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Anti-inflammatory functions of alverine via targeting Src in the NF-κB pathway

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Abstract: Alverine, a smooth muscle relaxant, is used to relieve cramps or spasms of the stomach and intestine. Although the effects of alverine on spontaneous and induced contractile activity were well known, its anti-inflammatory activity has not been fully evaluated. In this study, we investigated the anti-inflammatory effects of alverine *in vitro* and *in vivo*. The production of nitric oxide (NO) in RAW264.7 cells activated by lipopolysaccharide (LPS) or polyinosinic: polycytidylic acid [Poly (I:C)] was reduced by alverine. The mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α) was also dose dependently inhibited by treatment with alverine. In reporter gene assays, alverine clearly decreased luciferase activity mediated by the transcription factor NF-κB in MyD88- or TRIF-overexpressing HEK293 cells. Additionally, phosphorylation of NF-κB subunits and upstream signaling molecules, including p65, p50, AKT, IκBα, and Src was downregulated by 200 μM of alverine in LPS-treated RAW264.7 cells. Using immunoblotting and cellular thermal shift assays (CETSAs), Src was identified as the target of alverine in its anti-inflammatory response. In addition, HCl/EtOH-stimulated gastric ulcers in mice were ameliorated by alverine at doses of 100 and 200 mg/kg. In conclusion, alverine reduced inflammatory responses by targeting Src in the NF-κB pathway, and these findings provide new insights in the development of anti-inflammatory drugs.

Keywords: Anti-inflammatory effect; Alverine; Src; inflammatory mediators; NF-κB

1. Introduction

The immune response is a defense mechanism that evolved in higher organisms to protect them from pathophysiological events [1]. Inflammation, one of the important innate immune responses, abolishes pathogens, including viruses, fungi, and bacteria, within a few minutes or hours [2]. However, unregulated and prolonged inflammation can evoke excessive levels of inflammatory mediators and result in resistance to apoptosis and a diminished survival advantage, even leading to carcinogenesis or multiple organ failure. For these reasons, although inflammation is a normal part of the immune system, it has to be tightly regulated [3]. Macrophages play major roles in the activation, maintenance, and termination of inflammation by producing a wide range of biological activating molecules [4]. Lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, is a potent

activator of macrophage-derived inflammation. LPS is recognized by a complex of proteins made up of CD14, myeloid differentiation protein-2 (MD-2), and Toll-like receptor 4 (TLR4) [5]. LPS-stimulated macrophages express various inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, -6, and -12, through activation of transcriptional factors nuclear factor κB (NF-κB), and activator protein-1 (AP-1) [5,6].

Src kinase (Src) is non-receptor protein tyrosine kinase that participates in a diverse spectrum of biological responses such as gene transcription, cell adhesion, cellular metabolism, and cell proliferation [7]. Src also plays pivotal roles in innate immunity including recruitment and activation of immune cells, production of inflammatory cytokines, and regulation of vascular permeability [8]. For instance, phosphorylated Src activates phosphatidylinositol-3′ kinase (PI3K) by associating with the p85 subunit [9] and also increases translocation of c-Jun and p65, resulting in IL-6 induction [10].

Alverine (Fig. 1A, N-ethyl-3-phenyl-N-(3-phenylpropyl)propan-1-amine) is a small molecule drug developed by Dr. Reddy's Laboratories (UK). It is used as a smooth muscle relaxant and acts specifically on the muscles present in locations such as the alimentary tract and uterus. [11]. Alverine regulates rectal hypersensitivity by stabilizing 5-TH1A receptors as a selective antagonist of the 5-HT1A receptor subtype [12] and proportionally regulates Ca^{2+}-dependent and Ca^{2+}-independent contraction in the detrusor smooth muscle [13]. Interestingly, in a previous study [14], alverine was found to be one of the components included in *Prasiola japonica* ethanol extract. According to this report, the ethanol extract of *Prasiola japonica*, also known as freshwater laver, exerts anti-inflammatory effects by reducing NO production, suggesting that alverine might be responsible. However, the pharmacological effects of alverine on inflammation are as yet poorly understood. Therefore, we evaluated the potential inhibitory effects of alverine on inflammatory responses and propose reclassifying alverine as an anti-inflammatory agent.

2. Materials and Methods

2.1. Materials

Fetal bovine serum (FBS), DMEM, and RPMI 1640 were obtained from Thermo Fisher Scientific (Waltham, MA, USA). RAW264.7 cells and HEK293 cells were purchased from ATCC (Rockville, MD, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole], lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The total or phospho-specific antibodies against p50, p65, IκBα, IKKα/β, p85/PI3K, Src, Syk, AKT, HA, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and preparation of drugs

RAW264.7 cells were maintained in RPMI 1640 media supplemented with 100U/ml of penicillin/streptomycin and 10% FBS. The cells were incubated at 37°C and 5% COHEK (human embryonic kidney) 293 cells were cultured in DMEM media supplemented with 100U/ml of penicillin/streptomycin and 5% FBS. The cells were incubated at 37°C and 5% COThe stock solution (100 mM) of alverine was prepared using DMSO.

