. All assays were carried out in HEK-293 cells heterologously and stably expressing each of the eight human recombinant prostanoid receptors. For Ca^{2+} signals, hEP2, hEP4, hDP were co-expressed with a chimeric G protein, Gqs, which converts Gs signal to Gq Ca^{2+} signal, and hEP3 with a chimeric G protein, Gqi (Hata and Breyer, 2004). Each receptor-selective agonist induced Ca^{2+} signals with sub-nanomolar or nanomolar EC_{50} values. Subtype-selective compounds used here are PGE_2 for EP1, EP2, EP3 and EP4; BW245C for DP; 17-phenyl PGF2α for FP, carbacyclin for IP and U-46619 for TP.

Animals and treatments.

Female C57BL/6 mice at 8 weeks were purchased from Charles River. Animal housing and handling procedures were approved and performed according to the guidelines of Allergan's animal care and use committee. All the chemicals and reagents were obtained from Sigma Aldrich, USA, unless noted otherwise.

Colitis was induced in mice with 3 or 5% DSS in drinking water with or without indomethacin. DSS with the molecular weight of 8,000 is known to induce colitis at the proximal colon (Kitajima et al., 2000), the most affected region of IBD. The stock solution of indomethacin was prepared in DMSO with trace Tween 80, and was added to DSS solution to a final concentration of 4 mg/kg/d (Takeuchi et al., 1986; Kabashima et al., 2002). Normal control group was given drinking water containing only vehicle for 7 days.

AGN205203, the methyl ester of AGN205204, was chosen for the subcutaneous application here, because the ester is readily hydrolyzed to the parent acid in the plasma by endogenous esterases, but would provide more options for future applications such as subcutaneous depots and transdermal patches due to its more optimal hydrophobicity (cLog P=3.4) than the ionized parent acid. The stock solution of the compound was prepared in DMSO, and added to 0.9% normal saline to the final dose of 3 mg/kg/d. Osmotic pumps (the model 1002 Durect, USA) with the drug solution or the vehicle alone for the control and normal groups were implanted subcutaneously 2 days before the induction of colitis, so as to deliver the drug two days earlier as well as during the DSS-Indo treatment.

Body weight, stool consistency and occult blood in the stool were monitored daily (Kabashima et al., 2002). Cumulative consumption of food was measured over the study period. Diarrhea was scored as follows: 0, normal; 2, loose stools; 3, soft mud-like stool; 4, watery diarrhea. Hemoccult was estimated from a guaiac paper test (Colo-Screen; Helena Laboratories Corp., Beaumont, Texas, USA), and scored as follows: 0, normal; 2, trace positive; 3, strong positive; 4, gross bleeding (Kabashima et al., 2002; Krieglstein et

al., 2002). Mice were sacrificed on Day 7 following colitis induction. Spleen weight, colon length and weight with/without contents were measured. Colons were fixed in 10% neutral formalin for histological analysis.

Histology and immunological staining.

Colons were divided into proximal, middle and distal portions and embedded with paraffin. Sections of 5 μm thick were prepared and stained with hematoxylin and eosin (H&E) from each portion, and examined under microscopy. To observe goblet cells, Alcian Blue staining was used. Briefly, sections were incubated in 1% Alcian Blue in 3% acetic acid, pH 2.5, for 30 minutes and 0.1% nuclear Fast Red for 10 seconds. Alcian Blue-positive goblet cells were counted over the size of a grid (1mm grid with total 100 squares) under 600X magnification in 10 view fields randomly selected from each slide.

To assess cell apoptosis in colon, sections were processed with TUNEL staining according to manufacturer's instruction (CHEMICON International, Temecula, CA). Briefly, following deparaffinizing, slides were digested with proteinase K (20 μg/ml) at 37 °C for 10 minutes. Working strength terminal deoxynucleotidyl transferase was applied at 37 °C for 1hr after equilibration. Then anti-digoxigenin conjugated with fluorescence was added. Slides were also counterstained with 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI). The person who stained and evaluated the slides was blinded to treatments of the mice. From each section, 10 view fields were randomly chosen under 400X magnification through a fluorescence lens and a UV lens, respectively. Cells positive to TUNEL and DAPI were counted. The percentage of

TUNEL positive cells was calculated. One sample consists of the average of 10 view fields from a section.

Cell proliferation in colon was detected using proliferating cell nuclear antigen (PCNA) using a commercially available kit (Biomeda, Foster City, CA). Briefly, paraffin-embedded sections were de-paraffinized in xylene and rehydrated in ethanol. Antigen was retrieved with citrate buffer (pH 6.0) boiled for 5 minutes twice in microwave, cooled down at room temperature and blocked for endogenous peroxidase activity with 3% H_2O_2 in methanol for 10 minutes. Biotinylated mouse anti-rat PCNA antibody was applied and incubated 1 hr at room temperature, followed by streptavidin-peroxidase labeled horse anti-mouse second antibody for 30 minutes. DAB chromogen was applied finally. DAPI counterstaining was also employed. 10 views from each slide were randomly collected under 400X magnification. PCNA positive cells were counted.

Statistical Analysis.

Data are presented as the means with SEM. Data were analyzed using an unpaired two-tailed *t* test or one way ANOVA. A probability (*P value*) of less than 0.05 was considered significant.

Results

AGN205204, the parent acid of AGN205203, is an EP4-selective agonist from the 8-aza piperidone PGE series. In HEK-293 cells expressing recombinant hEP4, the drug bound hEP4 with a Ki of 81 nM from competition experiments with [^{3}H] PGE_2, and increased cAMP production with an EC_{50} of 0.08 nM. On the other hand, the drug at 10 μM showed no detectable FLIPR signals in HEK 293 cells heterologously expressing hEP1, hFP, hIP and hTP, and also in hEP2 (Gqs), hEP3 (Gqi), hDP (Gqs) where a chimeric G protein in parenthesis was co-expressed, either Gqi or Gqs, to convert Gi or Gs coupling to Ca^{2+} signal, respectively (Hata and Breyer, 2004).

