5-Aminosalicylic Acid Azo-coupled with a GPR109A Agonist Is a Colon-targeted Anti-colitic Codrug with a Reduced Risk of Skin Toxicity

Seongkeun Jeong[1], Hanju Lee[1], Soojin Kim[1], Sanghyun Ju[1], Wooseong Kim[1], Heeyeong Cho[2,3], Hyun Young Kim[2], Gwangbeom Heo[1], Eunok Im[1], Jin-Wook Yoo[1], In-Soo Yoon[1], and Yunjin Jung[1,*]

[1]College of Pharmacy, Pusan National University, Busan, Republic of Korea

[2]Biotechnology & Therapeutic Division, Korea Research Institute of Chemical Technology, Daejeon 305-343, Republic of Korea

[3]Korea University of Science and Technology, 141 Gajeong-ro, Yuseong, Daejeon 305-343, Republic of Korea

Running title: 5-ASA azo-coupled with a GPR109A agonist

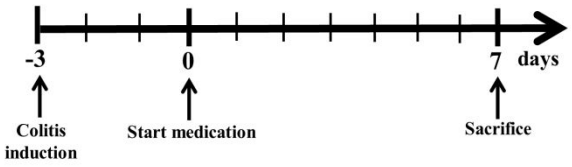
*To whom correspondence should be addressed:

Yuniin Jung

College of Pharmacy, Pusan National University, Busan 46241, Republic of Korea

Tel. 051-510-2527, Fax. 051-513-6754, Email: jungy@pusan.ac.kr

Supplementary data 1



1st experiment

	Group	Oral administration
	Normal	PBS
DNBS-induced colitis	Colitis control	PBS
DNBS-induced colitis	ASA-azo-NA	21.6 mg/kg (equivalent to 30 mg/kg of SSZ)
DNBS-induced colitis	SSZ	30 mg/kg
DNBS-induced colitis	PMT (physical mixture)	5-ANA (10.4 mg/kg, equivalent to 21.6 mg/kg of ASA-azo-NA) and 5-ASA (11.5 mg/kg, equivalent to 30 mg/kg of SSZ)

2nd experiment

	Group	Oral administration
	Normal	PBS
DNBS-induced colitis	Colitis control	PBS
DNBS-induced colitis	ASA-azo-NA	21.6 mg/kg (equivalent to 30 mg/kg of SSZ)
DNBS-induced colitis	SSZ	30 mg/kg
DNBS-induced colitis	ASA-azo-NA+MPZ	ASA-azo-NA and MPZ (2 mg/kg)
DNBS-induced colitis	SSZ+MPZ	SSZ and MPZ (2 mg/kg)
DNBS-induced colitis	MPZ	MPZ (2 mg/kg)

Supporting Information 1. Design of animal experiments

Score	Feature
0	normal appearance
1	localized hyperemia but no ulcer
2	linear ulcers without significant inflammation
3	2–4 cm site of inflammation and ulceration
4	serosal adhesion to other organs, 2–4 cm site of inflammation and ulceration
5	stricture, serosal adhesion involving several bowel loops, <4 cm site of inflammation and ulceration

Supporting Information 2. The modified scoring system of colonic damage score

Compounds	IR		^{1}H-NMR	
	Functional group	WL (cm^{-1})	Position	Chemical shift (ppm)
5-ANA	C=O, COOH in pyridine	1656	2-H	8.23 (s)
			4-H	7.39 (s)
			5-NH_2	5.58 (s)
			6-H	8.08 (s)
5-ASA	C=O, COOH in benzene	1651	3-H	6.67 (d)
			4-H	6.87 (d)
			6-H	7.18 (s)
ASA-azo-NA	C=O, COOH in pyridine	1712	2'-H	9.15 (s, 1H)
			4'-H	8.50 (s, 1H)
			6'-H	9.29 (s, 1H)
	C=O, COOH in benzene	1667	3-H	7.17 (d, J = 1.3 Hz)
			4-H	8.12 (d, J = 1.4 Hz)
			6-H	8.37 (s)