2.3. Determination of NO

RAW264.7 cells were plated in 96-well plates (1×10^6 cells/ml) and incubated at 37°C and 5% CO_2 for 18 h. After incubation, the cells were treated with alverine (0 to 200 μM) for 30 min and then further incubated with LPS (1 μg/ml) for 24 h. NO production was measured using Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfanilamide, 25% H_3PO_4). The inhibitory effects of alverine on NO production were detected by measuring the absorbance at 540 nm using a SpectraMax 250 microplate reader.

2.4. Cell viability test

RAW264.7 cells were plated in 96-well plates (1×10^6 cells/ml) and incubated at 37°C and 5% CO_2 for 18h. After incubation, the cells were treated with alverine (0 to 200 μM) and incubated for 24 h. Next, 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline, pH 7.4) was added to each well. After 3 h of incubation, 100 μl of MTT stop solution (15% SDS) was added to each well to solubilize the formazan, and the cells were incubated for 24 h. The effects of alverine on cell viability were determined by measuring the absorbance at 570 nm using a SpectraMax 250 microplate reader.

2.5. mRNA analyses using reverse transcriptase-polymerase chain reaction

RAW264.7 cells were treated with alverine (0 to 200 μM) for 30 min and then further incubated with LPS (1 μg/ml) for 6 h. Total RNA was isolated from the cells using TRIzol reagent (Gibco, Gaithersburg, MD, USA) following the manufacturer's instructions. The total RNA was then stored at -70°C until needed. PCR amplification was performed using pre-mix (Bio-D) as described previously [15], and the primers used are listed in Table 1.

Table Sequences of PCR primers used in this study.

Targets	Direction	Sequences (5′ to 3′)
iNOS	Forward	GGAGCCTTTAGACCTCAACAGA
	Reverse	TGAACGAGGAGGGTGGTG
COX-2	Forward	CACTACATCCTGACCCACTT
	Reverse	ATGCTCCTGCTTGAGTATGT
TNF-α	Forward	GCCTCTTCTCATTCCTGCTTG
	Reverse	CTGATGAGAGGGAGGCCATT
GAPDH	Forward	CAATGAATACGGCTACAGCAAC
	Reverse	AGGGAGATGCTCAGTGTTGG

2.6. Luciferase reporter gene assay

HEK293 cells (2×10^5 cells/ml) were co-transfected with either Myd88 or TRIF along with NF-κB-Luc DNA and β-galactosidase using the PEI (polyethylenimine) method in a 24-well plate. The cells were treated with alverine (0–200 μM) 24 h after transfection. After an additional 24 h, luciferase assays were performed using a Luciferase Assay System (Promega, Madison, WI, USA) as reported previously [16].

2.7. Immunoblotting

RAW264.7 cells (2×10^6 cells/ml) were treated with alverine (0–200 μM) for 30 min and then further incubated with LPS (1 μg/ml) for various amounts of time. The cells were collected in cold PBS and lysed in lysis buffer (20 mM Tris HCl, pH7.4, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 1 mM benzamide, and 2 mM PMSF) on ice. The cell lysates were then centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatant was stored at -20°C until further use. Immunoblotting assays was used to analyze the whole cells lysates as previously reported [17].

2.8. Cellular thermal shift assay

HEK293 cells (2.5×10^5 cells/ml) were transfected with Src using the PEI method in a 6-well plate. DMSO or alverine (200 μM) was added to the cells 24 h after transfection, and the cells were isolated with PBS. The resuspended cells were divided equally into 7 PCR tubes and heated for 3 min at 42 to 60°C. After cooling for 3 min at 25°C, the cells were lysed using liquid nitrogen, and this freeze-thaw cycle was repeated 3 times. The cell lysates were then centrifuged 13,000 rpm for 30 min at 4°C. The protein supernatants were analyzed using Western blotting, and the intensity of the bands was quantified using ImageJ software.

2.9. HCl/EtOH-induced acute gastritis

Five-week-old male ICR mice were purchased from ORIENT BIO (Seongnam, Korea). The mice had access to pelleted food (Samyang, Daejeon, Korea) and water *ad libitum*. All studies were performed according to the guidelines established by the Sungkyunkwan University Institutional Animal Care and Use Committee (Suwon, Korea; approval ID: SKKUIACUC2019-08-15-1). Inflammation of the stomach was induced with EtOH/HCl according to a previously published method [18] [19]. In brief, fasting ICR mice (n=4) were treated with alverine (0–200 mg/kg) administered orally twice per day for 3 days. One h after the final injection of alverine, the mice were dosed orally with 400 μl of 70% ethanol in 150 mM HCl to induce acute gastritis. After 1 h, each mouse was anaesthetized and sacrificed using CO_2.

