European Journal of Medicinal Chemistry 48 (2012) 36–44

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Structure–activity relationship of salicylic acid derivatives on inhibition of TNF-α dependent NFκB activity: Implication on anti-inflammatory effect of N-(5-chlorosalicyloyl)phenethylamine against experimental colitis

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ARTICLE INFO

Article history:
Received 10 August 2011
Received in revised form
2 November 2011
Accepted 16 November 2011
Available online 25 November 2011

Keywords:
Salicylic acid derivatives
Structure–activity relationship
Inflammatory bowel disease
NFκB

ABSTRACT

To develop a more potent NFκB inhibitor from salicylic acid which is known to inhibit activity of NFκB, a transcription factor regulating genes involved in immunity, inflammation and tumorigenesis, derivatives of salicylic acid (SA) where the 5 position, carboxyl or hydroxyl group was modified were treated in HCT116 cells transfected with an NFκB dependent luciferase gene and LPS-stimulated RAW264.7 cells. Amidation of the carboxylic group or substitution of chlorine at the 5 position increased the ability of SA to suppress the expression of NFκB dependent luciferase and inducible nitric oxide synthase, a product of an NFκB target gene. Moreover, simultaneous amidation and chlorination of SA (5-chlorosalicylamide; 5-CSAM) conferred an additive NFκB inhibitory activity on SA. To further enhance the inhibitory activity, N-modification was imposed on 5-CSAM. N-(5-chlorosalicyloyl)phenethylamine (5-CSPA), N-(5-chlorosalicyloyl)3-phenylpropylamine (5-CSPPA) and N-(5-chlorosalicyloyl)4-hydroxyphenylethylamine (5-CSHPA) showed greater potencies for inhibiting NFκB activity than other derivatives. Their IC_{50}s' in the luciferase assay measured 15 μM (5-CSPA), 17 μM (5-CSPPA) and 91 μM (5-CSHPA). Rectal administration of 5-CSPA ameliorated TNBS-induced rat colitis, which was more effective than a conventional drug, 5-aminosalicylic acid. These data may provide useful information for development of a therapeutic agent for treatment of diseases where NFκB plays a critical role in the pathogenic progresses.

1. Introduction

NFκB is an important transcription factor that regulates genes involved in immunity and inflammation [1]. Stimulation with pro-inflammatory cytokines such as TNF-α initiates an intracellular signaling cascade, resulting in the phosphorylation and subsequent degradation of IκB by the 26S-proteasome [2]. The degradation of IκBα releases NFκB allowing it to translocate into the nucleus and transactivates target genes such as cyclooxygenase-2 (COX-2), cytokines and chemokines that are pivotal mediators of the immune and inflammatory responses.

NFκB activity and levels of the pro-inflammatory cytokine TNF-α have been shown to be increased in the colon epithelial cells and mucosa of patients with inflammatory bowel disease (IBD) which comprises ulcerative colitis and Crohn's disease [3]. It is known that expression of TNF-α, which strongly activates NFκB, is itself up-regulated by NFκB. This provides a positive autoregulatory loop that amplifies the inflammatory response and perpetuates chronic intestinal inflammation [3]. For this reason, therapeutic intervention against TNF-α or NFκB activation has been used for treatment of inflammatory bowel disease (IBD) [4]. In fact, inhibition of NFκB activity has been suggested to be a major component of the anti-inflammatory activity of glucocorticoid and 5-aminosalicylic acid (5-ASA) both of which are frequently used for treatment of chronic intestinal inflammation [5,6]. Moreover, new strategies that specifically regulate NFκB activity using p65 (RelA) antisense oligonucleotides, proteasome inhibitors or adenoviral IκB expression vector show beneficial therapeutic effects in experimental colitis [7,8].

Abbreviation: IBD, inflammatory bowel disease; SA, salicylic acid; 5-CSAM, 5-chlorosalicylamide; 5-CSPA, N-(5-chlorosalicyloyl)phenethylamine; 5-CSPPA, N-(5-chlorosalicyloyl)3-phenylpropylamine; 5-CSHPA, N-(5-chlorosalicyloyl)4-hydroxyphenylethylamine; 5-ASA, 5-aminosalicylic acid; TNF, tumor necrosis factor; COX-2, cyclooxygenese-2; iNOS, inducible nitric oxide synthase; 5-DFPSA, diflunisal; 5-NSA, 5-nitrosalicylic acid; 5-CSA, 5-chlorosalicylic acid; ASP, acetylsalicylic acid; MSA, methylsalicylate; SAM, salicylamide; CINC, cytokine-induced neutrophil chemoattractant; TNBS, 2,4,6-trinitrobenzene sulfonic acid; MPO, myeloperoxidase.

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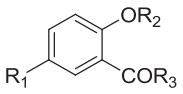
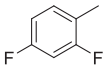
doi:10.1016/j.ejmech.2011.11.030

Table 1
Structures of salicylic acid derivatives.



Compd. name	R_1	R_2	R_3
SA	H	H	OH
5-NSA	NO_2	H	OH
5-ASA	NH_2	H	OH
5-CSA	Cl	H	OH
5-DFPSA		H	OH
MSA	H	CH_3	OH
SAM	H	H	NH_2
ASP	H	$COCH_3$	OH

SA: salicylic acid; 5-NSA: 5-nitrosalicylic acid; 5-ASA: 5-aminosalicylic acid; 5-CSA: 5-chlorosalicylic acid; 5-DFPSA: 5-(2,4 difluorophenyl)salicylic acid; MSA: methylsalicylate; SAM: salicylamide; ASP: acetylsalicylic acid.

Recent papers reported that NFκB inhibition is one of the anti-inflammatory mechanisms of salicylates such as sulfasalazine, salicylate, aspirin and 5-ASA [9–11]. Although the salicylates whose structures are different possess a common biological activity, there has been no prior study on structure–activity relationship on the NFκB inhibition activity. In the present work, derivatives of salicylic acid were purchased or prepared and structure–activity relationship of salicylic acid derivatives on inhibition of TNF-α dependent NFκB activity was investigated, which could provide valuable information for development of a new therapeutic agent for treatment of diseases where NFκB plays a critical role as a pathological mechanism. Based on the potential therapeutic application, the anti-inflammatory activity of one of the derivatives, 5-chlorosalicylic acid phenethylamide (5-CSPA), was evaluated in a TNBS-induced rat colitis model, which was compared to 5-ASA, the most widely used drug for the treatment of IBD.