WL: Wavelength

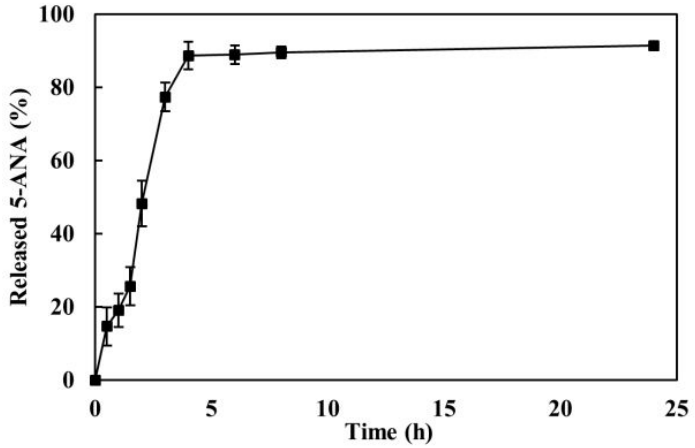
Supporting Information 3. IR and ^{1}H-NMR data

IR and ^{1}H-NMR data of 5-ANA, 5-ASA, and ASA-azo-NA

Compounds	Concentration (mM)	Calcium assay	
		% Activation	EC_{50} (μM)
5-ASA	1	-3.4 ± 1.1	-
	2	3.6 ± 1.4	
	10	1.6 ± 1.3	
NA	0.01	100 ± 3.2	0.052

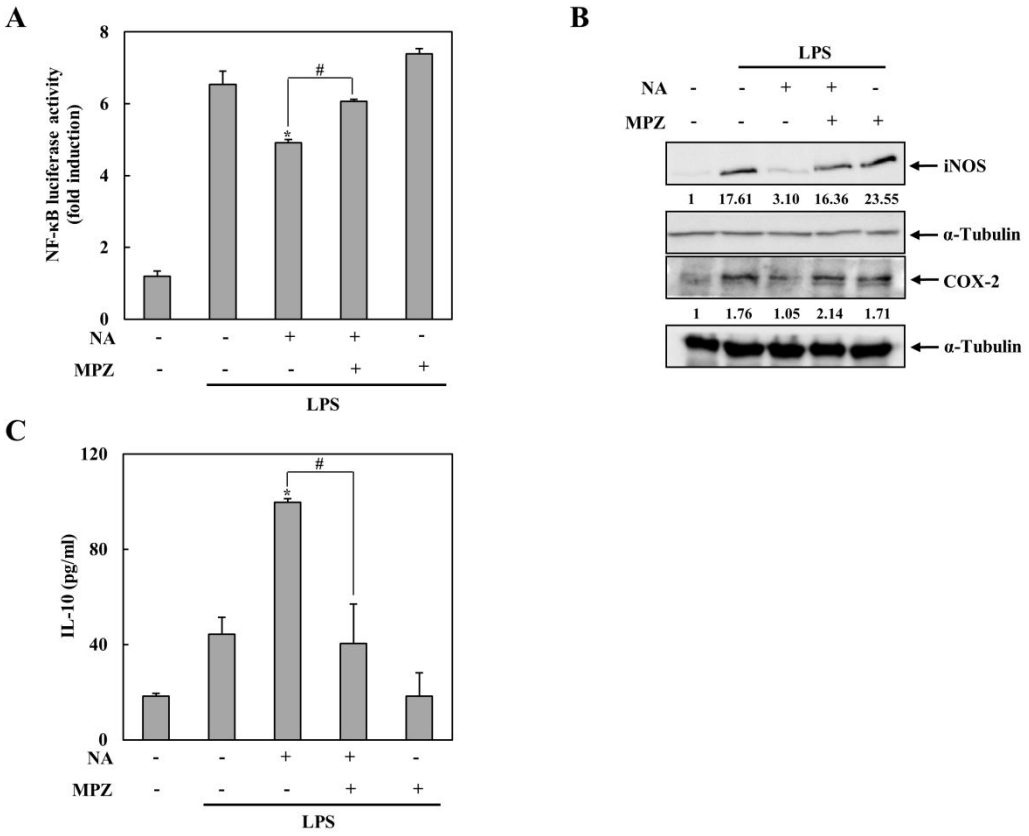
Supporting Information 4. Agonistic activity of 5-ASA on GPR109A

Agonistic activity of 5-ASA on GPR109A was measured using a calcium mobilization assay. Calcium flux was analyzed using CHO/Gα16 cells stably transfected with GPR109A cDNA clone. Activity of 5-ASA is presented as the percentage activation of NA (10 μM), which is taken as 100%.