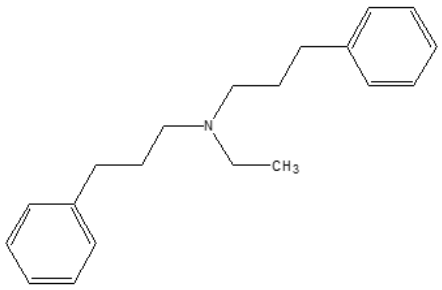
2.10. Statistical analysis

For the MTT and NO assays, ten wells were used in each experimental group to ensure the reliability of the results. For the luciferase assay, each experimental group has six parallel wells. In the PCR and western blotting analysis, each experimental group was tested in triplicate. Gastritis *in vivo* experiments were performed with six mice per group. All data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. For statistical comparison, the results were analyzed using either analysis of variance (ANOVA) with Scheffe's *post hoc* test, the Kruskal–Wallis test, or the Mann–Whitney U test. For all analyses, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Alverine inhibited NO production in macrophage-like RAW264.7 cells

To examine the effects of alverine on macrophage-mediated inflammatory responses, NO production was first investigated in alverine-treated RAW264.7 macrophage-like cells. NO production was induced by LPS and poly I:C, which are TLR4 and TLR3 ligands, respectively. Alverine dose-dependently blocked NO production in LPS- and Poly(I:C)-stimulated RAW264.7 cells (Fig. 1B and 1C). To exclude the possibility that this nitric oxide inhibitory effect was due to cytotoxicity, we evaluated the viability of RAW264.7 cells treated with alverine using an MTT assay. Alverine was not cytotoxic at any concentrations (Fig. 1D). L-NAME, a standard anti-inflammatory compound, also showed a suppressive pattern under the same NO production conditions in LPS - treated RAW264.7 cells without cytotoxicity (Fig. 1E and 1F).



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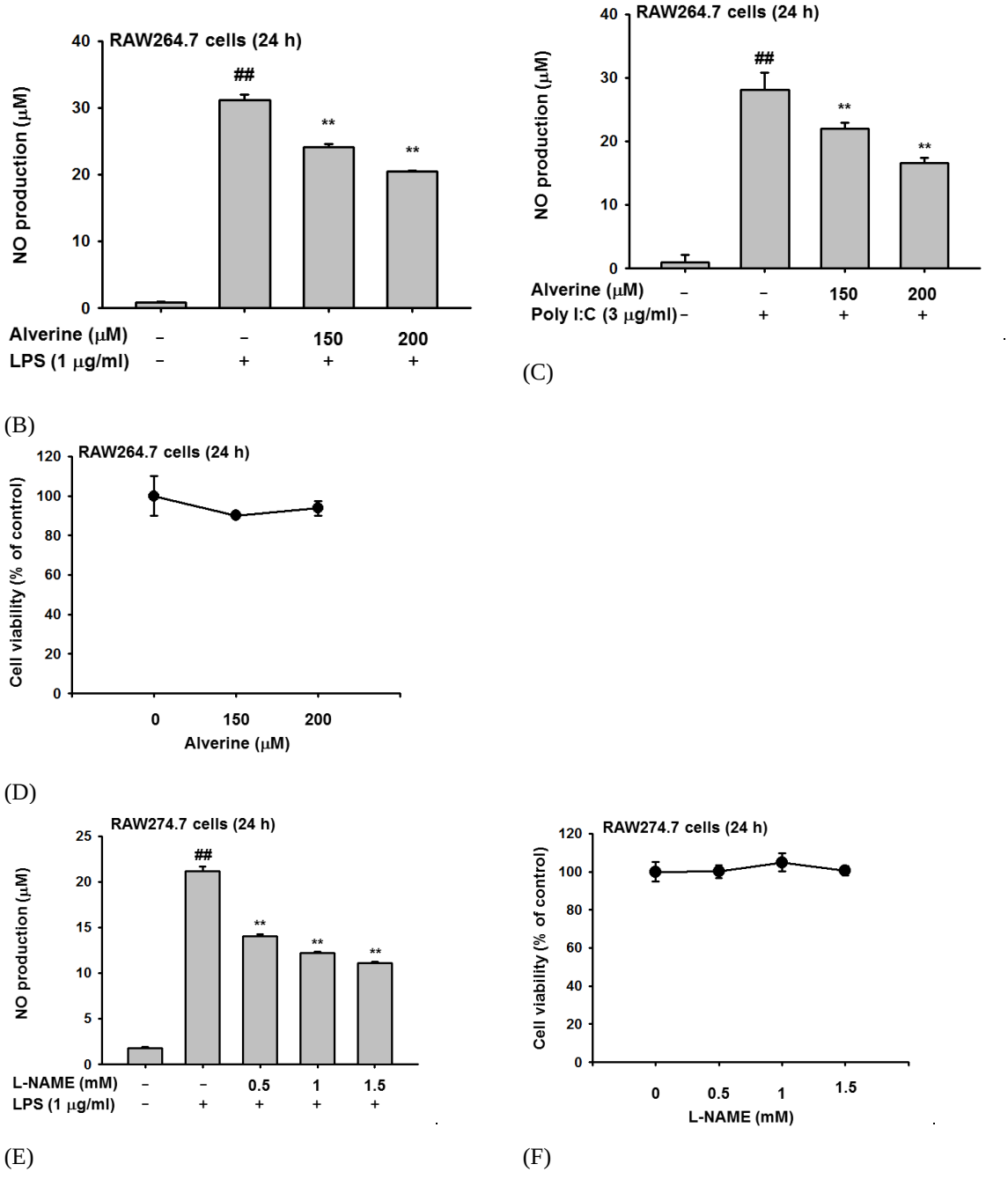