2. Results

2.1. Structure–activity relationship of salicylic acid derivatives on inhibition of TNF-α dependent NFκB activity

To investigate structure–activity relationship of salicylates on NFκB inhibition, salicylic acid derivatives (structures of the derivatives used are shown in Table 1), diflunisal (5-DFPSA), 5-nitrosalicylic acid (5-NSA), 5-aminosalicylic acid (5-ASA), 5-chlorosalicylic acid (5-CSA), acetylsalicylic acid (ASP), methylsalicylate (MSA) and salicylamide (SAM), were subjected to a luciferase assay using colon carcinoma HCT116 cells transfected with an NFκB dependent luciferase gene. As shown in Fig. 1A, stimulation with TNF-α induced approximately 18-fold luciferase activity in cells, which was attenuated to a varied degree by treatment with salicylates. While the potencies of 5-DFPSA, 5-CSA, MSA and SAM were greater than that of salicylic acid (SA), the other derivatives, ASP, 5-NSA and 5-ASA, showed similar or less potencies than that of SA. Because modifications of SA such as substitution of chlorine at 5 position or amidation of the carboxyl group elicited the positive effect on the potency, 5-CSA was amidated to afford 5-chlorosalicylamide (5-CSAM), expecting to further increase the potency of the inhibitory activity. As shown in Fig. 1B, consistent with the hypothesis, the ability of 5-CSAM to suppress the luciferase expression was greater than that of either 5-CSA or SAM. To confirm NFκB inhibition by the salicylates, SA, 5-CSA, 5-SAM and 5-CSAM, in cells, RAW264.7 cells were treated with LPS in the presence of the salicylates and inducible nitric oxide synthase (iNOS) protein expression, a target gene product of NFκB [12], was monitored. As shown in Fig. 1C, all the salicylates attenuated iNOS expression and their potencies were on the order of 5-CSAM > 5-

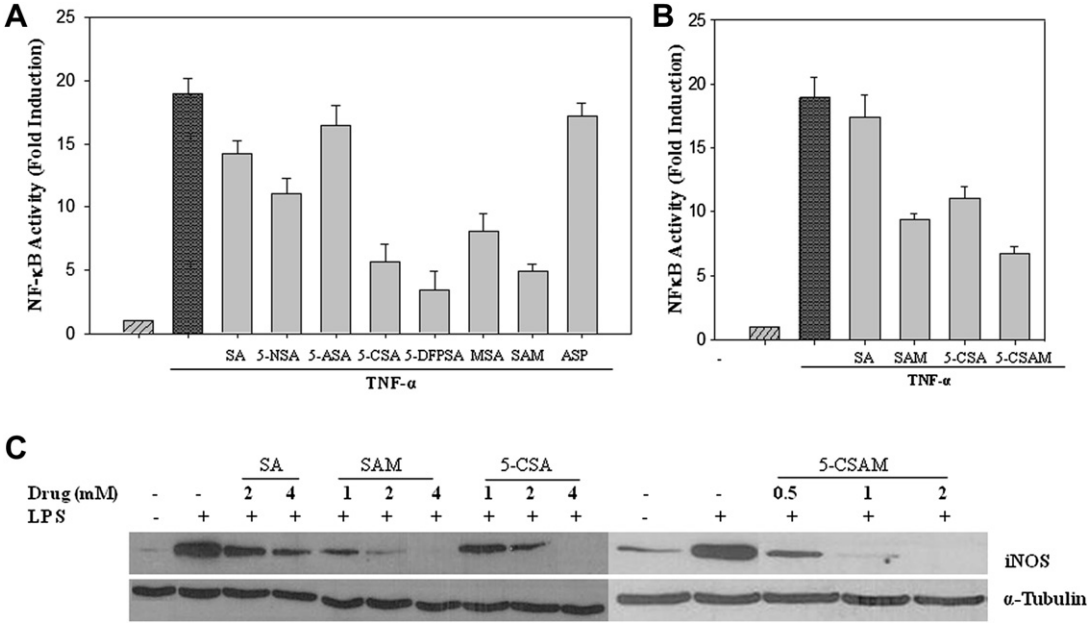


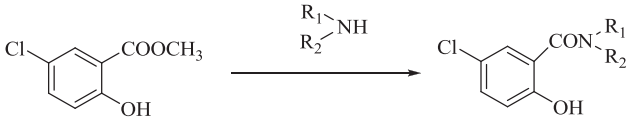
Fig. 1. Structure–activity relationship of salicylic acid derivatives on TNF-α mediated expression of NFκB dependent luciferase. A. HCT116 cells were cotransfected with NFκB dependent luciferase plasmid (0.4 μg) and CMV *Renilla* luciferase plasmid (4 ng) and subsequently treated with TNF-α (1 ng/ml) in the presence of salicylic acid derivatives (1 mM) for 6 h. Reporter activities were measured and normalized to CMV *Renilla* luciferase activity. SA: salicylic acid, 5-NSA: 5-nitrosalicylic acid, 5-ASA, 5-CSA, 5-DFPSA, MSA, SAM, ASP. B. The same experiment as in "A" was done in the presence of 0.5 mM of SA, 5-CSA, 5-CSAM or SAM. C. RAW264.7 cells were pretreated with various concentration of SA, 5-CSA, 5-CSAM or SAM for 1 h followed by stimulation with LPS (1 ng/ml) for 6 h. Levels of iNOS protein in the whole cell lysates were analyzed by Western blot.

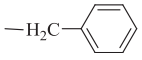
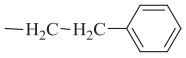
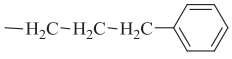
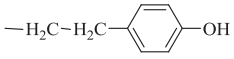
CSA/5-SAM > SA, which is in good agreement with the results of the luciferase experiments. Although 5-CSAM showed an enhanced inhibitory activity against NFκB, it still required approximately 0.34 mM for 50% inhibition of NFκB. For further improvement of therapeutic activity of 5-CSAM, 5-CSAM was modified to afford N-substituted derivatives. A variety of derivatives of 5-CSAM were prepared (structures of the derivatives are shown in Table 2). They were subjected to an NFκB dependent luciferase assay. Cells were stimulated with TNF-α in the presence of the derivatives. As shown in Fig 2A, of the derivatives, N-(5-chlorosalicyloyl)benzylamine **9**, N-(5-chlorosalicyloyl)phenethylamine (5-CSPA) **10**, N-(5-chlorosalicyloyl) 3-phenylpropylamine (5-CSPPA) **11**, N-(5-chlorotsalicyloyl) 4-hydroxyphenylethylamine (5-CSHPA) **12** and N-(5-chlorosalicyloyl)hexylamine **3** elicited greater potencies than 5-CSAM. To determine IC_{50} of 5-CSPA, 5-CSHPA and 5-CSPPA, the luciferase assay was performed at various concentrations of the derivatives. As shown in Fig. 2B, they inhibited NFκB dependent luciferase gene expression in a dose dependent manner and IC_{50} values measured 15, 17 and 91 μM for 5-CSPA, 5-CSPPA and 5-CSHPA, respectively. To confirm the NFκB inhibitory activity of the derivatives, LPS-mediated iNOS protein expression was monitored in the presence of 5-CSPA, 5-CSATY and 5-CSAPPA in RAW264.7 cells. Consistent with the results of the luciferase assay, the derivatives attenuated the expression of iNOS protein (Fig. 2C).