Mice were treated with 3% DSS with or without indomethacin (4 mg/kg/d) in drinking water. The 3% DSS treatment alone showed no considerable effects on body weight, loss of stool consistency and fecal blood (Fig. 2). Only the combined treatment with DSS and indomethacin led to considerable body weight loss, increased diarrhea score and colonic bleeding, as compared with mice treated with 3% DSS alone or normal control since day 2 (Fig. 2).

We investigated the effects of AGN205203, an EP4 agonist, on colitis in mice induced by 5% DSS-Indo. We observed a rapid body weight loss of 10% within 3 days, diarrhea and bloody stool (Fig. 3*A*, 3*C* and 3*D*). Subcutaneous administration of AGN205203 (3mg/kg/day) from Day (-2) to Day 7 abolished colitis symptoms; gaining body weight up to 4% instead of weight loss (Fig. 3*A*), lower diarrhea and hemoccult scores as compared with vehicle-treated mice (Fig. 3*C*, 3*D*). Food consumption was less in vehicle-treated mice than that in AGN205203-treated or normal control mice (Fig. 3*B*).

Overall, the mice treated with AGN205203 appeared much healthier than the vehicle-treated mice

Gross observations of the colon and spleen were performed on Day 7. The average colon length was 76.6 ± 1.4, 63.4 ± 0.8 and 75.2 ± 1.0 mm for the normal, vehicle- and AGN205203-treated mice, respectively. The ratio of colon weight to length which reflects colonic edema and water absorption was 5.9 ± 0.2, 7.5 ± 0.3 and 6.1 ± 0.2 for the normal, vehicle- and drug -treated mice, respectively ($P<0.0001$). The weight of spleen as normalized to the body weight was 0.41 ± 0.03, 1.42 ± 0.06 and 0.96 ± 0.05 % for the normal, vehicle- and AGN205203-treated mice, respectively ($P<0.0001$), and was inversely related to the hematocrit level as reported earlier (Morteau et al., 2000). Overall, AGN-205203 treatment reversed the phenotypes of colitis mice (vehicle-treated), such as significant shortenings of the colon, increased edema and decreased water absorption, and splenomegaly.

Tissues from isolated colons were processed for histological observation. Twenty tissue slides from each group were examined. Fig. 4A showed the representative sections. As shown on the top panel, H&E staining revealed considerable tissue damages in the vehicle-treated group (V), but not in the normal or AGN205203-treated groups. For example, the vehicle-treated colon shows a loss of columnar nuclei at the surface of crypts, the disappearance of the tight conjunctive epithelial cells, and the infiltration of mononuclear cells into eroded ulcerous mucous layers. The insert ‘a’ to the panel shows severely ulcerated regions with distorted crypts, destruction of cellular structure and some mononuclear cell infiltrations. On the bottom panel, Alcian Blue primarily stains mucus polysaccharide (blue color) and nuclei (pink). The staining revealed the depletion of

goblet cells (P<0.0001, Fig. 4*A* and 4*B*) and the loss of top crypt nuclei from the vehicle-treated mice. None of such damages was observed in the AGN205203-treated or control mice.

At cellular level, we also monitored apoptotic cells using TUNEL staining. Nearly 5 % of DAPI-stained cells were TUNEL-positive in colonic mucosal layers from DSS-Indo treated mice. With the drug treatment, however, TUNEL-positive cells decreased to less than 1.3 % (Fig. 5*B*). It should be noted that TUNEL-positive stained cells mainly located at the surface of mucous layer, with little staining at the bottom of crypts or other layers of the colons (Fig. 5*A*). We also monitored proliferation of epithelial cells, using proliferation cell nuclear antigen (PCNA) as a marker, since GI epithelial cells are turned over rapidly. PCNA is an intranuclear polypeptide whose synthesis reaches its maximum during the S phase of cell cycle. Generally, PCNA-positive cells were localized largely at crypt epithelium of the mucous layer, rarely found in sub-mucosal, muscular or serosa layer (Fig. 6*A*). With the DSS-Indo treatment, PCNA-positive cells are sparsely found only at the bottom of crypts, probably hinting an ongoing, spontaneous repair of epithelial damage, albeit not so robust. In contrast, the AGN205203 treatment abundantly produced PCNA-positive cells, which were localized not only at the bottom of crypts, but also at the top part of crypts. Overall, PCNA-positive cells in non-villus as well as villus crypts were significantly greater in number with AGN205203-treatment than those without the drug treatment (Fig. 6*B*). It appears that AGN205203 may accelerate the regenerative capability of epithelial cells and replenish cells at the crypts much faster. Taken together, the above results indicate that EP4 activation may contribute to the maintenance of intact mucosal barrier.

Discussion

Low dose of DSS treatment alone induced no obvious colitis symptoms in wild type mice (Morteau et al., 2000; Shichijo et al., 2005). In COX-1 or 2 knock out mice, on the other hand, DSS alone produced severe colitis (Morteau et al., 2000). These observations are also consistent with our current observations that only a combined treatment with DSS and indomethacin at a low dose (4mg/kg/d) produced significant retardation in body weight gain and bloody diarrhea. Also clinically, NSAIDs have been reported to trigger or worsen IBD patients (Thiefin and Beaugerie, 2005; Meyer et al., 2006). These results indicate some protective effects of prostanoids synthesized by COX against colitis development (Morteau et al., 2000). Particularly, PGE_2 has been reported to increase in colonic tissues upon DSS-treatment (Morteau et al., 2000), and seems to be the primary prostanoid contributing to such protective effects.