Figure Chemical structure of alverine and its anti-inflammatory effects in LPS-treated RAW264.7 cells. (A) Chemical structure of alverine. (B and C) RAW264.7 cells were stimulated with LPS (1 μg/ml) or Poly I:C (3 μg/ml) for 24 h in the presence or absence of alverine, and NO levels were determined by Griess assay. (D) The viability of RAW264.7 cells treated with alverine (0–200 μM) was measured using an MTT assay. (E and F) RAW264.7 cells were activated with LPS for 24 h in the presence of absence of L-NAME, a positive control. NO production was examined by Griess assay. ## $P < 0.01$ compared to the normal group, ** $P < 0.01$ compared to the control group (LPS or Poly I:C alone).

3.2. Alverine suppressed iNOS, COX-2, and TNF-α mRNA expression through NF-κB inhibition

The inhibitory effects of alverine on expression levels of pro-inflammatory genes were investigated using RT-PCR analyses in LPS-stimulated RAW274.7 cells. Alverine at 200 μM strongly inhibited mRNA expression of iNOS, COX-2, and TNF-α in RAW274.7 cells (Fig. 2A). To understand the molecular mechanism by which alverine regulates the expression of these genes, we examined

the influence of alverine on NF-κB, an inflammatory transcriptional factor. Luciferase assays were performed to analyze the activity of NF-κB in HEK293T cells. NF-κB-mediated luciferase activity in cells transfected with the inflammation-inducing genes MyD88 or TRIF was decreased by alverine in a dose-dependent manner (Fig. 2B and C). According to the MTT assay, alverine did not inhibit viability of HEK293 cells at concentrations between 0 to 200 μM, suggesting that the inhibitory effect of alverine on NF-κB-luc is not derived from cytotoxicity. Phosphorylation levels of p50 and p65, subunits of NF-κB, were also evaluated by immunoblotting assay. Only p-p50 level was diminished in the alverine-treated group (Fig. 2E). These results indicate that alverine exerts anti-inflammatory activity by specifically inhibiting NF-κB.