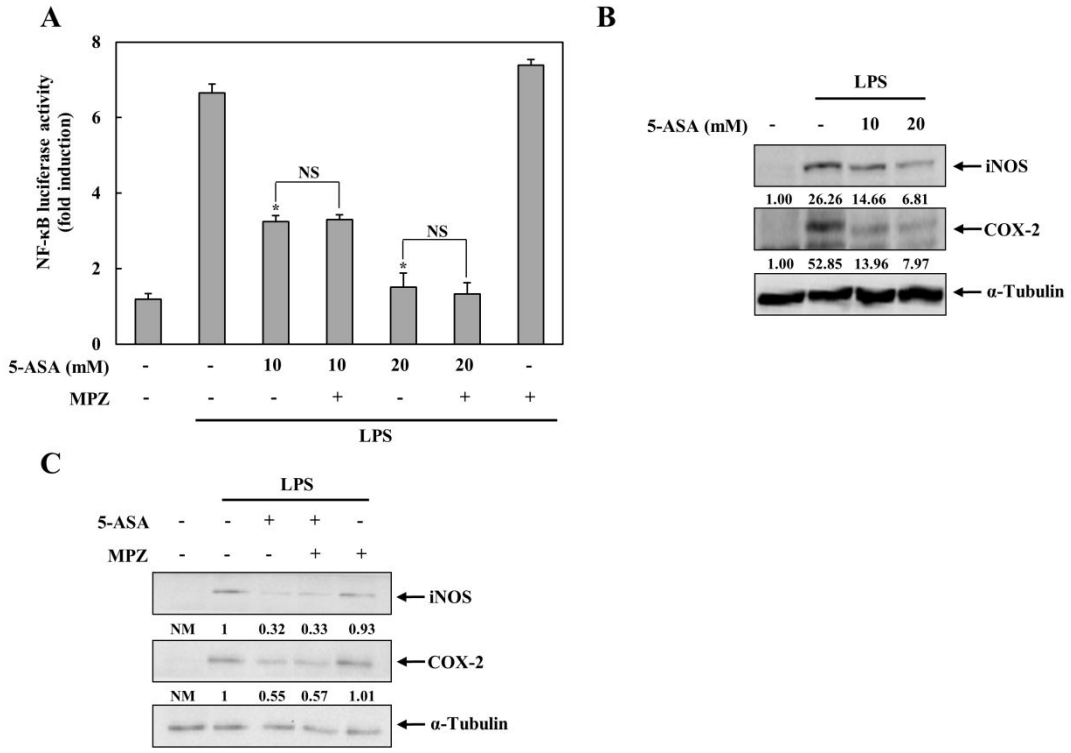
Supporting Information 5. Release profile of 5-ANA during incubation of ASA-azo-NA in cecal contents

ASA-azo-NA (1.0 mg/mL) was incubated in cecal contents suspended in IPS (10%). The concentration of 5-ANA was analyzed by HPLC at the indicated time points.



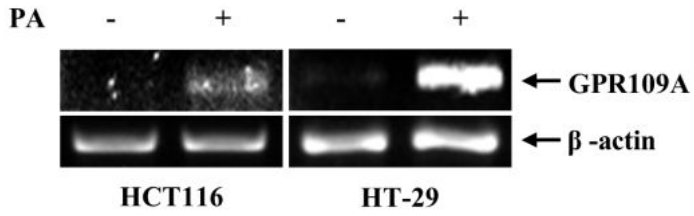
Supporting Information 6. Blockage of anti-inflammatory effects of NA by the GPR109A antagonist mepenzolate.