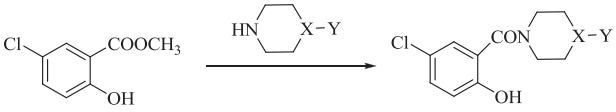
2.2. N-(5-chlorosalicyloyl)phenethylamine administered rectally ameliorates TNBS-induced colitis rats

To examine whether N-(5-chlorosalicyloyl)phenethylamine (5-CSPA) that was assessed as one of the most potent derivatives for NFκB inhibition is therapeutically active against diseases such as inflammatory bowel disease (IBD) whose pathological process is associated profoundly with increased NFκB activity [3], 5-CSPA was

Table 2
Synthetic reactions and structures of N-substituted 5-chlorosalicylamide (CSAM) derivatives.



Compd. no.	R_1	R_2	IC_{50} (μM)[a]
1	H	$-CH_3$	>250
2	CH_3	$-CH_3$	>250
3	H	$-(C_6H_{13})_n$	95
9	H		105
10	H		15
11	H		17
12	H		91



Compd. no.	X	Y	IC_{50}
4	O	–	>250
5	N	H	>250
6	N	$-CH_3$	>250
7	N		>250
8	C	H_2	>250

[a] IC_{50} was measured in an NFκB dependent luciferase assay.

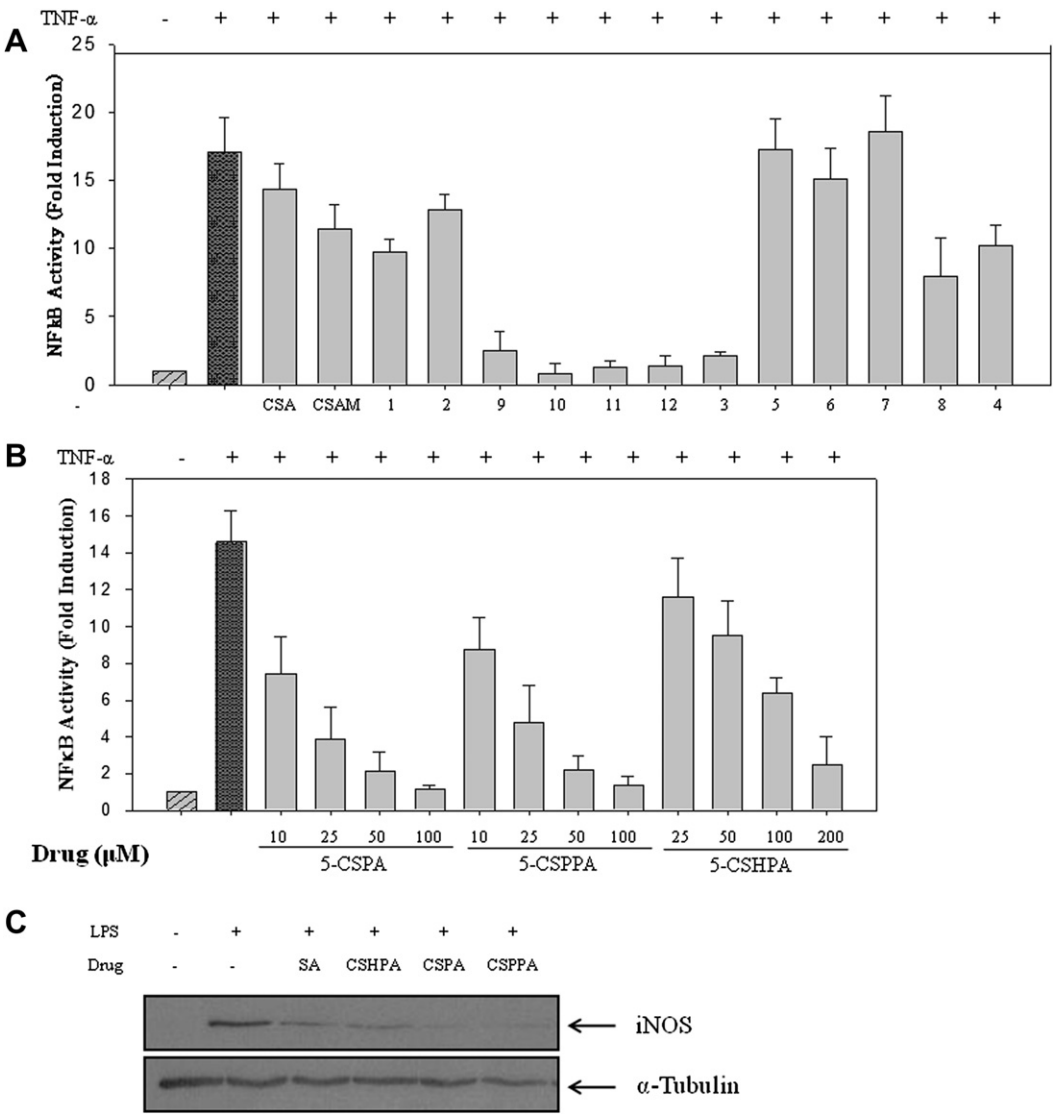


Fig. 2. Structure–activity relationship of N-substituted 5-CSAM derivatives on TNF-α mediated expression of NFκB dependent luciferase A. A. HCT116 cells were cotransfected with NFκB dependent luciferase plasmid (0.4 μg) and CMV *Renilla* luciferase plasmid (4 ng) and subsequently treated with TNF-α (1 ng/ml) in the presence of N-substituted 5-chlorosalicylamide derivatives (0.25 mM) for 6 h. Reporter activities were measured and normalized to CMV *Renilla* luciferase activity. B. The same experiment as in "A" was done in the presence of various concentrations of 5-CSPA, 5-CSPPA or 5-CSHPA. C. RAW264.7 cells were 1 h pretreated with various concentration of SA (5 mM), 5-CSPA (0.1 mM), 5-CSPPA (0.1 mM) or 5-CSHPA (0.25 mM) followed by stimulation with LPS (1 ng/ml) for 6 h. Levels of iNOS protein in the whole cell lysates were analyzed by Western blot.