PGE_2 interacts with four EP receptor subtypes (EP1-4). Earlier studies have shown that in human colon, both surface and lateral epithelium of crypts constitutively express the EP4 subtype while apex epithelia of crypts constitutively express the EP2 and EP3 subtypes (Takafuji et al., 2000). The EP1 subtype is not detected on colon epithelial cells (Morteau et al., 2000). Interestingly, only $EP4^{-/-}$ among the eight prostanoid receptor knock out mice is the most susceptible to DSS-induced colitis (Kabashima et al., 2002). Moreover, topical application of an EP4 agonist, ONO-AE1-329, reduces cytotoxic cytokine production, and ameliorates colitis symptoms in DSS colitis model (Nitta et al., 2002). Similarly, in this current study, we have shown that systemic application of a chemically and metabolically stable EP4 agonist, AGN205203,

completely eliminated colitis symptoms, such as body weight loss, reduced food intake, the shortening of colon, and severe diarrhea in the mouse IBD model. These results are consistent with the notion that PGE_2 via EP4 may ameliorate colitis symptoms.

Earlier studies with an ONO EP4 agonist in mice IBD models were largely focused on its ability to inhibit innate immune responses; namely, the inhibition of cytotoxic cytokine production (Kabashima et al., 2002; Nitta et al., 2002). More importantly, however, EP4 activation has been shown to induce anti-apoptotic activities in cultured gastric and intestinal epithelial cells (Hoshino et al., 2003; Joseph et al., 2005), which seem to be critical for preserving and mending mucosal barrier functions. Therefore, we further investigated morphological and biochemical changes in colonic tissues and cells. Histopathological examination showed that tissues from vehicle treated mice showed a marked loss of surface epithelia, visible erosion and ulcer formation, and infiltration of inflammatory cells. On the other hand, tissues from the AGN205203 treated mice showed a well preserved structure of mucous layer with no loss of surface epithelia, and no visible erosion and ulcer formation. Also of interest is our current observation that EP4 agonist treatment preserves not only epithelial, but also goblet cells, which are largely depleted in vehicle-treated mice. It has been reported that goblet cells from both human and experimental animals robustly express EP4, and its expression was further enhanced in colon mucosa during IBD (Northey et al., 2000; Takafuji et al., 2000; Nitta et al., 2002). Also known is that mucus secretion from goblet cells increases upon activation of EP4, but is inhibited by NSAIDs (Ohnishi et al., 2001; Shimamoto et al., 2005). So, EP4 agonists should bring about robust secretion of mucus through the activation of EP4 abundantly expressed in colonic goblet cells during colitis development,

and provides a semi-permeable, mechanical gel barrier between the lumen and the epithelium. Such actions of EP4 agonist certainly enhance mucosal barrier functions which are critical in minimizing the penetration of bacteria and toxins to the colon lining cells (Einerhand et al., 2002; Shaoul et al., 2004). Thus, depletion of goblet cells means the loss of such critical preventive functions in the colon, and may serve as a histological hallmark for ulcerative colitis in humans and experimental animals.

At cellular level, our current study demonstrated first time in vivo that EP4 agonist prevented colitis-induced epithelial apoptosis and promote cell proliferations. Previously, in cultured intestinal and gastric epithelial cells, PGE_2 has been reported to inhibit apoptosis and also NSAID-induced apoptosis (Joseph et al., 2005; Redlak et al., 2005). Mechanistically, several cellular kinases and transactivators have been proposed to mediate cell survival and cell growth by EP4 agonists, such as PKA, phosphatidylinositol 3-kinase (PI3K), the extracellular signal-regulated kinases (ERKs), and growth response factor-1 (EGR-1) (Fujino et al., 2003; Fugino & Regan, 2006). Further study would clarify the roles of these kinases in EP4 actions in colitis.

Some concerns exist, however, about a potential contribution of EP4 agonists to abnormal cell growth in the colon. In this study, AGN205203-treatment appears to preserve the normal morphology of crypts with no detectable change in the height of crypts as compared to those of the normal group. It seems that the drug reverses only DSS-Indo-induced epithelial cell death and colonic crypt reduction. On the other hand, it has been known from knockout mice studies that ablation of EP1, EP2 or EP4 reduced the formation of aberrant crypt foci in the colon, suggesting their potential contributions to colon carcinogenesis (Shoji et al., 2004). This could be also interpreted, however, that

EP4 activation may be necessary but not sufficient for colon carcinogenesis, and EP4-selective agonists may not have carcinogenic potentials without the activation of the other two subtypes of PGE_2 receptors.

Another obvious potential side-effect of PGE_2 analogs is diarrhea, due to the elevation of cAMP in epithelial cells, and the subsequent activation of cAMP-regulated chloride channels. Recently, however, the selective activation of EP4 has been reported to induce primarily Gi/PI3K/ERK- and much less Gs/cAMP-signalings (Fugino & Regan, 2006). It is reasonable, therefore, to speculate that EP4-selective agonists could induce diarrhea, but much milder than other non-selective PGE_2 analogs on the market. Future study is needed to clarify potential side effect profiles of EP4 agonists including diarrhea and abnormal cell growth in naïve animals.

In summary, IBD is triggered by disruption of epithelial integrity and inadequate repair. AGN205203, an EP4 agonist of the 8-aza piperidinone PGE series, when applied systemically, prevented goblet cell depletion, reduced epithelial cell apoptosis and enhanced epithelial proliferation, resulting in near normal functions and morphology of the colon in the presence of colitis inducing insults, DSS and indomethacin. We propose that AGN205203 would be therapeutically useful for prevention and treatment of IBD via monotherapy or/and combination therapy with drugs in market, particularly during the maintenance phase of IBD, the success of which depends on the restoration of mucosal barrier functions.