(A) RAW264.7 cells, transfected with an NF-κB-responsive luciferase plasmid, were pre-incubated with MPZ (100 μM) for 1 h, followed by treatment with NA (1 mM) for 2 h, and subsequently stimulated with LPS (1.0 mg/mL) for 6 h. Reporter activities were measured and normalized to the corresponding CMV *Renilla* activity. (B) RAW264.7 cells were pre-incubated with MPZ (100 μM) for 1 h, treated with NA (1 mM) for 2 h, and then stimulated with LPS for 6 h. The levels of iNOS and COX-2 proteins were assessed by western blot analysis. (C) RAW264.7 cells were pre-incubated with MPZ (100 μM), followed by treatment with NA (1 mM) for 2 h, and then stimulated with LPS for 24 h. IL-10 levels in the cell supernatants were analyzed by ELISA. The data in A, C represent the mean ± SD (n = 5). $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$.



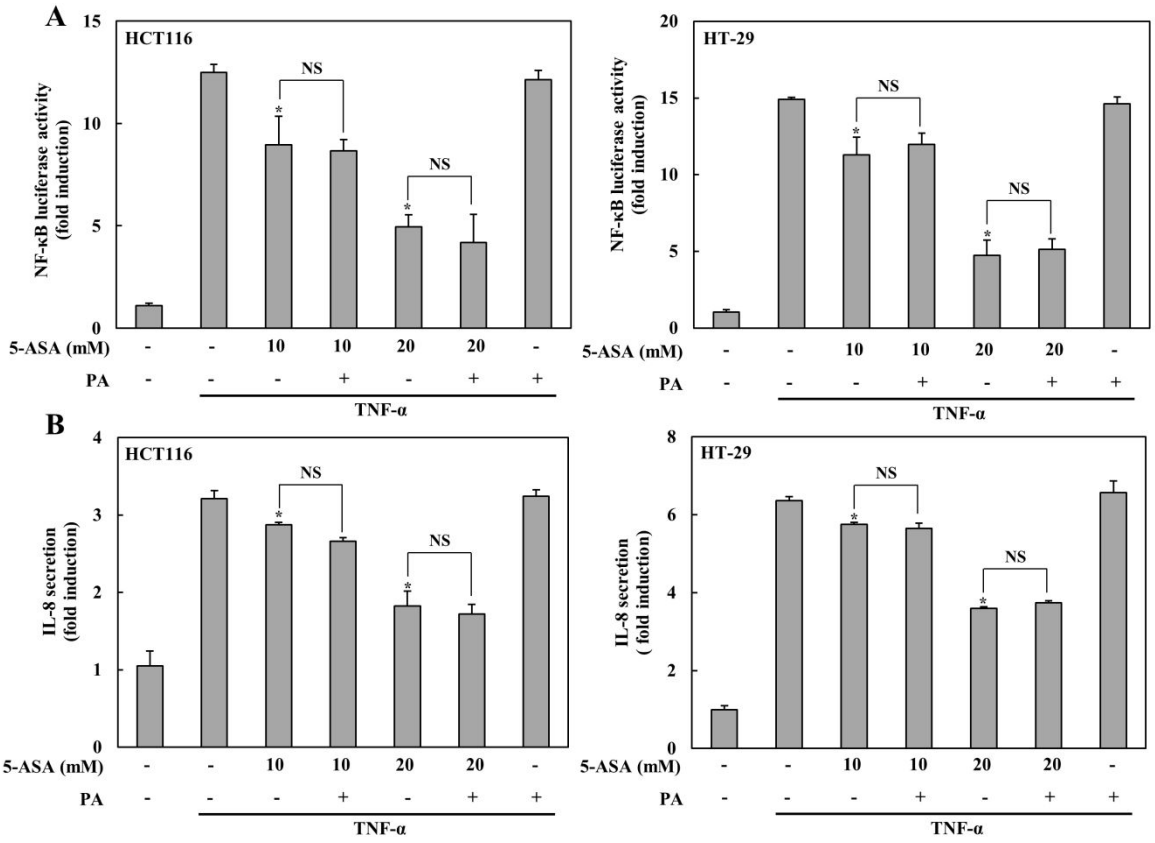
Supporting Information 7. NF-κB inhibitory activity of 5-ASA is not influenced by the GPR109A antagonist mepenzolate.