administered rectally to rats with colitis induced by TNBS once a day for 6 days, which was started 3 days after the induction of inflammation. The same experiment was done with 5-aminosalicylic acid that is the active agent of anti-IBD drugs, sulfasalazine and mesalazine, to compare potency and effectiveness. Rectal route instead of oral route was used to simplify analysis of the therapeutic effect by minimizing pharmacokinetic influence. Dose of 5-ASA was 30 mM in a buffer solution (500 μL) according to a previous report [13] and the doses of 5-CSPA were 100 and 300 μM in the same volume of the buffer. The anti-inflammatory activity of the drugs was assessed by colonic damage score (CDS) and myeloperoxidase (MPO) activity. Moreover, protein levels of pro-inflammatory NFκB target genes such as cyclooxygenase (COX)-2 and, cytokine-induced neutrophil chemoattractant (CINC)-3 were measured in the inflamed colonic tissues to examine whether the anti-inflammatory effect was mediated by inhibition of NFκB. As shown in Fig. 3A, in which CDS represent the extent of colonic injury by TNBS-induced inflammation, the normal colon showed no damage but the control colon, the inflamed colon with no medication, was severely damaged showing scab by the hemorrhagic necrosis of the mucosa, stricture and extensive serosal adhesion to other organs. Rectal administration of 5-CSPA significantly healed the damaged colon in a dose dependent manner, and 5-CSPA (300 μM) seemed more effective than 5-ASA (photographs of the colonic tissues are shown in Supplementary data). Fig. 3B showed that 5-CSPA lowered the level of MPO activity to about 60% (for 100 μM) and 35% (for 300 μM) of the control (group with no medication) and the effectiveness of 5-CSPA (300 μM) was greater than that of 5-ASA, which is in accordance with the recovery of the colonic damage. Expression of NFκB target gene products involved in inflammation, CINC-3 and COX-2 protein, was examined in the inflamed tissue. As shown in Fig. 4A and B, levels of the inflammatory mediators were elevated in the inflamed colonic tissue compared with the normal colonic tissue. Consistent with MPO and CDS results, 5-CSPA decreased the levels of the inflammatory mediators in the inflamed tissue in a dose dependent manner, and

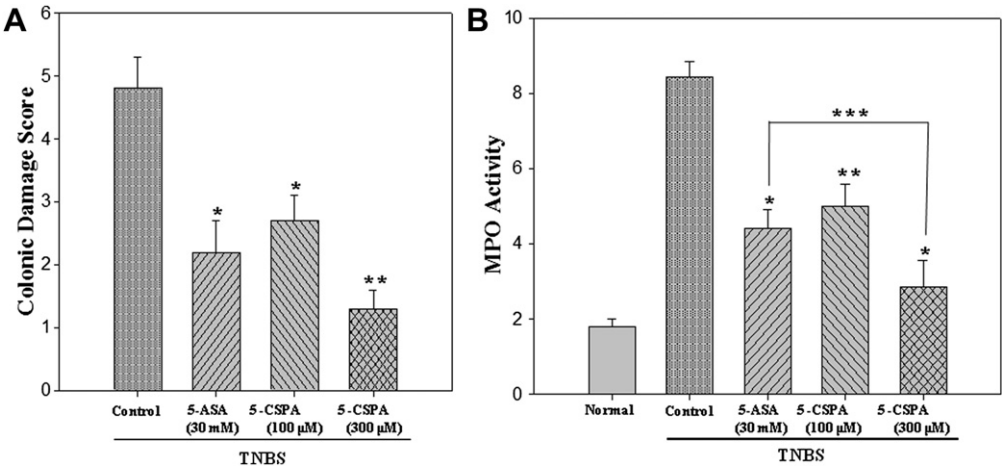


Fig. 3. Rectally administered 5-CSPA ameliorates TNBS-induced colitis of rats. A. Colonic damage score (CDS) was assigned for each rat by four independent observers who were blind to the experimental condition according to the modified CDS scoring system. The data are means ±S.E. ($n = 5$), $^{*}P < 0.05$ vs. control, $^{**}P < 0.001$ vs. control. B. MPO activities were measured in the distal colon segment (4 cm) as described under "Experimental part". The data are means ±S.E. (n = 5), $^{*}P < 0.005$ vs. control, $^{**}P < 0.01$ vs. control, $^{***}P < 0.05$.

the suppressive effect of 5-CSPA (300 μM) on the molecular indices was greater than 5-ASA.

3. Discussion

In this study, it was demonstrated that amidation or/and 5-chlorination of salicylic acid increased inhibitory activity of salicylic acid (SA) against NFκB, where simultaneous modification showed an additive effect on NFκB inhibition. N-monosubstituted derivatives of 5-chlorosalicylamide (5-CSAM), N-(5-chlorosalicyloyl) benzylamine, N-(5-chlorosalicyloyl)phenethylamine (5-CSPA), N-(5-chlorosalicyloyl) 3-phenylpropylamine, N-(5-chlorosalicyloyl) 4-hydroxyphenylethylamine and N-(5-chlorosalicyloyl)hexylamine, further increased the inhibitory activity. Furthermore, 5-CSPA, one

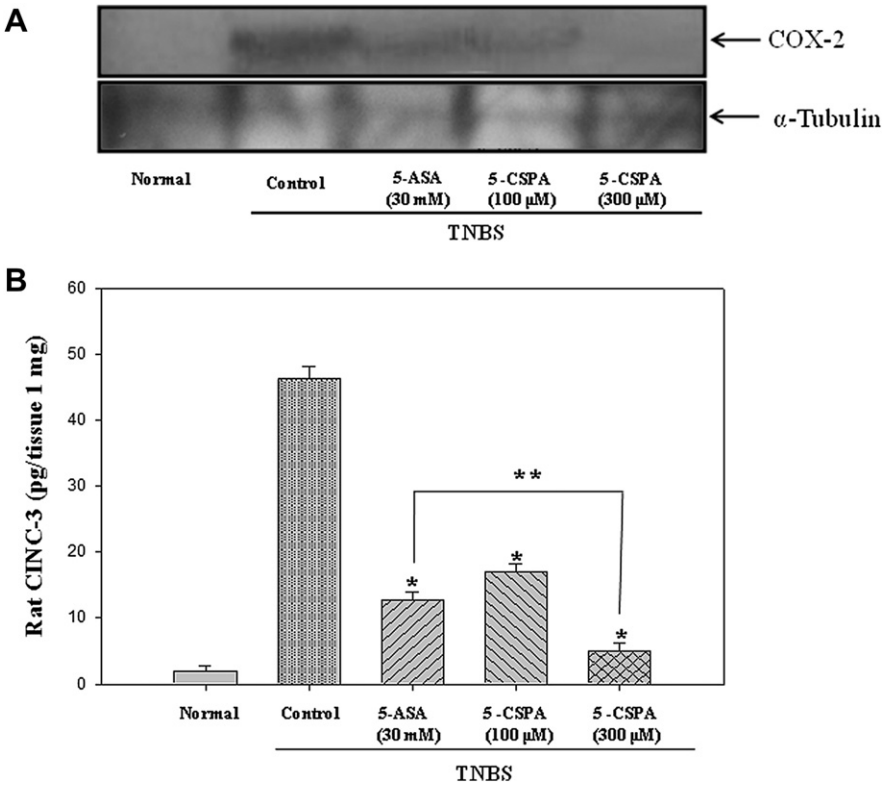


Fig. 4. Rectally administered 5-CSPA suppressed the expression of pro-inflammatory NFκB target gene products A. Tissues were homogenized with a dounce homogenizer and incubated on ice for 30 min. The homogenates were centrifuged at 13,000 rpm and 4 °C for 10 min. The supernatant was subjected to Western blot analysis and COX-2 protein was detected using a monoclonal anti-COX-2 antibody. B. The inflamed distal colon (1 g) was removed and mixed with RIPA buffer (3 mL) followed by homogenization. The homogenates were centrifuged at 13,000 rpm for 10 min and the supernatants (100 μL) were centrifuged again at 13,000 rpm and 4 °C for 10 min. An appropriate volume of the supernatants was subjected to CINC-3 ELISA. The data are means ±S.E. ($n = 5$), $^{*}P < 0.001$ vs. control, $^{**}P < 0.005$.

of the most potent derivatives, ameliorated TNBS-induced colitis of rats, which was more effective than 5-ASA, a conventional anti-IBD drug.