Acknowledgements

We are grateful to Yan Hai at Pathology department of Allergan for the excellent work in tissue sectioning and staining. We thank Sonal Vaziran from UCI for apoptosis study. We appreciate the discussion with Adelekan Oyejide, a pathologist at Allergan, Inc.

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Legends for Figures:

Fig. 1. Chemical structure of the piperidinone analog AGN205203.

Fig. 2. Induction of colitis by indomethacin in mice treated with 3% DSS (n=10/group). Mice were treated with either vehicle (N), 3% DSS or 3% DSS with indomethacin (3% DSS+I) for 7 days. A, percentage of body weight gain relative to initial body weight, * $P< 0.05$, or ** $P< 0.01$ versus 3% DSS+I. B, diarrhea score of the above-treated mice, * $P< 0.05$, or ** $P< 0.01$, or *** $P< 0.001$ versus 3% DSS and N. C, hemoccult score, ** $P<0.005$ versus 3% DSS and N.

Fig. 3. Clinical observation of colitis mice treated with EP4 agonist AGN205203. Mice were treated with either vehicle only (N), 5% DSS plus indomethacin and vehicle (D+I+V) or 5% DSS plus indomethacin and AGN205203 (D+I+AGN205203) (n=20/group). A, body weight loss as percentage of initial body weight. *** $P< 0.001$, D+I+V group verse N and D+I+AGN205203 groups. B, the average daily food intake relative to body weight in mice, *** $P<0.0001$ versus N and D+I+AGN205203. C, diarrhea score for all record points, ** $P<0.001$ between D+I+V and D+I+AGN205203. D, hemoccult score, * * $P<0.01$ versus N and D+I+AGN205203.

Fig. 4. Histopathological observation of colon treated with vehicle (N), 5% DSS plus indomethacin and vehicle (V), or 5% DSS plus indomethacin and AGN205203 (AGN205203). A, H&E staining of colon tissues, images from each group were shown

with 200X magnification (top panels). In the vehicle-treated colon, the loss of columnar nuclei of epithelial cells at the surface of crypts was common; some regions completely lost crypts and formed typical ulcers with mononuclear cells. The insertion (a) showed that severe damaged crypts had few enterocytes but mononuclear infiltrates. Bottom panels are representative images of Alcian Blue staining of goblet cells in colon tissues, with 200X magnification. In addition to the depletion of goblet cells, the top 1/3 or ½ of crypts showed pale or disappearance of nuclei in the vehicle-treated section. B, Quantitation of goblet cells from colon slides as shown in figure 5A. *** $P<0.0001$ versus N and AGN205203, n=20.

Fig. 5. Immunofluorence staining of colon sections from vehicle and AGN205203-treated mice drinking 5% DSS and indomethacin water. A, representative images of TUNEL staining of colon mucous layer; bottom part of each image faces colon lumen; DAPI images are corresponding to TUNEL images; 200X magnification. B, Quantitation of TUNEL-positive cells from sections as shown in figure 6A. *** $P<0.0001$ versus AGN205203, n=20.

Fig. 6. Immunohistochemical staining of colon sections from vehicle and AGN205203-treated mice drinking 5% DSS and indomethacin water. A, representative images of PCNA staining of colon mucous layer. B, PCNA positive cells per crypt at 200X magnification. *** $P<0.0001$, n=10.

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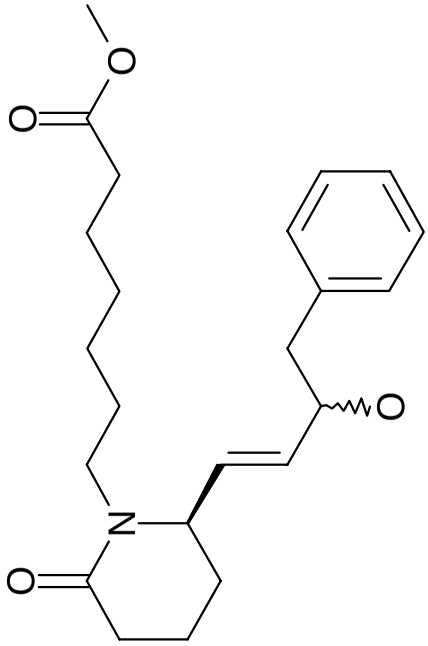


