A Thesis for the Degree of Master of Science

Anti-inflammatory Effects of Orobol, an Enzyme Converted Product of Genistein, on the IL-8 Production in HT-29 Colon Cells and the Dextran Sodium Sulfate (DSS)-Induced Acute Colitis Symptoms in C57BL/6 mice.

장 세포에서의 IL-8 생산과 마우스 모델에서의 DSS로 유도되는 급성 장염에 대한 오로볼의 항염증 효과 규명

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ABSTRACT

Inflammatory bowel disease (IBD) is a pathologic conditions of the gastrointestinal tract in humans. IBD has been suggested that that inflammatory responses play major roles in the pathogenesis though the etiology of IBD still remains unclear. Soy isoflavones are attractive source of food materials with anti-inflammation and gut-healty effects. After fermentation or bioconversion, the majority of soy isoflavones are transformed to their metabolites such as genistein, daidzein and so on. Recently, metabolites have been studied in their improved bioactivity. Orobol (7,8,3',4'-tetrahydrowyisoflavone) is a metabolite of genistein. It has been known to anti-cancer effect for human ovarian and breast cancer. Although genistein exerts beneficial anti-inflammatory effects on colitis, orobol has not been studied about colitis yet. Interleukin-8 (IL-8), which is a major chemokine, plays a critical role in the inflammation including colitis. IL-8, derived epithelial cell, has been demonstrated to support neutrophil infiltration in various types of mucosal inflammation including IBD.

In this study, I investigated the anti-inflammatory effect of orobol, a major metabolite of genistein in soybean. I found that oral administration of orobol decreased DSS-induced colitis symptoms such as weight loss, diarrhea, bloody fecal, and damaged colon tissue in C57BL/6 mice. Also, orobol reduced the IL-8 production and IL-8 mRNA in HT-29 cells. In luciferase assay, orobol inhibited the transactivation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is a major of transcription factor of IL-8. In a 359 whole human kinases, MAPK-interacting Ser/Thr kinase 1 (MNK1), which plays critical roles in various cytokine receptors signaling, was inhibited activity by orobol (IC_{50} = 33.43 nM) and suggested as a direct target of orobol. Additionally, orobol decreased tumor necrosis factor alpha (TNF-α)-induced phosphorylation of eukaryotic initiation factor 4E (eIF4E) which is downstream of MNK1. Overall, orobol showed the anti-inflammatory effects in DSS-induced colitis mouse model. Also, orobol suppressed TNF-α-induced IL-8 production by directly targeting MNK1 in HT-29 colon cells. Thus, orobol could

be a beneficial agent for reducing inflammation related to IBD.

Keywords: Orobol; Kinase profiling; MNK1; IL-8; TNF-α; Inflammatory bowel disease

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CONTENTS

Ⅰ. INTRODUCTION

Inflammatory bowel diseases (IBD) are defined as chronic inflammation of the gastrointestinal tract. Intestinal epithelial cells have a defense function of cytokines and chemokines which are produced by external stimuli, such as tumor necrosis factor alpha (TNF-α) [1]. TNF-α, a key inflammatory cytokine produced mainly by macrophages, has been shown to play a central role in the inflammatory cascade [2]. Also, TNF-α is known to produce several cytokines and chemokines such as interleukin-8 [3]. Interleukin-8 (IL-8) is a major chemokine in human that is rapidly induced in intestinal epithelial cells after stimulation by external stimuli and secreted basolaterally [4]. IL-8, derived epithelial cell, has been demonstrated to support neutrophil infiltration in various types of mucosal inflammation including IBD. [5, 6]. Actually, in IBD patients, IL-8 level was increased in the affected tissues during the active phase and decreased when patients went into remission [7]. Therefore, inhibition of IL-8 expression is one of the excellent strategies for preventing IBD.

MAPK-interacting Ser/Thr kinase 1 (MNK1) is activated

downstream of the p38 MAPK and MEK/ERK signaling pathways. Also, these kinase control phosphorylation of the eukaryotic initiation factor 4E (eIF4E) [8]. MNK1 regulates eIF4E phosphorylation in response to external stimuli [9]. The phosphorylation of eIF4E at serine 209 by MNK1 has been shown to initiate oncogenic mRNA translation and indirectly enhance the NF-κB complex activation [10]. MNK1 regulated cytokines such as IL-8, TNF-α, and IL-1β have also been shown to positively regulating anti-inflammation, cancer progression, and chemo-resistance [8, 11]. Overall, MNK1 mediates signals important for pro-inflammatory responses, raising the possibility that targeting MNK1 may provide a potential novel therapeutic approach for the treatment of IBD [8].

Soy isoflavones are commonly consumed and it is well documented for their beneficial effects. After fermentation or bioconversion, the majority of soy isoflavones are transformed to their metabolites which have improved bioactivity on cancer, obesity, and gut-related disease. Orobol (7,8,3',4'-tetrahydrowyisoflavone) is a metabolite of genistein produced in human liver microsomes after soy consumption [12]. Orobol

occurred in fermented soybean is produced from *Tritirachium* or *Aspergillus niger* [13]. Furthermore, it could be produced by hudroxylation of genistein with tyrosinase from *Bacillus megaterium* [14]. It has been known to anti-cancer effect for human ovarian and breast cancer [15, 16]. However, the effect of orobol for inflammatory bowel disease and its molecular target has not been studied yet.