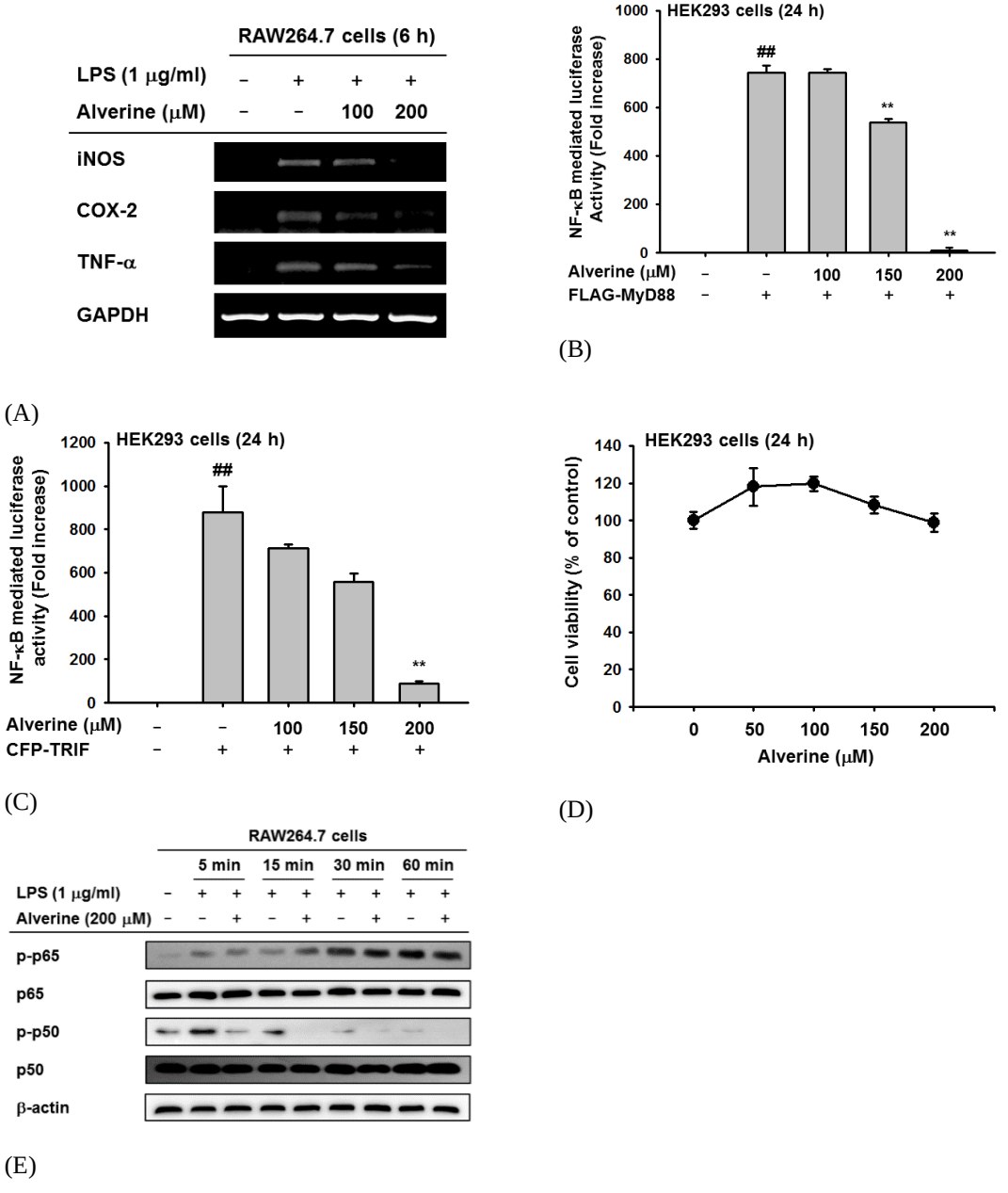


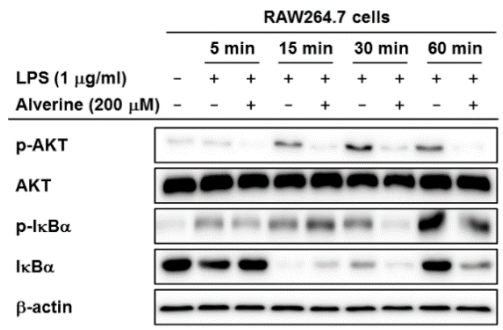
Figure Effects of alverine on inflammatory gene expression and transcriptional activation of NF-κB. (A) RAW264.7 cells were treated alverine (0–200 μM) and then stimulated with LPS (1 μg/ml) for 6 h. mRNA expression levels of iNOS, COX-2, and TNF-α were evaluated using reverse transcription PCR. (B and C) NF-κB promoter binding activity was determined using a reporter gene assay in HEK293T cells. The cells were transfected with FLAG-MyD88 (B) or CFP-TRIF (C) plasmid constructs for 24 h followed by treatment with alverine (0–200 μM) for an additional 24 h. Luciferase activity was evaluated using a luminometer and normalized to that of β-galactosidase. (D) The viability of HEK293T cells treated with alverine (0–200 μM) was determined using the MTT

assay. (E) The phosphorylated forms and total forms of NF-kB subunits (p60 and p50) were determined in LPS-stimulated RAW264.7 cells by western blotting. ## $P < 0.01$ compared to the normal group, ∗∗ $P < 0.01$ compared to the control group (TRIF or MyD88 alone).

3.3. Alverine regulated upstream NF-κB signaling proteins by targeting Src kinase in LPS-activated RAW264.7 cells

To figure out the target molecule of the alverine, we studied the effect of alverine on the upstream signaling cascade for the activation of NF-κB in LPS-treated RAW264.7 cells. The phosphorylation of IκBα, a key regulator of NF-κB, was significantly decreased at 30 and 60 min by alverine (200 μM) (Fig. 3A). Alverine also downregulated AKT phosphorylation at all time points in LPS-stimulated RAW264.7 cells (Fig. 3A). It has been reported that the phosphorylation of IκBα is modulated by p85 and Src in TLR4-stimulated conditions [20,21]. Therefore, we measured the activation of p85 and Src at earlier time points. The phosphorylated p85 level in LPS-stimulated macrophages was suppressed by alverine (200 μM) at 5 min. On the other hand, alverine inhibited phosphorylation of Src kinase from 2 min after LPS stimulation (Fig. 3B), suggesting that Src among the NF-κB signal proteins might be a target molecule of alverine.