Figure 1

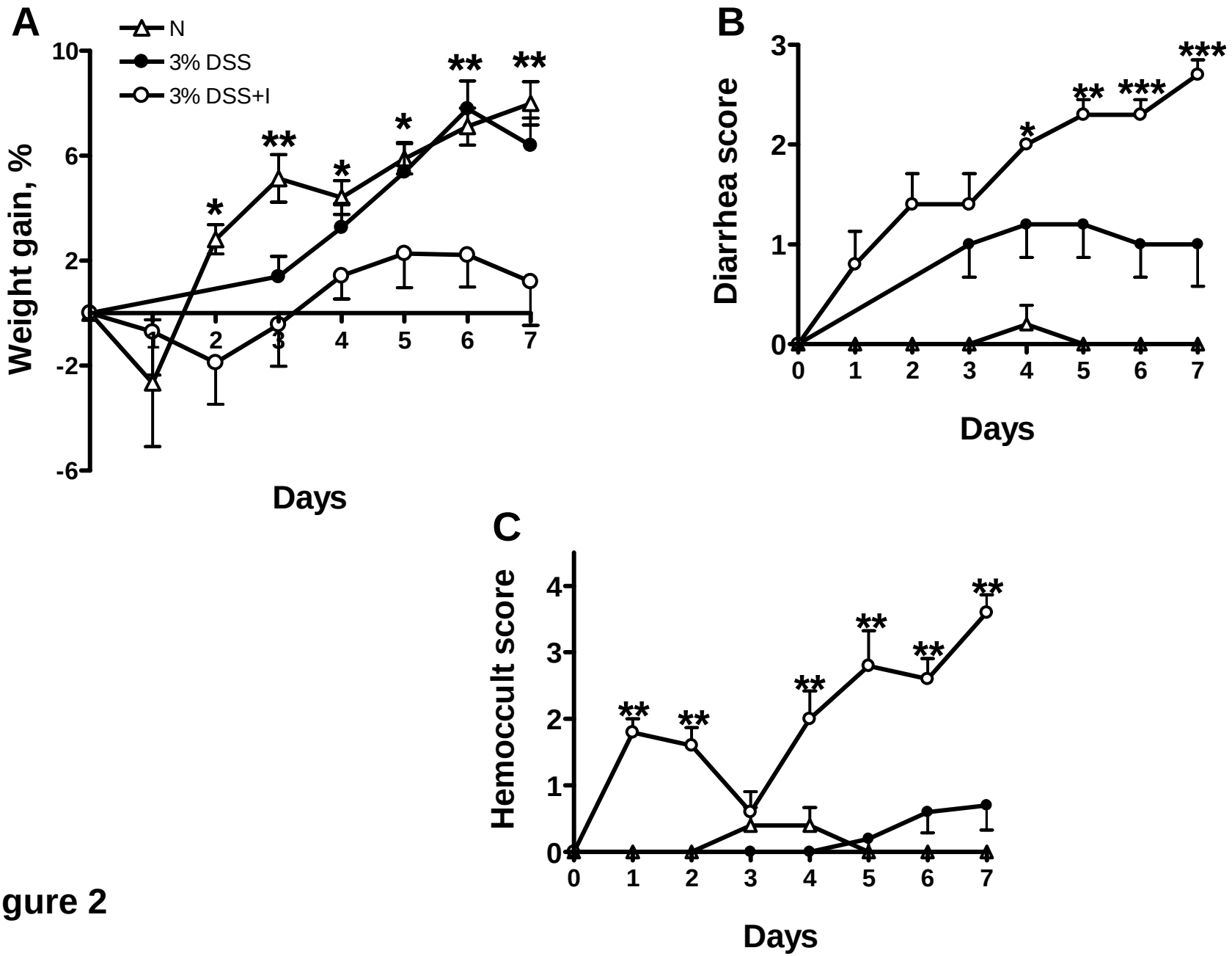
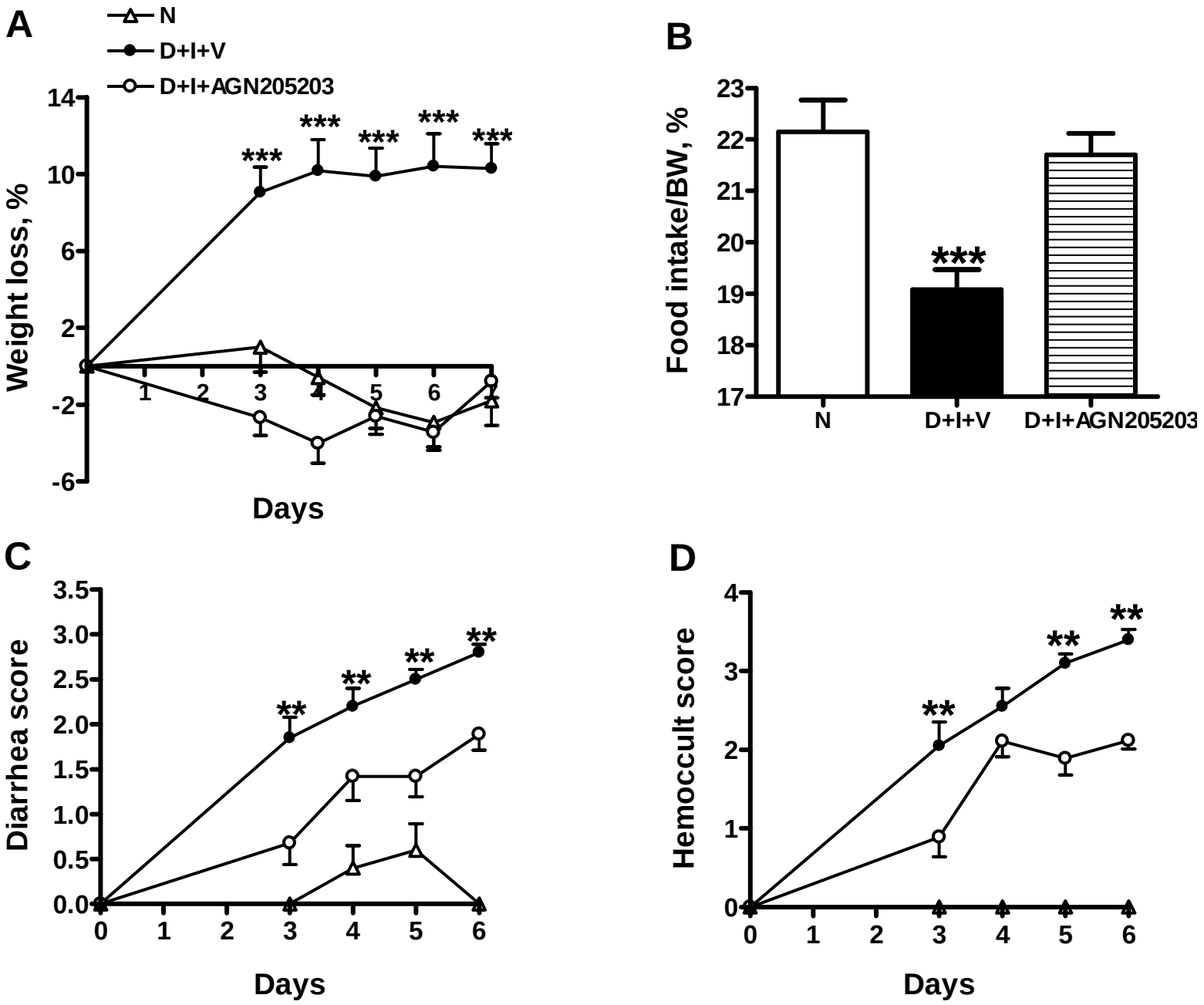