In this study, I investigated the anti-inflammatory effect of orobol. Orobol attenuated several DSS-induced acute colitis symptoms including body weight loss, diarrhea, fecal blood, and improved colon tissue damage. Also, orobol effectively reduced IL-8 production induced by TNF-α in human epithelial cell (HT-29 cell) compared to genistin and genistein. It inhibited NF-κB transactivation, a major of transcription of IL-8. Furthermore, after kinase profiling, I suggested MNK1 as a direct target of orobol and tried to prove it.

Ⅱ. MATERIALS AND METHODS

2.1. Chemicals and reagents

Orobol was gently provided for Prof. Byung-Gee Kim laboratory (Seoul National University, Seoul, Korea). Orobol were o-hydroxylated by genistein with > 95% conversion yield. It was biotransformed by tyrosinase expressed from Bacillus megaterium [13]. Genistin was purchased from Chem Faces (Hubei, Wuhan, CN). Genistein was purchased from LC Laboratory (Woburn, MA, US). Dextran sulfate sodium salt powder was purchased from MP bio (Santa Ana, CA, US). Mc Coy's 5A medium was purchased from Gibco (Grand Island, NY, US). Fetal bovine serum (FBS) was bought from Seradigm (Radnor, PA, US). Penicillin-Streptomycin Solution was purchased from Mediatech, Inc. (Manassas, VA). The enzyme-linked immunosorbent assay (ELISA) kits for interleukin-8 was purchased from R&D systems Inc. (Minneapolis, MN, USA). Antibodies against phosphorylated MNK-1 and total MNK1 were purchased from Cell signaling (Danvers, MA, US). The antibody against phosphorylated eIF4E was obtained from Epitomics (Cambridge, MA, US) and total eIF4E was purchased from Santacruz

(Dallas, TX, US). 3-[4,5-dimethylatiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) powder was purchased from USB Co. (Cleveland, OH). Protein assay reagent kits were obtained from Bio-Rad Laboratories (Hercules, CA). SYBR Premix Ex Taq™ were purchased from TAKARA Bio Inc. (Otsu, Japan), and the primers for Real-time PCR were obtained from Bioneer (Daedeok, Daejeon, KR). The dual-luciferase reporter assay system was obtained from Promega (Madison, WI, USA).

2.2. Animals design and scoring

Male C57BL/6 mice (7 weeks old) were purchased from Japan SLC (Hamamatsu, Shinzuoka, Japan). Animals were housed in an air-conditioned room (23 ± 3℃, 50 ± 10% humidity) with a 12 h light/dark cycle. These mice were acclimated for 1 week prior to study and had free access to food and water. After an acclimatization period, the mice were divided into 5 groups (8-9 mice in each group), Normal, DSS, DSS-Genistin, DSS-Genistein, and DSS-Orobol group to compare effects of anti-colitis.

To investigate effect of genistin, genistein, and orobol, the mice were orally administered with 50 $mgkg^{-1}$ body weight (BW) of

sample, which was dissolved in 3% dimethyl sulfoxide (DMSO) and 97% PEG 300, or vehicle(3% DMSO and 97% PEG 300) for 7 days. Colitis was induced by 2.5% DSS for 6 days. The body weight and water intake were measured daily. All experimental protocols were approved by the Institutional Animal Care and Use Committee (No. 2015-0401) of the Biomedical Research Institute, Seoul National University Hospital (Association for Assessment and Accreditation of Laboratory Animal Care accredited facility).

Diarrhea scoring and bleeding scoring were performed on day 13 as modified criteria according to the method previously described [17]. Diarrhea score and bleeding score were each divided into 5 grades of diarrhea score: (0: normal stool; 1: soft stool; 2: very soft stool; 3: watery stool) and bleeding score (0: normal colored stool; 1: brown stool; 2: mildly bloody stool; 3: bloody stool).

2.3. Tissue sample preparation

At the end of the experiment, mice were sacrificed by isoflurane (Piramal, PA, USA). The colon was removed from each mouse, and two sections were dissected. , One of the section (from the cecum) was used for total protein isolation. Another section was

used for histological assessment. These tissue samples were fixed in 4% formaldehyde solution and then fixed by paraffin.

2.4. Tissue staining

To observe the morphology of tissues, Hematoxylin and eosin (H&E) staining was performed. Mouse colon tissues fixed by paraffin were cut serial sections (4 μm) and then stuffed onto slides. After deparaffinizing, tissue sections were re-hydrated and stained with hematoxylin solution for 5 minutes. After this step, slides were washed and stained in counterstain in eosin Y solution for 30 seconds. Next, the slides were dehydrated through 95% alcohol and washed in absolute alcohol, 5 minutes each. Lastly, the slides were incubated in xylene. Tissue sections were examined at 100×−200× magnification using an Olympus AX70 light microscope (Tokyo, Japan).

2.5 Evaluation of tissue damage

To investigate colon tissue damage, the thickness of the mucosa and muscularis propria was calculated as mean value of three different points per mouse on uniform horizontal cross

sections of colon crypts using ImageJ [18].

2.6. Cell culture and treatments

HT-29 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). HT-29 cells were cultured in Mc Coy's 5A medicum with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin at 37 ° C and 5 % CO_2. HT-29 cells were treated with genistin, genistein, and orobol, which was dissolved in 100% DMSO, for 1 hour before the addition of 10 ng/ml human recombinant TNF-α (PuroSpec, Ness-Ziona, IL).