(A) RAW264.7 cells, transfected with an NF-κB-responsive luciferase plasmid, were pre-incubated with MPZ (100 μM) for 1 h, treated with 5-ASA for 2 h, and then stimulated with LPS (1.0 mg/mL) for 6 h. Reporter activities were measured and normalized to the corresponding CMV *Renilla* activity. (B, C) RAW264.7 cells were pre-treated with 5-ASA for 2 h in the absence (B) or presence (C) of MPZ (100 μM) and then stimulated with LPS for 6 h. The levels of iNOS and COX-2 proteins were assessed by western blot analysis. The data in A represent mean ± SD (n = 5). $^{*}P < 0.05$ vs control, NS: not significant, NM: not measured.



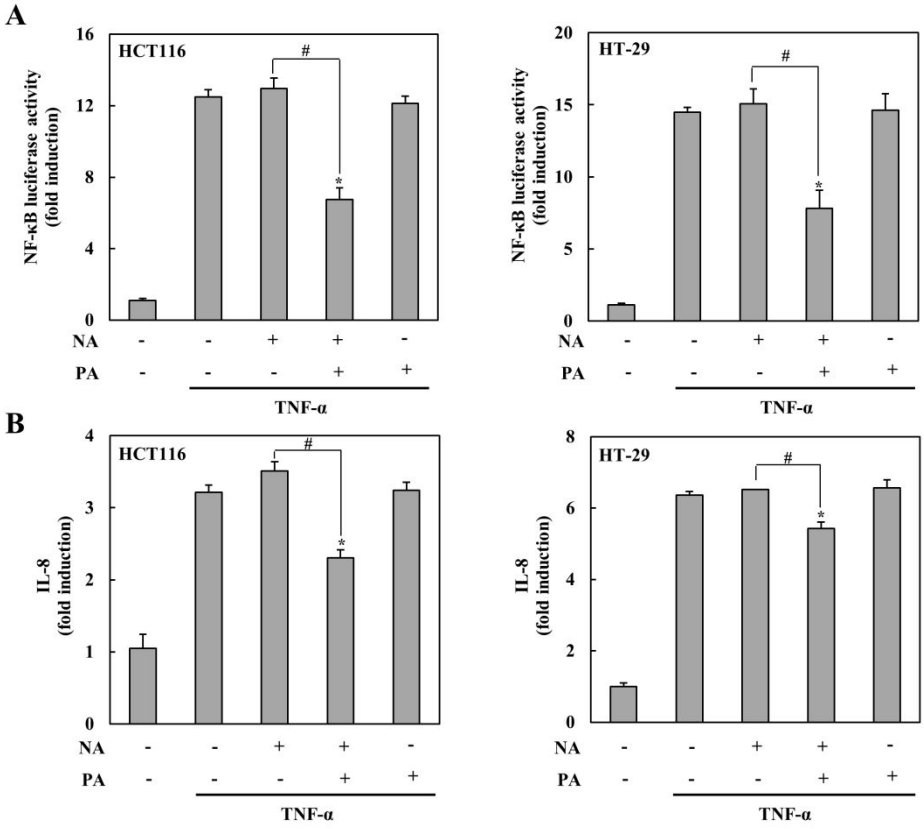
Supporting Information 8. Re-expression of GPR109A in PA-treated colon carcinoma cells

HCT116 (left panel) and HT-29 (right panel) cells were treated with PA (1 mM) for 24 h and the expression of *GPR109A* was analyzed by RT-PCR.



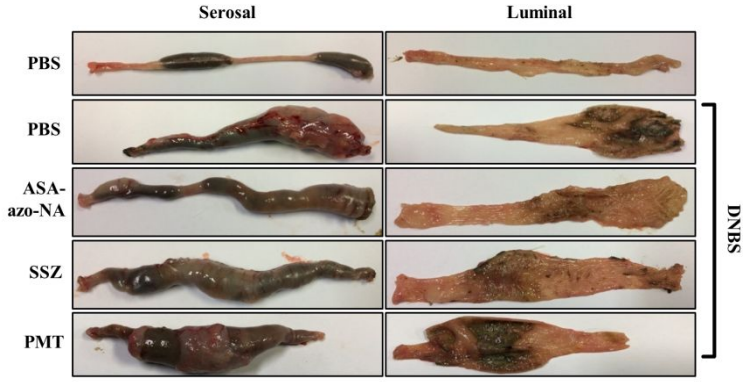
Supporting Information 9. 5-ASA-mediated inhibition of NF-κB is not influenced by pre-treatment with PA.