To explore whether alverine can modulate Src kinase, Src was auto-phosphorylated and activated by overexpression of the Src plasmid in HEK293T cells. As we expected, the phosphorylation of Src and p85 was clearly suppressed by alverine in a dose-dependent manner (Fig. 3C). Since drug binding can lead to significant thermal stabilization of the protein, the interaction between alverine and Src was evaluated by cellular thermal shift assay (CETSA) assay. Src kinases in the control group were mostly degraded at 57 and 60°C, while Src was still detected in alverine-treated cells (Fig. 3D). The specific domain of Src that interacts with alverine was investigated by overexpressing Src domain deletion mutants such as Src-dSH2 and Src-dSH3 in HET 293T cells. Alverine reduced Src phosphorylation induced by Src-WT and Src-dSH3 but did not affect Src phosphorylation by Src-dSH2, implying that alverine interacts with the SH2 domain of Src kinase (Fig. 3E and 3F).



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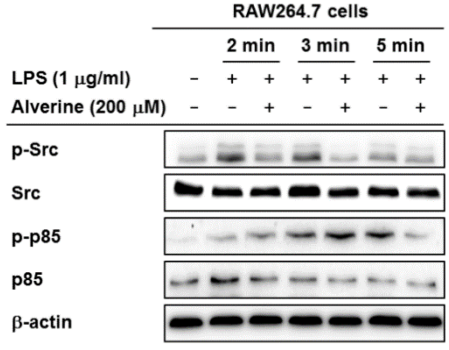

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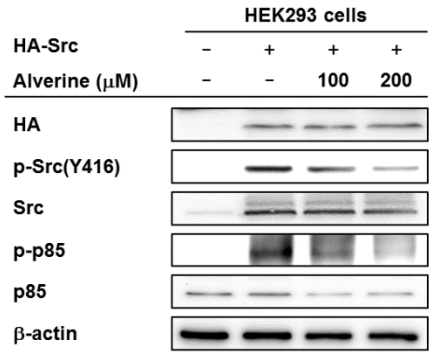

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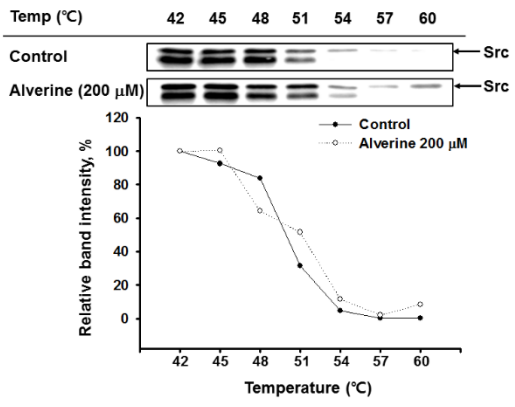

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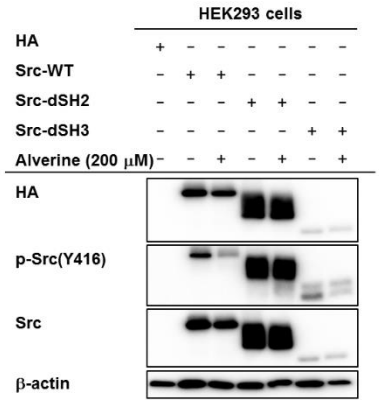

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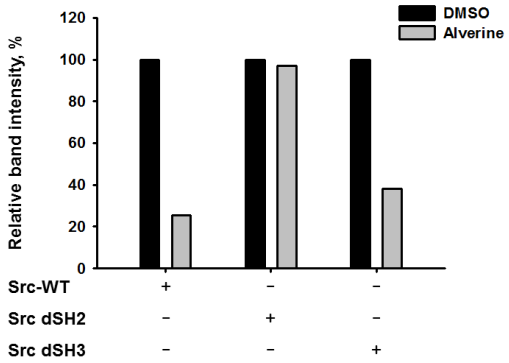
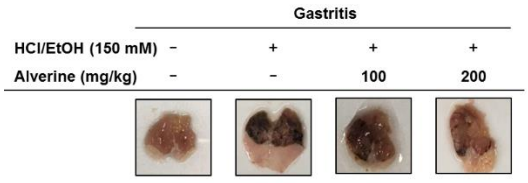


(F)

Figure Src as a specific target of alverine among the upstream proteins of NF-κB signaling. (A and B) RAW264.7 cells were treated with alverine (0–200 μM) and activated with LPS (1 μg/ml) for the indicated times. The levels of the phosphorylated and total forms of NF-κB singling proteins including AKT, IκBα, p85, and Src were measured using western blotting. (C) HEK293T cells overexpressing HA-Src were treated with alverine (0–200 μM). The total and phosphorylated protein levels of Src, p85, HA, and β-actin were evaluated using western blotting. (D) CETSA was performed in HEK293T cells treated with alverine (200 μM) or DMSO (control). (E and F) HEK293T cells overexpressing either WT Src or Src with a deletion of the SH2 or SH3 domain treated with alverine (200 μM). Immunoblotting was performed to determine the total and phosphorylated protein levels of Src, HA, and β-actin (E). The band intensity of p-Src was quantified using ImageJ (F).