2.7. Enzyme-linked immunosorbent assay

HT-29 cells cultured on the 24-well plate were pre-incubated with genistin, genistein, and orobol for 1 h, and then exposed to a serum-free McCoy's 5A medium containing 10 ng/ml TNF-α for 3 h. After incubation, the level of IL-8 in the cell supernatant was determined, using commercially available enzyme-linked immunosorbent assay kits (R&D systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. RNA isolation and real-time quantitative PCR

HT-29 cells were treated with orobol for 1 h and exposed to a serum-free McCoy's 5A medium containing 10 ng/ml TNF-α for 3 h. Samples were harvested in RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After reverse transcript with oligo-dT primers using a PrimeScriptTM 1st strand cDNA synthesis Kit (Takara Bio Inc.), Real-time quantitative RT-PCR was performed using IQ SYBR (Bio-Rad Laboratories) and 2 μl of cDNA in triplicate with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Before PCR amplification, the primers were denatured at 95 ℃ for 3 min. Amplification was made up of 44 cycles at 95 ℃ for 10 sec, 60 ℃ for 30 sec, and 72 ℃ for 30. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). cDNA was probed by the following primer: IL-8 forward (5'-AGA GTG ATT GAG AGT GGA CC-3'); IL-8 reverse (5'-ACT TCT CCA CAA CCC TCT G-3'); GAPDH forward (5'-CAG GGC TGC TTT TAA CTC TGG TAA A-3');

GAPDH reverse (5'-GGG TGG AAT CAT ATT GGA ACA TGT AA-3').

2.9. Luciferase assay for NK-κB transactivation

The lentiviral expression vectors, including pGF-NF-κB-mCMV-EF1-Puro (System Biosciences, CA), and packaging vectors, including pMD2.0G and psPAX, were purchased from Addgene Inc (Cambridge, MA). pGF-NF-κB-mCMV-EF1-Puro vectors and the packaging vectors (pMD2.0G and psPAX) were transfected into HEK293T cells using jetPEI following the manufacturer's instructions. The transfection medium was changed into 24 h after transfection and the cells were then cultured for 24 h. The viral particles were harvested by filtration using a 0.45 mm syringe filter, then combined with 10 μg/mL polybrenes (MERK Millipore) and infected into 60 % confluent HT-29 cells overnight. The cell cultured medium was replaced with fresh complete growth medium for 24 h before the cells were selected for using puromycin (Sigma-Aldrich) over 24 h. The selected cells were then used for further experiments. HT-29 cells were cultured for 24 h and then starved to serum-free Mc Coy's 5A medium for 16-24 h. After starvation, HT-29 cells

were treated with or without various concentrations of orobol for 1 h, followed by TNF-α 10 ng/ml induction for 3 h. Cell extracts were prepared for reporter lysis buffer (Promega). The extracts were used for luciferase assay. NF-κB activity in HT-29 cells were determined by using a luciferase assay kit (Promega), as described by the manufacturers.

2.10. Western blot

HT-29 cells were cultured for 24 h, and then the cells were incubated in serum-free Mc Coy's 5A medium for 16-24 h. After that, the cells were treated with or without various concentrations of orobol (5, 10, 20 μg/ml) for 1 h, followed by TNF-α 10 ng/ml induction. The media was harvested on ice, and then centrifuged at 14,000 rpm for 10 min. The protein concentration was measured using a protein assay reagent kits as described by the manufacturer. The cells were lysed with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1 % Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 % glycerol and protease inhibitor cocktail tablet]. The

proteins were separated electrophoretically using a 12 % SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (MERK Millipore). The membrane was blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, London, UK) after hybridization with the HRP-conjugated secondary antibody (Life technologies, Waltham, MA).

2.11. Kinase profiling and kinase assay

Kinase profiling and kinase assay were conducted by kinase assay services (Reaction Biology Corporation, Malvern, PA). Kinases are incubated with substrate and required cofactor. The reaction is initiated by the addition of the compound in dimethyl sulfoxide (DMSO) and 33P-ATP (specific activity 10 μCi/μl). After incubation for 120 min at room temperature, reaction is spotted onto P81 ion exchange paper (GE healthcare) and washed extensively in 0.75 % phosphoric acid. Kinase activity data was expressed as the percent remaining kinase activity in test samples compared to vehicle (DMSO) reactions.

2.12. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan and p values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

3.1. Oral administration of orobol decreased DSS-induced acute colitis in C57BL/6 mice.

The structures of genistin, genistein, and orobol showed in Figure 1A. To investigate anti-colitis effects of orobol, C57BL/6 mice were orally administrated genistin, genistein and orobol 50 $mgkg^{-1}$ BW for 7 days. Then, mice was provided 2.5% DSS through water bottles during 6 days with sample or vehicle. I measured body weight daily. Once 2.5% DSS intake began, body weight started to decrease. On day 6 after provided 2.5% DSS, the body weight of the DSS group had decreased significantly. Orobol treatment recovered weight loss compared to DSS group ($p < 0.05$). But, DSS-genistin group and DSS-genistein group had no improvement compared to DSS group [p vale; DSS vs Genistin ($p = 0.087$) and DSS vs Genistein ($p = 0.152$)] (Fig. 1B). Also, Orobol group were significantly improvement the diarrhea compared to DSS group ($p < 0.05$; Fig. 1C) and decreasing tendency in fecal blood symptoms ($p = 0.10$; Fig. 1D). Taken together, orobol attenuated DSS-induced body weight loss, diarrhea, and fecal blood symptoms.