Figure 2

Figure 3



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A

N V AGN205203

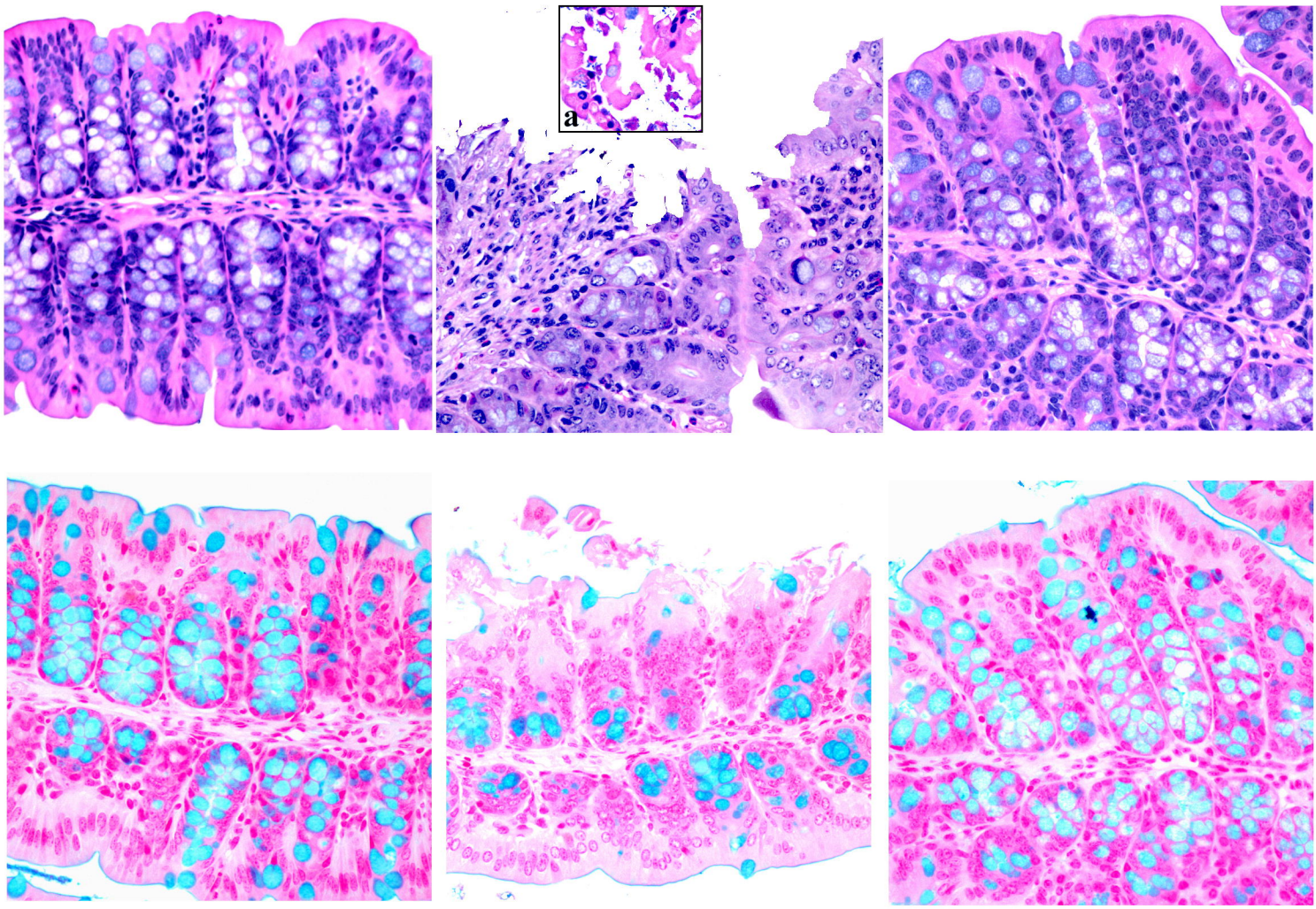

Figure 4A

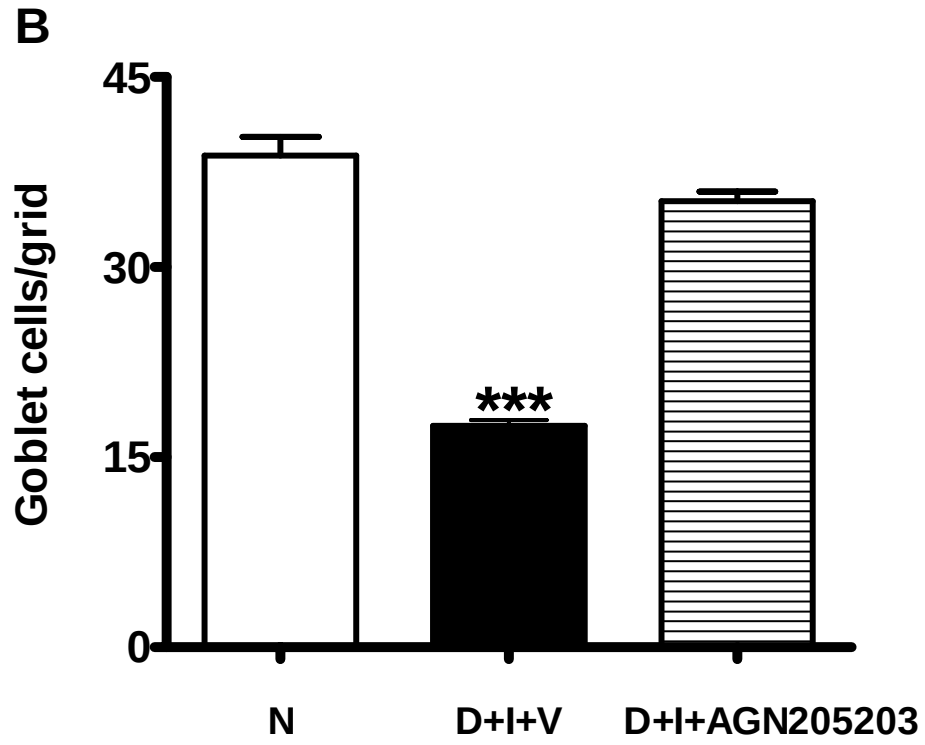