3.4. Alverine exerted anti-inflammatory effects in an HCL/EtOH-induced gastritis mice model.

To determine the anti-inflammatory activity of alverine in animals, *in vivo* gastritis was induced by injecting HCl/EtOH into mice. Alverine ameliorated the ulcerative lesions in a dose-dependent manner (Fig. 4A). Similar to the *in vitro* results, iNOS and TNF-α mRNA levels in stomach tissue lysates of gastritis mice were also lower in the alverine administration group (Fig. 4B). Additionally, we assessed whether alverine modulated the NF-κB signaling pathway in the stomach tissues of gastritis-induced mice. Alverine clearly suppressed the levels of phosphorylated p65 and IκBα in stomach tissue lysates (Fig. 5C).



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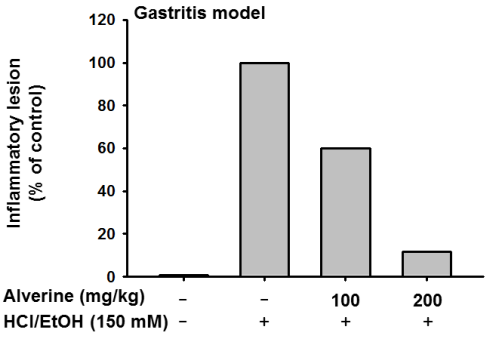

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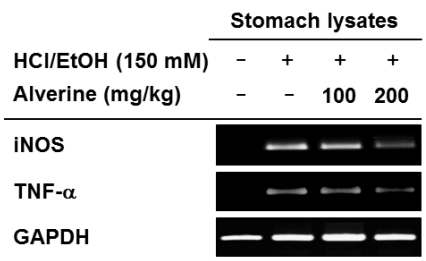

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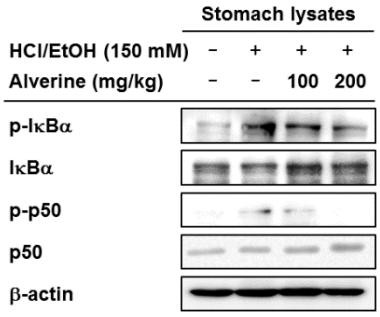

(D)

Figure Anti-inflammatory effects of alverine in an *in vivo* acute gastritis model. (A and B) Mice were pretreated with alverine (0–200 mg/kg) orally twice per day for 3 days then injected orally with EtOH/HCl (150 mM). After 1 h, gastric inflammatory lesions were photographed using a digital camera (A). Gastritis lesions in the stomachs were quantified using ImageJ software (B). (C) mRNA expression levels of iNOS, TNF-α, and GAPDH in stomach tissues were examined using RT-PCR. (D) Total and phosphorylated protein levels of IκBα, p65, p50, and β-actin were analyzed using a Western blot assay.

4. Discussion

A previous study explored the active ingredients and pharmacological properties of freshwater laver *Prasiola japonica* [14]. In particular, LPS-stimulated NO production was shown to be effectively inhibited by *Prasiola japonica* ethanol extract. Interestingly, alverine was identified as an active ingredient in the solvent fractions (chloroform, butanol, and water) obtained from *Prasiola japonica* ethanol extract. Alverine is a smooth muscle relaxant and is accordingly used for irritable bowel syndrome, which is a painful diverticular disease of the colon, and primary dysmenorrhea [22] [23]. However, the pharmacological effects of alverine on inflammatory processes are not fully understood. Therefore, we investigated the ameliorating effects of alverine on inflammation and associated cellular and molecular mechanisms.

To demonstrate the anti-inflammatory activities of alverine, RAW264.7 cells were treated with LPS and Poly I:C, which activated TLR4 and TLR3, respectively [24] [25]. Alverine strongly inhibited the production of NO in LPS- and Poly (I:C)-stimulated RAW274.7 cells without significant cytotoxicity at concentrations up to 200 μM (Fig. 1A, B, and C). In addition, alverine decreased mRNA expression levels of inflammatory genes such as iNOS, COX-2, and TNF-α in LPS-stimulated RAW264.7 cells (Fig. 2A). These results imply that alverine exhibits anti-inflammatory properties. In particular, alverine is expected to have a broad impact in suppressing inflammatory responses, as it reduces NO production induced by both TLR3 and TLR4.