Figure 1

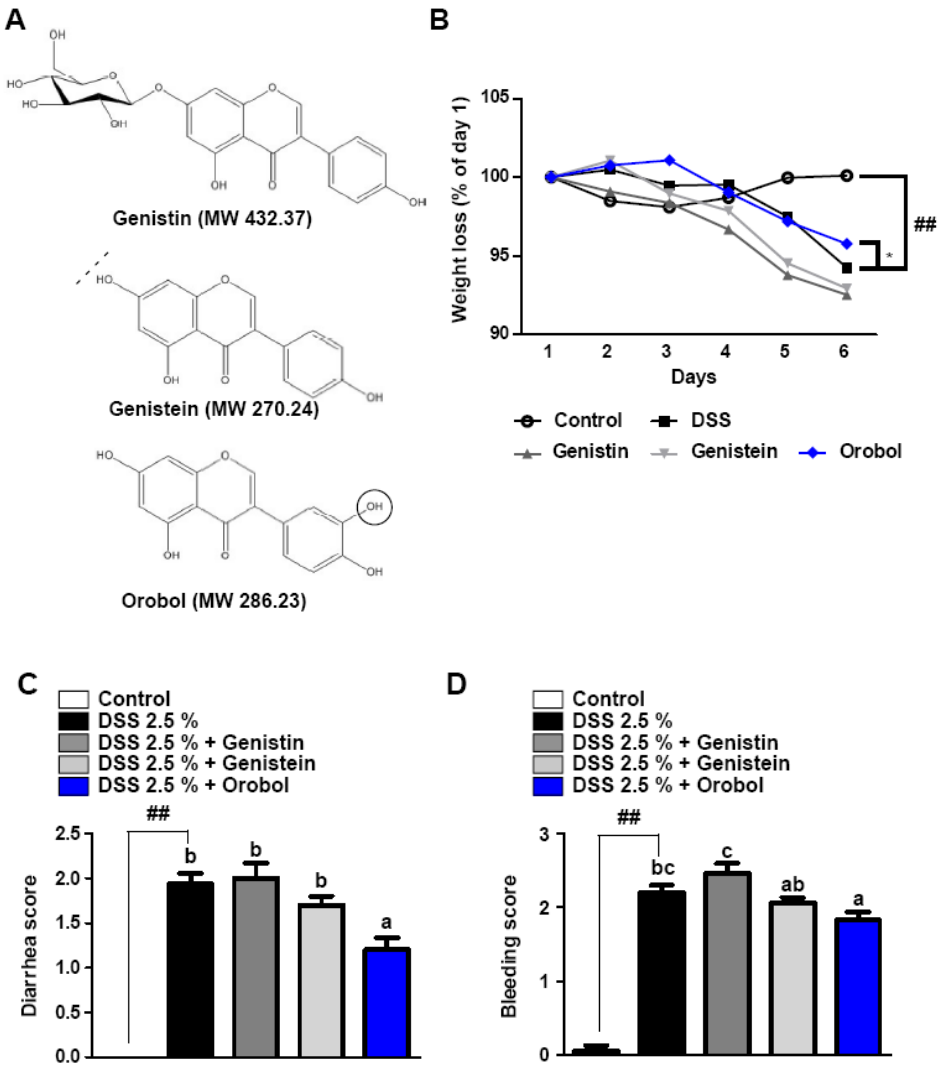
A
Genistin (MW 432.37)
Genistein (MW 270.24)
Orobol (MW 286.23)
B
Weight loss (% of day 1)
105
100
95
90
1
2
3
4
5
6
Days
##
*
Control
DSS
Genistin
Genistein
Orobol
C
Control
DSS 2.5 %
DSS 2.5 % + Genistin
DSS 2.5 % + Genistein
DSS 2.5 % + Orobol
##
Diarrhea score
2.5
2.0
1.5
1.0
0.5
0.0
b
b
b
a
D
Control
DSS 2.5 %
DSS 2.5 % + Genistin
DSS 2.5 % + Genistein
DSS 2.5 % + Orobol
##
Bleeding score
3
2
1
0
bc
c
ab
a

Figure 1. Effects of orobol on dextran sodium sulfate-induced acute colitis C57BL/6J mice.

A, Chemical structures of genistin, genistein, and orobol. *B,* Data for weight changes after starting 2.5% DSS treatment are expressed as the mean percentage change from the starting body weight. Each value is the mean ± SEM (n = 5). *C* and *D,* Symptoms of colitis induced by DSS are scored with scoring criteria. Each value is the mean ± SEM (n = 6). The vehicle was 3% DMSO and 97% PEG 300. The sharps (##) indicate a significant difference between the control group and the DSS group ($p < 0.01$). The star (*) indicate a significant difference between the orobol group and the DSS group ($p < 0.05$) by ANOVA test. Means with letters (a-b) within a graph are significantly different from each other at $p < 0.05$.

3.2. Orobol attenuated DSS-induced damage of colon tissue in C57BL/6 mice.

In general, DSS induced inflammation in the distal colon and severe mucosal damage [19]. To identify the improvement effect of orobol on colon tissue, I stained tissues of mouse distal colon in the way of Hematoxylin and eosin staining and Masson's trichrome staining respectively. Control and orobol groups were less damaged and disrupted than other DSS-induced groups (Fig. 2A). Also, orobol showed a decreasing tendency in thickness of mucosa and muscularis propria compared to other DSS-induced groups (Fig. 2B). Taken together, orobol attenuated damage of colon tissue induced by DSS.