Inhibitory activity of SA against NFκB could be enhanced by chemical modification such as 5-substitution or/and amidation. This is shown by the data demonstrating that the potencies of 5-CSA and salicylamide (SAM) for inhibiting NFκB was greater than SA and simultaneous modification to produce 5-CSAM further increased the inhibitory potency, which suggests that the structural effect of each modification is additive. Although substitution of 2,4 difluorophenylgroup at 5 position of SA, namely diflunisal, afforded an equivalent inhibitory effect to that of chlorine, the modification was not adopted as a lead for further modification because its solubility became too low on modification of the carboxylic group of diflunisal. N-modification of 5-CSAM was found to further increase the potency for NFκB inhibition. The direction, N-substitution, was taken by previous papers demonstrating that the biological activities (including NFκB inhibition) of caffeic acid (3,4 dihydroxycinnamic acid) are enhanced by esterification with alkyl or arylalkyl alcohols [12,14]. It seems that N-monosubstituted derivatives elicit greater potency than N-disubstituted derivatives. However, it is not certain whether the difference is derived from pharmacokinetic or pharmacodynamic factors. This observation needs to be confirmed by testing more derivatives in elaborated conditions where the pharmacokinetic factors are well-controlled.

5-CSPA elicited a substantial anti-inflammatory activity against TNBS-induced colitis of rats as shown in the data demonstrating the improvement of colonic damage score and attenuation of MPO activity. The therapeutic activity of 5-CSPA seems to be relevant to inhibition of NFκB in the inflamed tissue. NFκB inhibition in the inflamed tissue is supported by showing that 5-CSPA substantially suppressed the expression of pro-inflammatory NFκB target gene products such as COX-2 and CINC-3, which is in line with the in vitro results of the NFκB luciferase assay and iNOS expression. This hypothesis on a molecular mechanism of therapeutic action is consistent with the recent findings that NFκB inhibition is a promising therapeutic target for treatment of IBD [4,15]. The data showing that 5-CSPA (300 μM) was more effective at ameliorating the rat colitis than 5-ASA (30 mM) imply that 5-CSPA is much more potent than 5-ASA. Although molecular mechanisms of 5-ASA underlying anti-IBD activity are still controversial [16], previous papers provide a number of evidence that NFκB inhibition is crucial for its therapeutic activity [6,9]. Therefore, their difference in therapeutic effectiveness may come from that in the inhibitory potency on NFκB. Recently, peroxisome proliferator-activated receptor-r (PPAR-r), which is involved in the regulation of colon

Table 3
Physical data of N-substituted CSAM derivatives.

Compd. no.	Mp (°C)	IR (Nujol, −C=O, cm^{-1})	^{1}H NMR (DMSO-d_6, ppm) E.A (calculated/found)	M.S. m/z $[M+H]^+$
1	183–185	1640	2.80 (s, 3H, CH_3), 6.83 (d, J = 8.8 Hz, 1H, H-3), 7.29 (dd, J = 8.7, 2.7 Hz, 1H, H-4), 7.82 (d, J = 3.0 Hz, 1H, H-6) C, 51.77; H, 4.34; N, 7.55/C, 51.53; H, 4.31; N, 7.57	196
2	166–168	1583	2.78–2.92 (br d, 6H, N(CH_3)$_2$), 6.86 (d, J = 8.8 Hz, 1H, H-3), 7.10 (d, J = 2.6, 1H, H-6), 7.23 (dd, J = 8.8, 2.7 Hz, 1H, H-4) C, 54.15; H, 5.05; N, 7.02/C, 54.23; H, 5.07; N, 7.05	210
3	65	1635	0.84 (t, J = 7.1 Hz, 3H, CH_3), 1.22–1.31 (m, 6H, $CH_2CH_2(CH_2)_3CH_3$, 1.51 (p, J = 7 Hz, 2H, $CH_2CH_2(CH_2)_3CH_3$, 3.25 (q, J = 5.9 Hz, 2H, $NCH_2CH_2(CH_2)_3CH_3$), 6.91 (d, J = 9.0 Hz, 1H, H-3), 7.41 (dd, J = 8.8, 2.7 Hz, 1H, H-4), 7.93 (d, J = 2.7, 1H, H-6) C, 61.05; H, 7.09; N, 5.48/C, 61.16; H, 7.04; N, 5.44	256
4	218–221	1570	3.16–3.58 (br m, 8H, morpholine), 6.86 (d, J = 8.8 Hz, 1H, H-3), 7.15 (d, J = 2.7 Hz, 1H, H-6), 7.24 (dd, J = 8.7, 2.5 Hz, 1H, H-4) C, 54.67; H, 5.00; N, 5.80/C, 54.74; H, 5.03; N, 5.76	253
5	187–188	1574	3.05–3.98 (br m, 8H, piperazine), 6.84 (d, J = 8.6 Hz, 1H, H-3), 7.09 (d, J = 2.7 Hz, 1H, H-6), 7.18 (dd, J = 8.8. 2.7 Hz, 1H, H-4) C, 54.89; H, 5.44; N, 11.64/C, 54.81; H, 5.47; N, 11.59	241
6	164–165	1576	2.16 (s, 3H, N(CH_3)), 2.28 (br m, 4H, N(CH_2)$_2$), 3.33 (br t, 4H, OCN(CH_2)$_2$), 6.85 (d, J = 8.8 Hz, 1H, H-3), 7.1 (d, J = 2.7 Hz, 1H, H-6), 7.2 (dd, J = 8.6, 2.9 Hz, 1H, H-4) C, 56.58; H, 5.94; N, 11.00/C, 56.77; H, 5.98; N, 11.05	255
7	200–202	1572	3.13 (br m, 4H, N(CH_2)$_2$), 3.32 (br m, 4H, OCN(CH_2)$_2$), 6.89 (d, J = 8.8 Hz, 1H, H-3), 7.18 (d, J = 2.7 Hz, 1H, H-6), 7.27 (dd, J = 8.5, 2.7 Hz, 1H, H-4),6.79 (t, J = 7.3 Hz, 1H, H-4′), 6.93 (d, J = 8.1 Hz, 2H, H-2′,6′), 7.21 (t, J = 8.3 Hz, 2H, H-3′,5′) C, 64.46; H, 5.41; N, 8.84/C, 64.71; H, 5.38; N, 8.89	317
8	192–194	1565	1.46–1.56 (m, 6H, (CH_2)$_3$, 3.13–3.53 (br d, 4H, N(CH_2)$_2$), 6.85 (d, J = 8.7 Hz, 1H, H-3), 7.09 (d, J = 2.7 Hz, 1H, H-6), 7.21 (dd, J = 8.8, 2.7 Hz, 1H, H-4) C, 60.13; H, 5.89; N, 5.84/C, 60.31; H, 5.85; N, 5.83	251
9	146–147	1638	4.50 (d, J = 5.9 Hz, 2H, NCH_2), 6.94 (d, J = 8.8 Hz, 1H, H-3), 7.4 (dd, J = 8.8, 2.4 Hz, 1H, H-4), 7.98 (d, J = 2.7 Hz, 1H, H-6), 7.25 (m, 1H, H-4′), 7.32 (m, 4H, H-2′, 3′, 5′, 6′) C, 64.25; H, 4.62; N, 5.35/C, 64.41; H, 4.64; N, 5.32	272
10	120–121	1639	2.84 (t, J = 7.1 Hz, 2H, NCH_2CH_2), 3.51 (m, 2H, NCH_2CH_2), 6.92 (d, J = 8.8 Hz, 1H, H-3), 7.4 (dd, J = 8.8, 2.7 Hz, 1H, H-4), 7.9 (d, J = 2.4 Hz, 3H, 6-H), 7.19 (t, J = 8.5, 1H, H-4′), 7.22–7.30 (m, 4H, H-2′, 3′, 5′, 6′) C, 65.34; H, 5.12; N, 5.08/C, 65.42; H, 5.09; N, 5.05	286
11	99–100	1638	1.83 (p, J = 7.3 Hz, 2H, $NCH_2CH_2CH_2$), 2.62 (t, J = 7.9 Hz, 2H, $NCH_2CH_2CH_2$), 3.30 (q, J = 6.8 Hz, 2H, $NCH_2CH_2CH_2$), 6.92 (d, 8.8 Hz, 1H, H-3), 7.41 (dd, J = 8.8, 2.7 Hz, 1H, H-4), 7.9 (d, J = 2.4 Hz, 1H, H-6), 7.15 (t, J = 7.4 Hz, 1H, H-4′), 7.20–7.28 (m, 4H, H-2′, 3′, 5′, 6′) C, 66.32; H, 5.57; N, 4.83/C, 66.60; H, 5.55; N, 4.85	290
12	176–178	1638	2.71 (t, J = 7.3 Hz, 2H, NCH_2CH_2), 3.44 (q, J = 6.4 Hz, 2H, NCH_2CH_2), 6.91 (d, 8.8 Hz, 1H, H-3), 7.41 (dd, J = 8.8, 2.7 Hz, 1H, H-4), 7.89 (d, J = 2.5 Hz, 1H, H-6), 6.67 (d, J = 8.5 Hz, 2H, H-2′, 6′), 7.01 (d, J = 8.6 Hz, 2H, H-3′, 5′) C, 61.76; H, 4.84; N, 4.80/C, 61.77; H, 4.81; N, 4.79	292