(A) HCT116 (left panel) or HT-29 (right panel) cells, transfected with an NF-κB-responsive luciferase plasmid for 24 h in the presence or absence of PA (1 mM), were treated with 5-ASA for 2 h and then stimulated with TNF-α (10 ng/mL) for 6 h. Luciferase activities were monitored and normalized to the corresponding CMV *Renilla* activity. (B) HCT116 (left panel) or HT-29 (right panel) cells were pre-incubated with PA (1 mM) for 24 h and treated with 5-ASA for 2 h, followed by stimulation with TNF-α for 6 h. IL-8 levels in cell supernatants were measured by ELISA. The data in A and B represent the mean ± SD (n = 5). $^{*}P < 0.05$ vs control, NS: not significant.



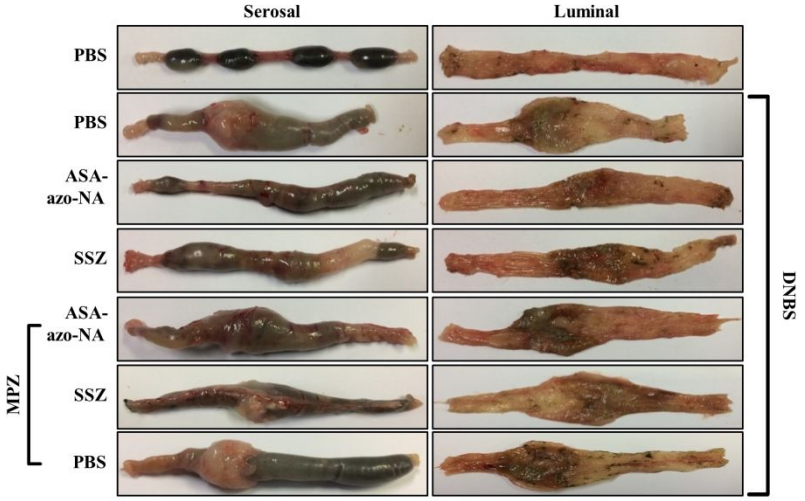
Supporting Information 10. NA inhibits NF-κB activity in PA-treated colon carcinoma cells.

(A) HCT116 (left panel) or HT-29 (right panel) cells, transfected with an NF-κB-responsive luciferase plasmid for 24 h in the presence or absence of PA (1 mM), were treated with NA (1 mM) for 2 h and then stimulated with TNF-α (10 ng/mL) for 6 h. Luciferase activities were monitored and normalized to the corresponding CMV *Renilla* activity. (B) HCT116 (left panel) or HT-29 (right panel) cells were pre-incubated with PA (1 mM) for 24 h and treated with NA (1 mM) for 2 h, followed by stimulation with TNF-α for 6 h. IL-8 levels in cell supernatants were measured by ELISA. The data in A and B represent the mean ± SD (n = 5). $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$.



Supporting Information 11. Images showing the serosal and luminal sides of distal colons

Three-days after the induction of colitis, SSZ (30.0 mg/kg), rats were administered ASA-azo-NA (21.6 mg/kg, equivalent to 30.0 mg/kg of SSZ), or a physical mixture (PMT) of 5-ANA (10.4 mg/kg) with 5-ASA (11.5 mg/kg, equivalent to 21.6 mg/kg of ASA-azo-NA) suspended in PBS (1 mL) by oral gavage once per day, and then sacrificed on the 7th day of treatment.



Supporting Information 12. Images showing the serosal and luminal sides of distal colons

Three-days after the induction of colitis, rats were administered SSZ (30 mg/kg), ASA-azo-NA (21.6 mg/kg, equivalent to 30 mg/kg of SSZ), or MPZ (2 mg/kg) suspended in PBS (1 mL) by oral gavage once per day and then sacrificed on the 7th day of treatment.