Figure 2

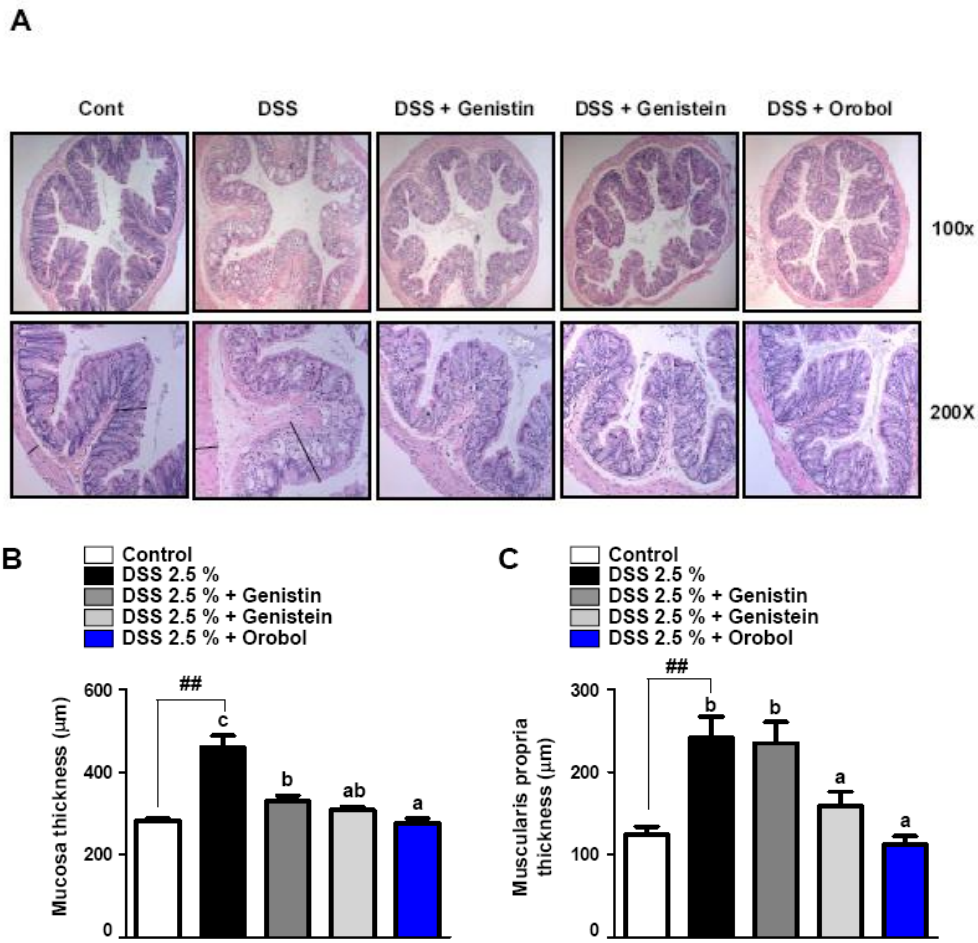
A
Cont
DSS
DSS + Genistin
DSS + Genistein
DSS + Orobol
100x
200X
B
Control
DSS 2.5 %
DSS 2.5 % + Genistin
DSS 2.5 % + Genistein
DSS 2.5 % + Orobol
Mucosa thickness (μm)
600
400
200
0
##
c
b
ab
a
C
Control
DSS 2.5 %
DSS 2.5 % + Genistin
DSS 2.5 % + Genistein
DSS 2.5 % + Orobol
Muscularis propria thickness (μm)
300
200
100
0
##
b
b
a
a

Figure 2. Effects of orobol on the histological changes in the distal colon of mice treated with DSS.

A, Distal colon sections were stained with hematoxylin & eosin (H&E). *B and C,* The thickness of mucosa and muscularis propria were quantified using the image J software analysis as described in materials and methods (Open source Java-written program, NIH, USA). Means with letters (a-c) within a graph are significantly different from each other at $p < 0.05$. Data represent the means ± SEM (n=3).

3.3. Orobol significantly decreased TNF-α-induced IL-8 overexpression and NF-κB transactivation in HT-29 cells.

IL-8, which is a potent leukocyte chemotactic and activating cytokine, is produced by intestinal cells. I investigate the most effective isoflavones for repressing IL-8 secretion in HT-29 cells induced by TNF-α. Genistin and genistein were used as a compared isoflavones, As a result, orobol showed the greatest inhibitory effect among the isoflavones (Fig. 3A). Also orobol significantly decreased IL-8 protein secretion on HT-29 cells (Fig. 3B). And the level of IL-8 mRNA was decreased it in dose-dependent manner (Fig. 3C).

Accumulating evidence indicate that the transcription of IL-8 gene requires the activation of NF-κB [20]. To determine whether the suppressive effects of orobol on the TNF-α-induced IL-8 production was due to the suppression of the NF-κB promoter, I investigated the effect of orobol on the activity of NF-κB promoter by using a luciferase reporter assay. The levels of NF-κB transactivation in the presence of orobol on HT-29 cells were significantly decreased (Fig. 3D). Overall, these results indicate that orobol effectively suppressed both TNF-α-induced IL-8 production and NF-κB transactivation level.