CSAM: 5-chlorosalicylamide.

inflammation [17], is identified as a therapeutic target of 5-ASA for treatment of intestinal inflammation [18]. Thus, it would be interesting to investigate that N-substituted derivatives including 5-CSPA can activate the nuclear receptor.

It is not sure that the therapeutic activity of 5-CSPA administered rectally could be reproduced upon administration via oral route. A drug administered orally must come through absorption, distribution, metabolism and excretion to reach its drug target. Considering pharmacokinetic factors and the potency in animal study, 5-CSPA still needs to be polished to develop to a therapeutic agent for treatment of IBD. However, 5-CSPA could be subjected to a preclinical study by adoption of a colon-specific drug delivery system, which might present a similar therapeutic situation to rectal administration. Colon-specific delivery of 5-CSPA implies that 5-CSPA administered orally is delivered to the large intestine without significant loss in the upper intestine leading to greater concentration at the large intestine and lower concentration in the blood, generally resulting in increasing therapeutic effectiveness and lowering systemic side effects [19]. Development of a colon-specific drug delivery system of 5-CSPA using pharmaceutical formulation and a prodrug approach is under study.

4. Conclusion

Our data may provide useful information for development of a therapeutic agent for treatment of diseases where NFκB plays a critical role in the pathogenic progresses.

5. Experimental part

5.1. Reagents

Recombinant human TNF-α was obtained from R & D systems (MN, USA). LPS and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma–Aldrich (MO, USA). Salicylic acid (SA), 5-nitrosalicylic acid (5-NSA) and 5-aminosalicylic acid (5-ASA) were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Acetylsalicylic acid (ASP), 5-chlorosalicylic acid (5-CSA), diflunisal (DFA), methylsalicylate (MSA), salicylamide (SAM), methylamine, dimethylamine, benzylamine, phenethylamine, 3-phenpropylamine, (4-hydroxyphenyl)ethylamine, hexylamine, piperidine, morpholine, piperazine, 1-methylpiperazine and 1-phenylpiperazine were purchased from Sigma–Aldrich. Reaction solvents were obtained from Junsei chemical Co. (Tokyo, Japan). All other chemicals were reagent-grade, commercially available products. IR spectra were recorded on a Varian FT-IR spectrophotometer (Varian, CA, USA), respectively. ^{1}H NMR spectra were taken on a Varian AS 500 spectrometer. Elemental analysis was carried out by an Elemental Analyzer System (Profile HV-3, Manchester, UK). Mass spectra were obtained using an Agilent QQQ 6460 mass spectrometer (Agilent Technologies, CA, USA). The animal protocol used in this study has been reviewed and approved by the Pusan National University–Institutional Animal Care and Use Committee (PNU-IACUC) on their ethical procedures and scientific care.

5.2. Synthesis

5.2.1. Methyl 5-chlorosalicylate (MCSA)

5-Chlorosalicylic acid (1 g) was dissolved in methanol (15 mL) and concentrated H_2SO_4 (approximately 20 μL) was added, which was evaporated after 1 h reflux. The residue dissolved in ethylacetate was washed with 5% $NaHCO_3$ and subsequently dried over anhydrous Na_2SO_4, which was subjected to flash evaporation to obtain MCSA. Yield: 0.96 g (89%); mp: 46 °C; IR (Nujol): 1682 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): $\delta = 3.86$ (s, 3H, CH_3), 7.01 (d, $J = 8.8$ Hz, 1H, H-3), 7.53 (dd, $J = 8.4$, 2.7 Hz, 1H, H-4), 7.69 (d, $J = 2.4$ Hz, 1H, H-6); $C_8H_7O_3Cl_1$ (186.59) calcd: C, 51.49; H, 3.78; found: C, 51.82; H, 3.72.