Figure 4B

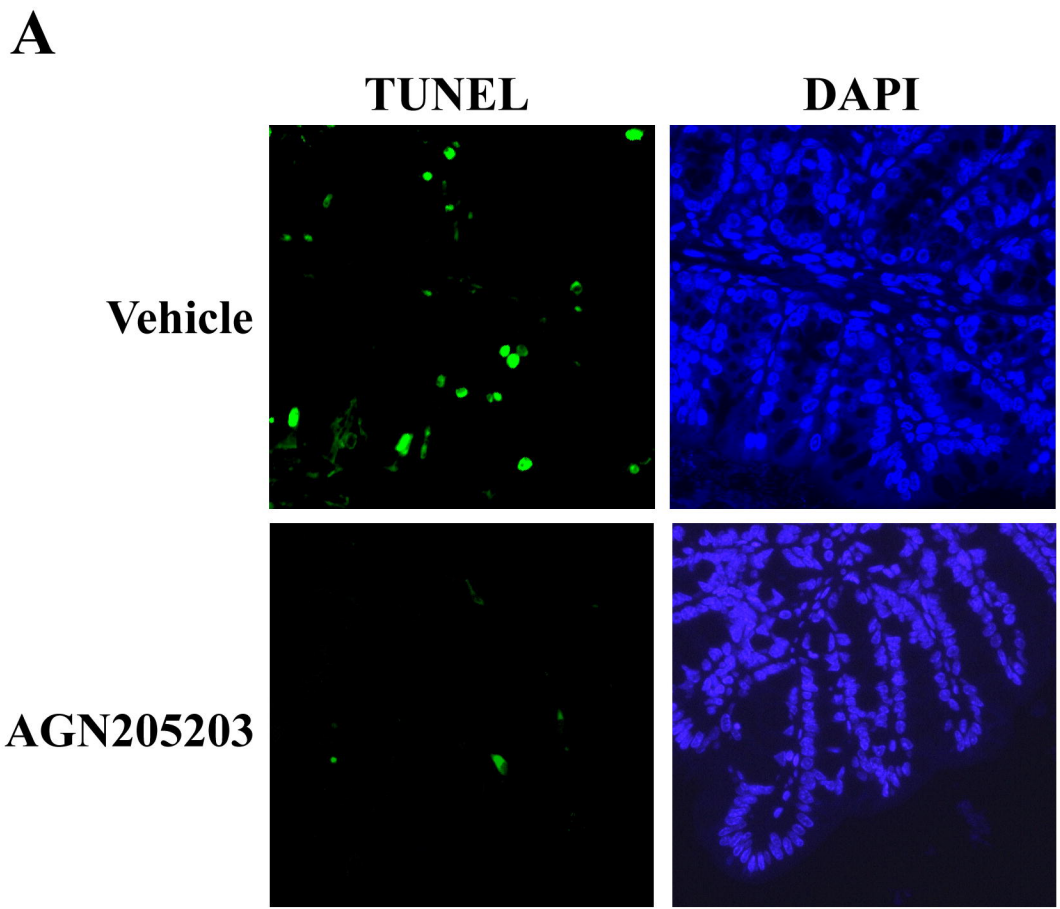

Figure 5A

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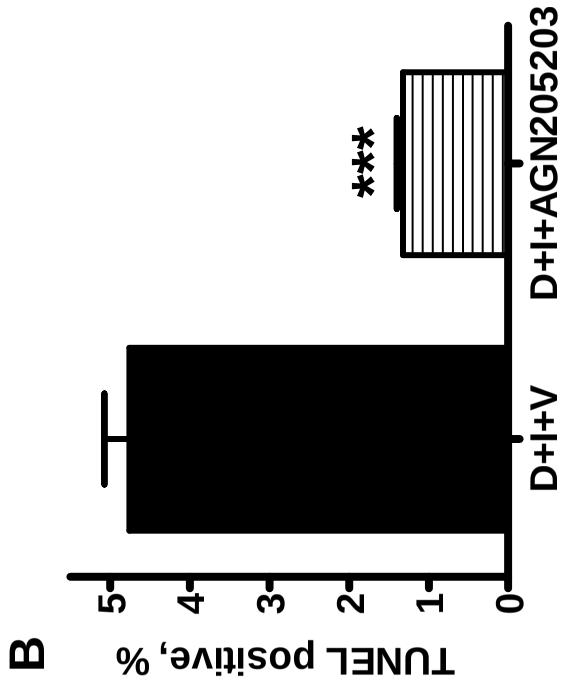