Figure 3

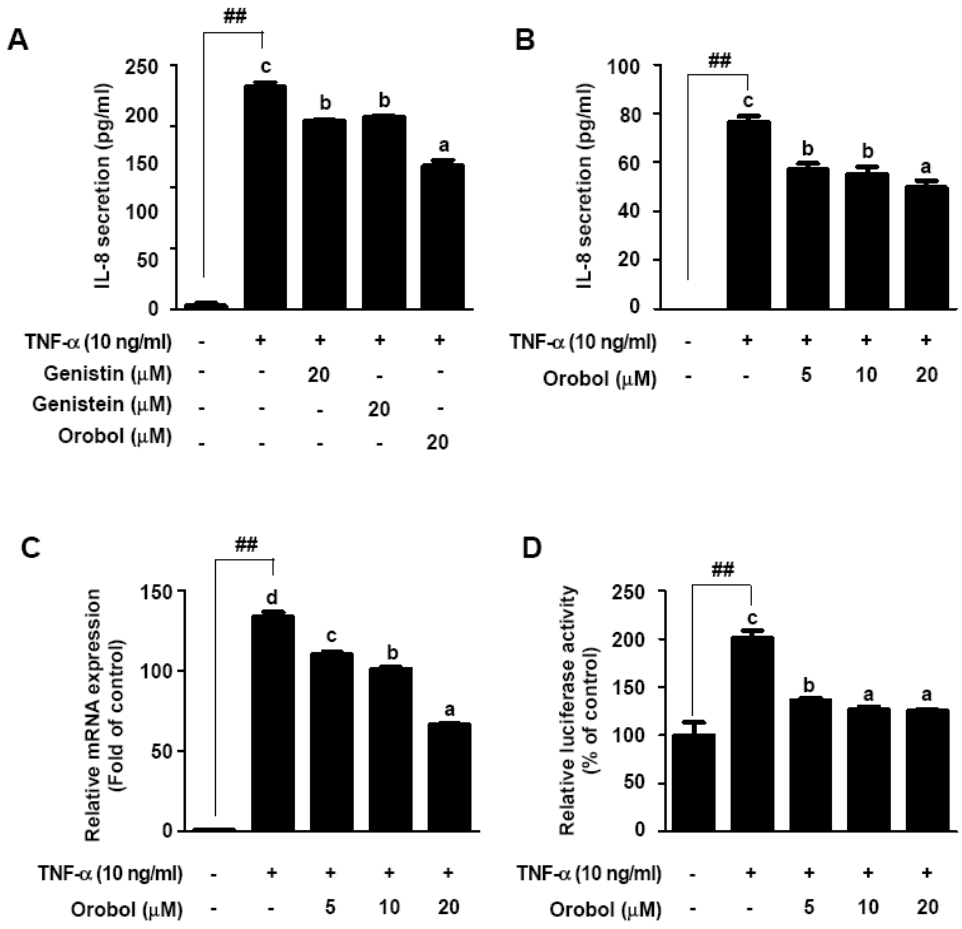
A
##
IL-8 secretion (pg/ml)
250
200
150
100
50
0
c
b
b
a
TNF-α (10 ng/ml) - + + + +
Genistin (μM) - - 20 - -
Genistein (μM) - - - 20 -
Orobol (μM) - - - - 20
B
##
IL-8 secretion (pg/ml)
100
80
60
40
20
0
c
b
b
a
TNF-α (10 ng/ml) - + + + +
Orobol (μM) - - 5 10 20
C
##
Relative mRNA expression (Fold of control)
150
100
50
0
d
c
b
a
TNF-α (10 ng/ml) - + + + +
Orobol (μM) - - 5 10 20
D
##
Relative luciferase activity (% of control)
250
200
150
100
50
0
c
b
a
a
TNF-α (10 ng/ml) - + + + +
Orobol (μM) - - 5 10 20

Figure 3. Inhibitory effects of orobol on TNF-α-induced IL-8 production and NF-κB transactivation in HT-29 cells.

A and B, Cells were pretreated with genistin, genistein and orobol at the indicated concentrations for 1 h, and then further treated with 10 ng/ml TNF-α for 3 h at 37 ℃. The culture medium was collected, and IL-8 secretion was determined by ELISA. Data (n=3) represent the means ± SD. *C,* IL-8 mRNA levels for the orobol group were analyzed by real-time quantitative PCR. Cells were pretreated with orobol at the indicated concentrations for 1 h, and then further treated with 10 ng/ml TNF-α for 3 h at 37 ℃. Data (n=3) represent the means ± SD. *D,* NF-κB transactivation ability of orobol was measured using a luciferase reporter gene assay as described in the Materials and Methods. Cells were pretreated with orobol at the indicated concentrations for 1 h, and then further treated with 10 ng/ml TNF-α for 3 h at 37 ℃. Data (n=3) represent the means ± SD. Means with letters (a-d) within a graph are significantly different from each other at $p < 0.05$.

3.4. Orobol inhibits MNK1 kinase activity and suppressed TNF-α-induced eIF4E phosphorylation in HT-29 cells.

Based on the previous study, MNK1 expresses and releases the inflammatory mediators such as IL-8 [20]. To elucidate the effect of orobol on MNK1 function, I examined the MNK1 kinase activity treated with orobol at 2 μM. Orobol significantly attenuated the activity of MNK1 kinase with the half maximal inhibitory concentration (IC_{50}) (IC_{50} = 33.43 nM) (Fig. 4A). Next, I investigated the effects of downstream of MNK1 signal with orobol in HT-29 cells. eIF4E is known as downstream of MNK1 and phosphorylates it. Also, eIF4E-mediated translation indirectly enhances the NF-κB activation [10]. Thus, I examined an inhibitory effect of orobol on phosphorylation of eIF4E. Orobol effectively suppressed TNF-α-induced phosphorylation of eIF4E (Fig. 4B). These results indicate that orobol inhibited MNK1 activity and its downstream signal.