5.2.2. 5-Chlorosalicylamide (CSAM)

Methyl 5-chlorosalicylate (0.5 g) was dissolved in 25% ammonia solution (10 mL) and stirred at RT for 2 days. The reaction mixture was cooled down in an ice bath to afford CSAM as white precipitate. Yield: 0.35 g (78%); mp: 200–202 °C; IR (Nujol): 1655 (C=O) cm^{-1}; ^{1}H NMR (DMSO-d_6): $\delta = 6.90$ (d, $J = 8.9$ Hz, 1H, H-3), 7.43 (dd, $J = 8.4$, 2.4 Hz, 1H, H-4), 7.94 (d, $J = 3.0$ Hz, 1H, H-6); $C_7H_6N_1O_2$ Cl_1 (171.58) calcd: C, 49.00; H, 3.52; N, 8.16; found: C, 49.12; H, 3.55; N, 8.15.

5.2.3. N-substituted 5-chlorosalicylamide derivatives

Methyl 5-chlorosalicylate (0.5 g) was dissolved in methanol (15 mL) followed by addition of 10 fold molar concentration of methylamine, dimethylamine, hexylamine, piperidine, piperazine, morpholine, 1-methylpiperazine, 1-phenylpiparzine, benzylamine, phenethylamine, 3-phenylpropylamine or (4-hydroxyphenyl)ethylamine, which was evaporated after stirring at RT for 36 h. The residue was dissolved in ethylacetate, which was washed with 1 M HCl and 5% $NaHCO_3$ and subsequently dried over anhydrous Na_2SO_4. Flash evaporation of ethylacetate afforded N-(5-chlorosalicyloyl) methylamine **1** (yield: 0.46 g, 88%), N-(5-chlorosalicyloyl)dimethylamine **2** (yield: 0.47 g, 85%), N-(5-chlorosalicyloyl)hexylamine **3** (yield: 0.58 g, 85%), N-(5-chlorosalicyloyl)morpholine **4** (yield: 0.51 g, 76%), 4-(5-chlorosalicyloyl)1-phenylpiperazine **7** (yield: 0.54 g, 64%), N-(5-chlorosalicyloyl)piperidine **8** (yield: 0.52 g, 78%), N-(5-chlorosalicyloyl)benzylamine **9** (yield: 0.64 g, 89%), N-(5-chlorosalicyloyl)phenethylamine **10** (yield: 0.62 g, 81%), N-(5-chlorosalicyloyl) 3-phenylpropylamine **11** (yield: 0.64 g, 83%) or N-(5-chlorosalicyloyl) 4-hydroxyphenylethylamine **12** (yield: 0.61 g, 79%) as white powders, respectively. To obtain 4-(5-chlorosalicyloyl) piperazine **5** and 4-(5-chlorosalicyloyl)1-methylpiperazine **6**, the reaction mixtures were evaporated at 90 °C followed by the washing process (distilled water was used instead of 1 M HCl) and subsequent flash evaporation, which afforded the final products as white powder (yield of **5**: 0.32 g, 51%; yield of **6**: 0.44 g, 65%). The physical data and IC_{50} values measured in an NFκB-dependent luciferase assay and structures of N-substituted CSAM derivatives were shown in Tables 2 and 3.

5.3. Cell lines

Human colon carcinoma HCT116 cells (ATCC, MD, USA) and murine macrophage RAW264.7 cells (ATCC) were grown in DMEM medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Hyclone).

5.4. Luciferase assay

Cells were plated in 6-well plates to be 50–60% confluent on the day of transfection with NFκB dependent luciferase plasmid (0.5 μg, a gift from Dr. M. Birrer, NCI) and cytomegalovirus (CMV) *Renilla* luciferase plasmid (4 ng, Promega, WI, USA). Fugene (Roche, CA, USA) was used as a transfection reagent. Twenty-four hours post-transfection, cells were treated with TNF-α in the presence of the reagent at the indicated concentrations in the figure legends. Cells were lysed 6 h later and luciferase activities were measured and normalized to CMV *Renilla* luciferase activities using a Dual Luciferase reporter assay system (Promega).

5.5. Immunoblot analysis

Cells were lysed and cell lysates were prepared as described previously [20]. To prepare tissue total lysates, the inflamed distal colon was removed and mixed with 3 ml of ice cold RIPA buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 150 mM NaCl, 0.3 μM Aprotinin, 1 μM Pepstatin and 1 mM PMSF) per gram of tissue. Tissues were further disrupted and homogenized with a homogenizer and incubated on ice for 30 min. Tissues were centrifuged at 13,000 rpm and 4 °C for 10 min. Supernatants were removed and centrifuged again. Protein concentration in the supernatants was determined by the BCA method. The extracts were transferred to a fresh tube and stored at −80 °C until used. Cell or tissue extracts were electrophoretically separated using 7.5 or 10%. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, NH, USA). COX-2 protein in tissues homogenates was detected using a monoclonal anti-COX-2 antibody (Santa Cruz, CA, USA). iNOS protein was detected in whole cell lysates (30–40 μg) using a polyclonal anti-iNOS antibody (Santa Cruz). Signals were visualized using the Supersignal chemiluminescence substrate (Pierce, IL, USA). Experiments were performed in duplicate and equivalent loading was confirmed by probing the blots with anti-α-tubulin antibody (Santa Cruz).

5.6. TNBS-induced inflammation

Inflammation was induced by the method of Morris et al. [21] and Yano et al. [22]. Briefly, before induction of colitis, rats were starved for 24 h but had free access to water. The rats were lightly anesthetized with ether. A rubber cannula (OD, 2 mm) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexture. TNBS dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 ml/rat).

5.7. Evaluation of TNBS-induced colitis

Three days after induction of inflammation, N-(5-chlorosalicyloyl)phenethylamine (100, 300 μM, 500 μL) or sodium 5-aminosalicylate (30 mM, 500 μL) dissolved in PBS was administered rectally once a day and the rats were sacrificed after the treatment for 6 days. A gross colonic damage score (CDS) was calculated according to the criteria set forth previously [21,22]. The modified scoring system is: 0, normal appearance; 1, localized hyperemia but no ulcer; 2, linear ulcers without significant inflammation; 3, 2–4 cm site of inflammation and ulceration without scab; 4, serosal adhesion to other organs, 2–4 cm site of inflammation and ulceration with scab; 5, stricture, serosal adhesion involving several bowel loops, <4 cm site of inflammation and ulceration with scab. Four independent observers blinded to the treatment did the assessment of colonic damage score. Using the distal colon (4 cm), MPO activity was measured as described previously [23]. The distal colon segment, which was kept at −80 °C, was finely minced in a vial containing 1 ml of 0.5% (HTAB, pH 6.0) and homogenized using a polytron homogenizer on ice. After homogenization, the homogenizer was rinsed with HTAB solution. HTAB was added to adjust the pooled homogenates to 100 mg tissue per ml which was sonicated for 10 s, frozen-thawed three times, and centrifuged at 13,000 rpm at 4 °C. The supernatants, 0.1 ml, were combined with 2.9 ml of 0.050 M phosphate buffer solution (pH 6.0) containing 0.167 mg/ml *o*-dianisidine hydrochroride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured by an UV-2101 PC spectrophotometer (Shimadzu, Tokyo, Japan) for 5 min at 25 °C. One unit of myeloperoxidase activity is defined as that degrading 1 μmol of peroxide per minute at 25 °C.