Figure 5B

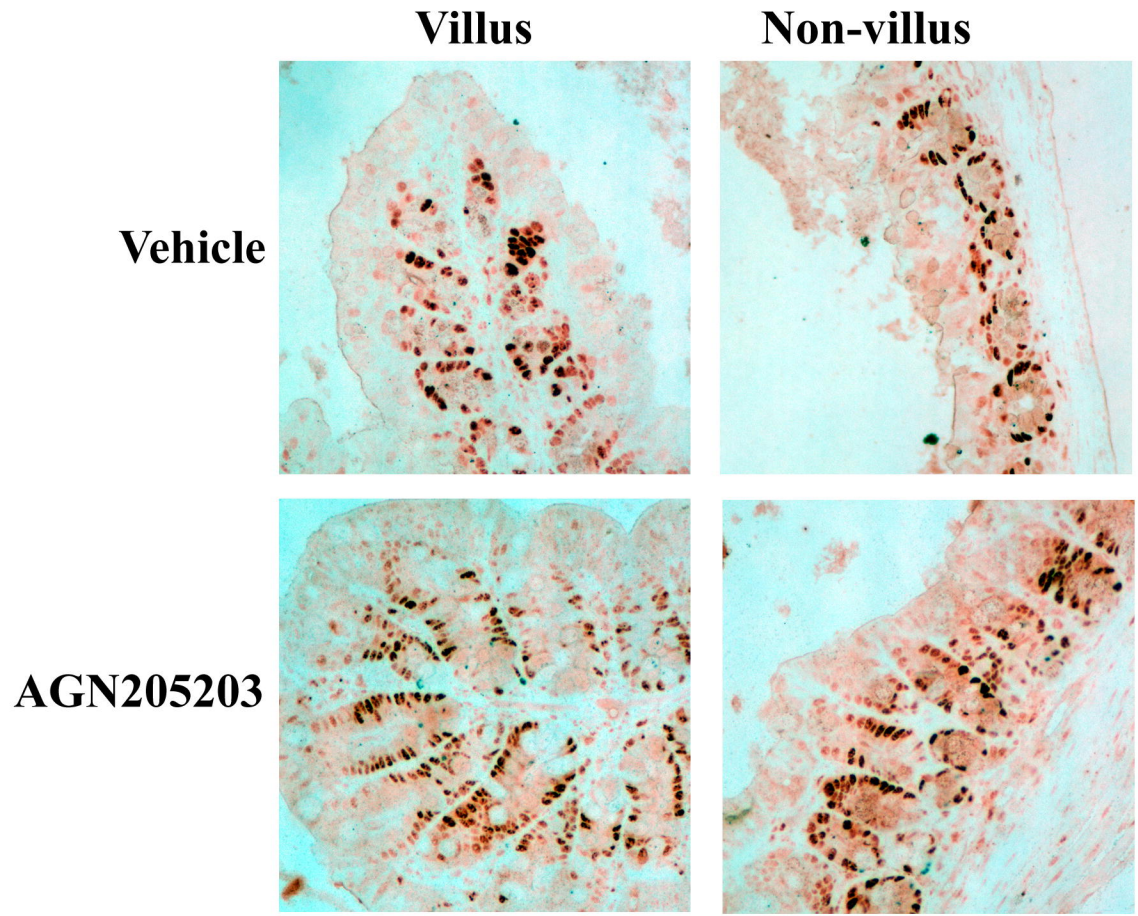

Figure 6A

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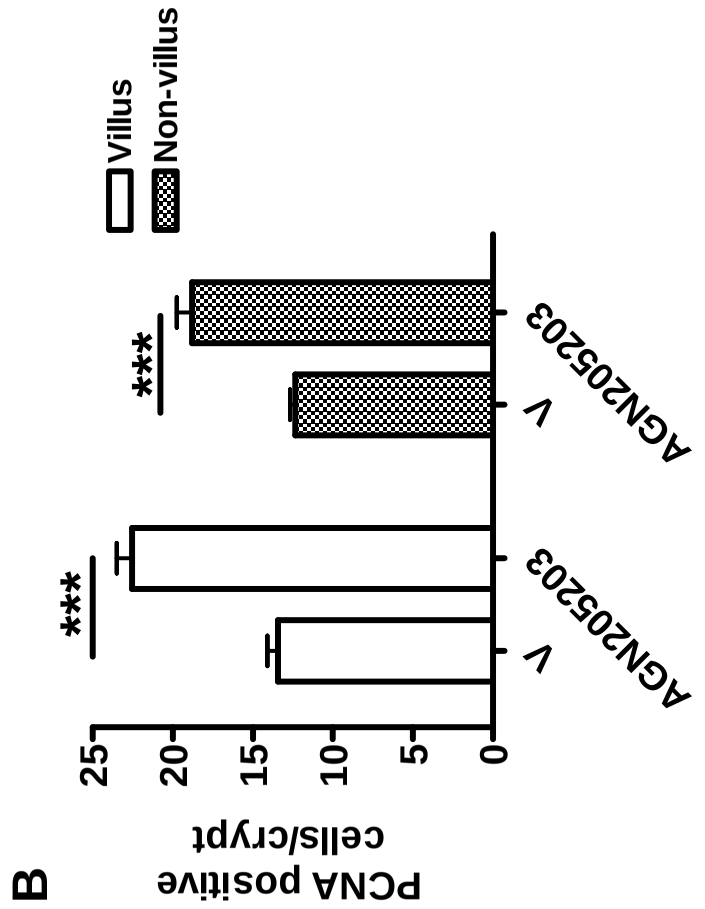

Figure 6B