Figure 4

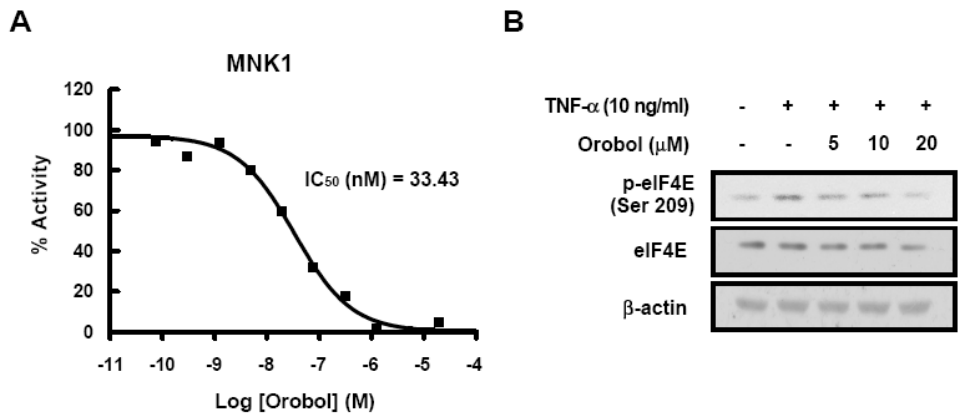

Figure 4. Inhibitory effects of orobol on MNK1 kinase activity.

A, Orobol was tested for MNK1 kinase inhibitory activity from ten concentrations with 2-fold serial dilutions starting at 20 μM. B, Effect of orobol on TNF-α-induced phosphorylation of eIF4E in HT-29 cells. After orobol treatment and 10 ng/ml TNF-α, the cells were lysated as described in the Materials and Methods. Phosphorylated and total form of indicated proteins were determined by western blotting as described in the Materials and Methods. β-actin was used as a loading control.

Figure 5

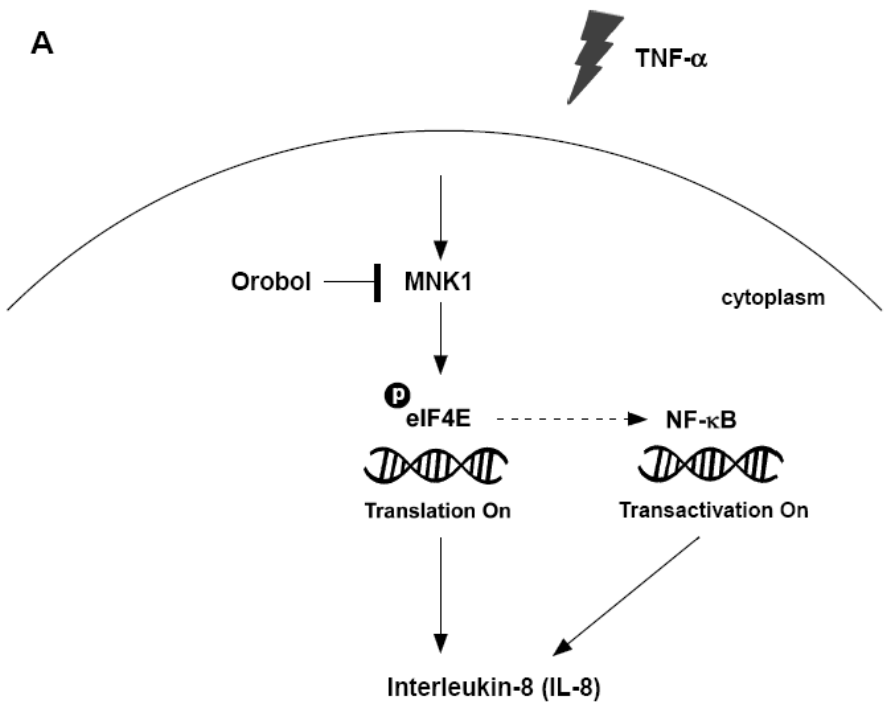

Figure 5. Proposed mechanism of orobol.

Orobol inhibited on TNF-α-induced IL-8 production by directly suppressing MNK1 activity in HT-29 cells.

Ⅳ. DISCUSSION

Crohn's disease (DC) and ulcerative colitis (UC) was s major forms of inflammatory bowel disease (IBD) which represent the presence of chronic inflammation involving various parts of the intestinal tract. [21-26]. Therapy of IBD has improved and expanded as understanding of the disease mechanisms. Pharmacologic agents are the mainstays of therapy such as aminosalicylates, anti-TNF-α agent, or steroids [26-30]. The specific target agents, such as TNF-α, have emerged and provided great clinical effects [26]. To understand physiological effect in colitis, I used DSS-induced colitis mice model and TNF-α induced IL-8 production on HT-29 cells.

The DSS-induced colitis model displays many similar symptoms to human ulcerative colitis (UC), such as diarrhea, bloody feces, weight loss and tissue damage [19]. Also, dietary factors such as curcumin, histidine, glutamine and taurine were reported to have anti-inflammatory effects in DSS-induced colitis models [31]. In addition, genistein exerts beneficial anti-inflammatory effects in a rodent model of chronic colitis [32].