5.8. ELISA for cytokine-induced neutrophil chemoattractant (CINC)-3

To measure CINC-3 levels in the tissue, the inflamed distal colon (1 g) was removed and mixed with RIPA buffer (3 mL) followed by homogenization. The homogenates were centrifuged at 13,000 rpm and 4 °C for 2 min. The supernatants (100 μL) transferred to a fresh microtube and then were centrifuged at 13,000 rpm and 4 °C for 10 min. An appropriate volume of the supernatants was subjected to CINC-3 ELISA (R&D Systems, MN, USA).

5.9. Statistical analysis

The results are expressed as the mean ± S.D. One-way ANOVA followed by Tukey's (HSD) test was used for testing the difference between data. Differences with $p < 0.05$ were considered significant. The XLSTAT® Software (Addinsoft, Inc, NY, USA) was used for the statistical analysis.

Acknowledgments

This work was supported by a grant of the Korea Healthcare technology R & D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A080640) awarded to Y.J.

Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.11.030.

References

[1] Q. Li, I.M. Verma, NF-kappaB regulation in the immune system, Nat. Rev. Immunol. 2 (2002) 725–734.
[2] K. Tanaka, T. Kawakami, K. Tateishi, H. Yashiroda, T. Chiba, Control of IkappaBalpha proteolysis by the ubiquitin-proteasome pathway, Biochimie 83 (2001) 351–356.
[3] M.F. Neurath, C. Becker, K. Barbulescu, Role of NF-kappaB in immune and inflammatory responses in the gut, Gut 43 (1998) 856–860.
[4] Y. Yamamoto, R.B. Gaynor, Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer, J. Clin. Invest. 107 (2001) 135–142.
[5] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis, Science 270 (1995) 286–290.
[6] F. Yan, D.B. Polk, Aminosalicylic acid inhibits IkappaB kinase alpha phosphorylation of IkappaBalpha in mouse intestinal epithelial cells, J. Biol. Chem. 274 (1999) 36631–36636.
[7] M.F. Neurath, S. Pettersson, K.H. Meyer zum Buschenfelde, W. Strober, Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-kappa B abrogates established experimental colitis in mice, Nat. Med. 2 (1996) 998–1004.
[8] E.M. Conner, S. Brand, J.M. Davis, F.S. Laroux, V.J. Palombella, J.W. Fuseler, D.Y. Kang, R.E. Wolf, M.B. Grisham, Proteasome inhibition attenuates nitric oxide synthase expression, VCAM-1 transcription and the development of chronic colitis, J. Pharmacol. Exp. Ther. 282 (1997) 1615–1622.
[9] G.C. Kaiser, F. Yan, D.B. Polk, Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes, Gastroenterology 116 (1999) 602–609.
[10] M.J. Yin, Y. Yamamoto, R.B. Gaynor, The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta, Nature 396 (1998) 77–80.
[11] C.K. Weber, S. Liptay, T. Wirth, G. Adler, R.M. Schmid, Suppression of NF-kappaB activity by sulfasalazine is mediated by direct inhibition of IkappaB kinases alpha and beta, Gastroenterology 119 (2000) 1209–1218.
[12] Y. Lee, D.H. Shin, J.H. Kim, S. Hong, D. Choi, Y.J. Kim, M.K. Kwak, Y. Jung, Caffeic acid phenethyl ester-mediated Nrf2 activation and IkappaB kinase inhibition are involved in NFkappaB inhibitory effect: structural analysis for NFkappaB inhibition, Eur. J. Pharmacol. 643 (2010) 21–28.

[13] Y. Jung, H.H. Kim, H. Kim, H. Kong, B. Choi, Y. Yang, Y. Kim, Evaluation of 5-aminosalicyltaurine as a colon-specific prodrug of 5-aminosalicylic acid for treatment of experimental colitis, Eur. J. Pharm. Sci. 28 (2006) 26–33.
[14] D. Choi, J. Han, Y. Lee, J. Choi, S. Han, S. Hong, H. Jeon, Y.M. Kim, Y. Jung, Caffeic acid phenethyl ester is a potent inhibitor of HIF prolyl hydroxylase: structural analysis and pharmacological implication, J. Nutr. Biochem. 21 (2010) 809–817.
[15] I. Atreya, R. Atreya, M.F. Neurath, NF-kappaB in inflammatory bowel disease, J. Intern. Med. 263 (2008) 591–596.
[16] P. Del Soldato, M. Campieri, C. Brignola, G. Bazzocchi, P. Gionchetti, G.A. Lanfranchi, M. Tamba, A possible mechanism of action of sulfasalazine and 5-aminosalicylic acid in inflammatory bowel diseases: interaction with oxygen free radicals, Gastroenterology 89 (1985) 1215–1216.
[17] L. Dubuquoy, C. Rousseaux, X. Thuru, L. Peyrin-Biroulet, O. Romano, P. Chavatte, M. Chamaillard, P. Desreumaux, PPARgamma as a new therapeutic target in inflammatory bowel diseases, Gut 55 (2006) 1341–1349.
[18] C. Rousseaux, B. Lefebvre, L. Dubuquoy, P. Lefebvre, O. Romano, J. Auwerx, D. Metzger, W. Wahli, B. Desvergne, G.C. Naccari, P. Chavatte, A. Farce, P. Bulois, A. Cortot, J.F. Colombel, P. Desreumaux, Intestinal antiinflammatory effect of 5-aminosalicylic acid is dependent on peroxisome proliferator-activated receptor-gamma, J. Exp. Med. 201 (2005) 1205–1215.
[19] Y. Jung, Y.M. Kim, What should be considered on design of a colon-specific prodrug? Expert Opin. Drug Deliv. 7 (2010) 245–258.
[20] N.C. Andrews, D.V. Faller, A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells, Nucleic Acids Res. 19 (1991) 2499.
[21] G.P. Morris, P.L. Beck, M.S. Herridge, W.T. Depew, M.R. Szewczuk, J.L. Wallace, Hapten-induced model of chronic inflammation and ulceration in the rat colon, Gastroenterology 96 (1989) 795–803.
[22] H. Yano, F. Hirayama, M. Kamada, H. Arima, K. Uekama, Colon-specific delivery of prednisolone-appended alpha-cyclodextrin conjugate: alleviation of systemic side effect after oral administration, J. Control. Release. 79 (2002) 103–112.
[23] M.W. Son, J.I. Ko, H.M. Doh, W.B. Kim, T.S. Park, M.J. Shim, B.K. Kim, Protective effect of taurine on TNBS-induced inflammatory bowel disease in rats, Arch. Pharm. Res. 21 (1998) 531–536.