Interleukin-8 (IL-8) is a major chemokine. IL-8, derived epithelial cell, has been demonstrated to support neutrophil infiltration in various types of mucosal inflammation including IBD. In previous study, IL-8 expression in mouse model is the patchy distribution of the neutrophil infiltrates in the caecum and colon that corresponded to the areas of IL-8 expression [33].

In this study, orobol was shown to attenuate DSS-induced colitis symptoms such as weight loss, diarrhea, fecal bloody and thickness of mucosa and muscularis propria compared to genistin and genistein. Also, I found out that orobol decreased IL-8 secretion and NF-κB transactivation on HT-29 cells.

Orobol is hydroxylated ingredients of genistein which is a most abundant isoflavone in soybean [34]. Though limitation of the contents, only few industrial usage and researches had been accomplished [13, 15, 16]. Recently, bioconversion strategy of producing orobol has been studied via using tyrosinase as a monophonol monooxygenase. The region-selective-hydroxylation of genistein was performed by whole *E. coli* cell biotransformation with heterologously expressed tyrosinase from *Bacillus megaterium* [14]. Orobol has an additional hydroxyl group at the 3'

position of genistein. Once the chemical has additional hydroxyl group at the 3' position of cheminals, its interactions with kinases could be stronger. The hydroxyl group could form a hydrogen bond with the backbone carbonyl group. And the hydrophobic site acts as the inhibitor sandwiched in the binding site [35, 36]. Thus, orobol is expected to interact stronger with the hinge region of MNK1 than that of genistein and orobol could be effectively inhibit MNK1 kinase activity. In conclusion, orobol is expected to see an increase in anti-inflammatory effects because of hydroxylation.

In this study, I found that MNK1 is a direct molecular target of orobol. Also, orobol was shown to inhibit phosphorylation of eIF4E and NF-κB transactivation. MNK1 is play critical roles and ubiquitously expressed in various cytokine receptors signaling which have important functions as mediators of pro-inflammatory cytokine production [8]. The phosphorylation of eIF4E at serine 209 by MNK1 has been shown to initiate oncogenic mRNA translation. Also, NF-κB complex activation is enhanced indirectly via translation of eIF4E [10]. Taken together, these findings show that MNK1 positively acts as a modulator of colitis and MNK1 might be a potential target of IBD.

In conclusion, this study demonstrated that orobol attenuated DSS-induced colitis *in vivo* and inhibited TNF-α-induced IL-8 production *in vitro*. These results indicate that orobol can be an important anti-inflammatory component, and may lead to prevention of colitis.

In further study, it is required how orobol structurally binds MNK1 and regulates its function. Furthermore, although data in this paper support the biological activity and mechanisms of orobol *in vitro* and *in vivo*, clinical studies are required to further validate its anti-inflammatory of colitis effect.

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Ⅵ. 국문 초록

염증성 장 질환은 인간의 위장관에 나타나는 병리학적 현상으로 비록 아직까지 명확한 원인은 밝혀져 있지는 않지만, 염증 반응이 주요한 병인으로 알려지고 있다. 콩 내의 생리활성 물질인 이소플라본은 항염증 효과와 장 건강 효능을 갖기에 유용한 식품 소재이다. 발효 혹은 생물과정을 통해 콩 이소플라본은 그들의 대사체들로 전환이 이루어지는데, 최근 대사체들의 개선된 생리활성 연구가 이루어지고 있다. 오로볼은 genistein의 대사체로 난소암과 유방암에 항암효과가 있다고 보고되고 있다. 최근 연구에 의하면, genistein이 대장염에 효능이 있다고 알려져 있으나 오로볼은 아직 대장염 효능에 있어서 연구된 바가 없다. 인터루킨-8(IL-8)은 염증성 장 질환 환자에게서 과다 분비되는 대표적인 chemokin으로 장 상피세포에서 분비된 IL-8은 호중구 세포의 침투를 증진시켜 장내 염증을 유발한다. 본 연구에서는 콩 이소플라본 genistein의 대사체인 오로볼이 대장 내 염증을 예방 및 완화할 수 있음을 제시하였다. 본 연구 결과, 콩 발효 대사체인 오로볼은 Dextran sodium sulfate (DSS)로 유도된 장염 마우스 모델에서 체중 감소, 설사, 혈변 및 장 조직 붕괴를 억제하는 것을 확인하였다. 또한, 대장 세포 모델을 통해 IL-8의 단백질 발현 역시 저해되었고, IL-8의 주요 전사인자인 NF-

kB를 효과적으로 억제하였다. 오로볼의 신호전달 표적 단백질을 규명하기 위하여 인체 내 존재하는 359 종의 kinase와의 결합을 확인하는 Kinase profiling을 수행하였다. 그 결과, 오로볼이 MAPK-interacting Ser/Thr kinase 1 (MNK1)의 활성을 억제하는 것을 확인하였다. 또한 오로볼은 MNK1의 하위 인자이자 간접적으로 NF-kB을 활성 시키는 인자인 Eukaryotic initiation factor 4E (eIF4E)의 인산화를 억제하였다.

본 연구 결과를 통해 오로볼이 DSS로 유도한 장염 완화 및 작용 기작을 밝힘으로써 염증성 장 질환을 완화 및 예방하는데 화학적 효능을 가지고 있음을 규명하였으며, 이를 통해 염증성 장 질환을 개선하는 신규 천연물로서 오로볼의 가능성을 제시하였다.

주요어: 오로볼; 카이네이즈 프로파일링; MNK1; IL-8; TNF-α; 염증성 장 질환

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