Next, we sought to figure out the molecular mechanisms underlying the anti-inflammatory activity of alverine. It has been reported that gene expression of iNOS, COX-2, and TNF-α is mainly modulated by NF-κB and AP-1 transcriptional factors [26,27]. In addition, MyD88 and TRIF are known as essential adaptor molecules in the activation of TLR-mediated NF-κB and AP-1 signaling [28]. Therefore, NF-κB and AP-1 reporter gene assays were performed in MyD88 and TRIF-overexpressing HEK293T cells. Alverine suppressed MyD88- and TRIF-dependent NF-κB luciferase activity (Fig. 2B and 2C, respectively), independent of the AP-1-mediated pathway (data not shown). Since phosphorylation of p65 and p50 are involved in NF-κB stabilization and promoter activity [29], p-p65 and p-p50 levels were also determined. Consistent with the reporter gene assay results, phosphorylation of p50 was also diminished by alverine. In TLR-mediated NF-κB signaling, p85-AKT pathway activation by various stimuli induces phosphorylation and degradation of IκBα, resulting in NF-κB activation [30]. Thus, we investigated the effect of alverine on LPS-induced phosphorylation of p85, AKT, and IκBα. Alverine inhibited all of these enzymes (Fig.3A and B), implying that alverine specifically regulates the NF-κB-signaling pathway.

Due to the importance of NF-κB in inflammatory responses, the strategy for inhibiting this pathway has attracted attention in the development of anti-inflammatory drugs [31]. For example, glucocorticoids, such as dexamethasone and prednisone, can reduce the inflammatory response by increasing IκBα expression [32]. In addition, there are considerable reports indicating that the molecular target of nonsteroidal anti-inflammatory drugs (NSAIDs) is at least partially NF-κB [33,34,35]. In a similar vein, we concluded that alverine exerts anti-inflammatory activity through inhibition of the NF-κB pathway and therefore may be used as an anti-inflammatory drug. Indeed, the potential of alverine as an anti-inflammatory drug was investigated in HCl/EtOH-triggered gastritis models. Alverine reduced inflammatory gene expression as well as redness and bleeding in the gastric mucosa (Fig. 4A and B). In addition, phosphorylation of IκBα and p50 was suppressed by alverine administration in stomach lysates of gastritis mice (Fig. 4C). These results provide evidence that alverine can be used to treat inflammatory diseases by inhibiting NF-κB signaling.

Our next concern was to identify direct targets of alverine among NF-κB signaling molecules. Treatment with LPS has long been known to cause rapid induction of tyrosine phosphorylation in macrophages [36]. It has been also reported that Src family kinases are activated after LPS treatment [37,38,39], and ligands for other TLRs besides LPS induce tyrosine phosphorylation of Src kinase substrates such as Vav1, Pyk2, Cbl, Syk and paxillin [40,41,42,43,44,45]. In addition, phosphorylated Src is known to induce PI3K/Akt-NF-κB pathway activation in macrophages [46]. These previous studies imply that Src kinase contributes to activation of the LPS-induced NF-κB signaling pathway. Thus, we assessed the effect of alverine on LPS-induced Src phosphorylation. According to the immunoblotting results, alverine suppressed phosphorylation of p85 at 5 min, but phosphorylation of Src was inhibited from 2 min after LPS stimulation (Fig. 3B). Given the timing of responses, we suspected that Src was a target molecule of alverine. To test our hypothesis, we overexpressed Src kinase, leading to autophosphorylation and activation of Src. As shown in Fig. 3C, alverine inhibited auto-phosphorylated Src, indicating that the alverine targets Src directly, and not upstream enzymes of Src kinase. Next, CETSA analysis was performed to evaluate the binding of Src and alverine in cells. CETSA is a useful method for quantifying the association of a drug with targets inside cells on the basis of binding-induced stabilization of the protein [47]. As we expected, Src was present in a more stable form at high temperatures in the alverine treatment group compared to the control group, implying that alverine regulates Src phosphorylation and activation by binding directly to Src (Fig. 3D). Src kinase is composed of four domains: the unique region and the SH3, SH2, and kinase domains [48]. To determine the alverine binding domain of Src, WT-Src or SH2- or SH3-domain deletion Src were overexpressed in HEK293T cells. The levels of phosphorylated Src in Src-dSH3 and Src-WT plasmids transfection groups were reduced by alverine; however, phosphorylation of SH2-domain deletion Src was constant with and without alverine (Fig. 3E). These data imply that alverine binds to the SH2 domain of Src and disturbs its phosphorylation, leading to regulation of inflammatory responses through modulation of the NF-κB pathway. Since NF-κB is involved in normal physiological events, such as epithelial differentiation and cell growth, long-term systemic application of a direct NF-κB inhibitor can cause significant side effects [31,49]. However, alverine can be expected to reduce the side effects observed in typical NF-κB inhibitors because alverine inhibits inducible NF-κB signaling by suppressing pathogen-stimulated Src kinase rather than basal NF-κB.

In conclusion, we have established the anti-inflammatory functions of alverine in LPS-stimulated macrophages and an *in vivo* gastritis model. Alverine repressed production of inflammatory mediators and gene expression of cytokines, including iNOS, COX-2, and TNF-α, by suppressing NF-κB transcriptional activity. In addition, alverine inhibited NF-κB signaling by directly targeting Src kinase (Fig. 5). Collectively, our findings suggest that alverine is a potential anti-inflammatory drug candidate with relatively fewer side effects than other NF-κB inhibitors.

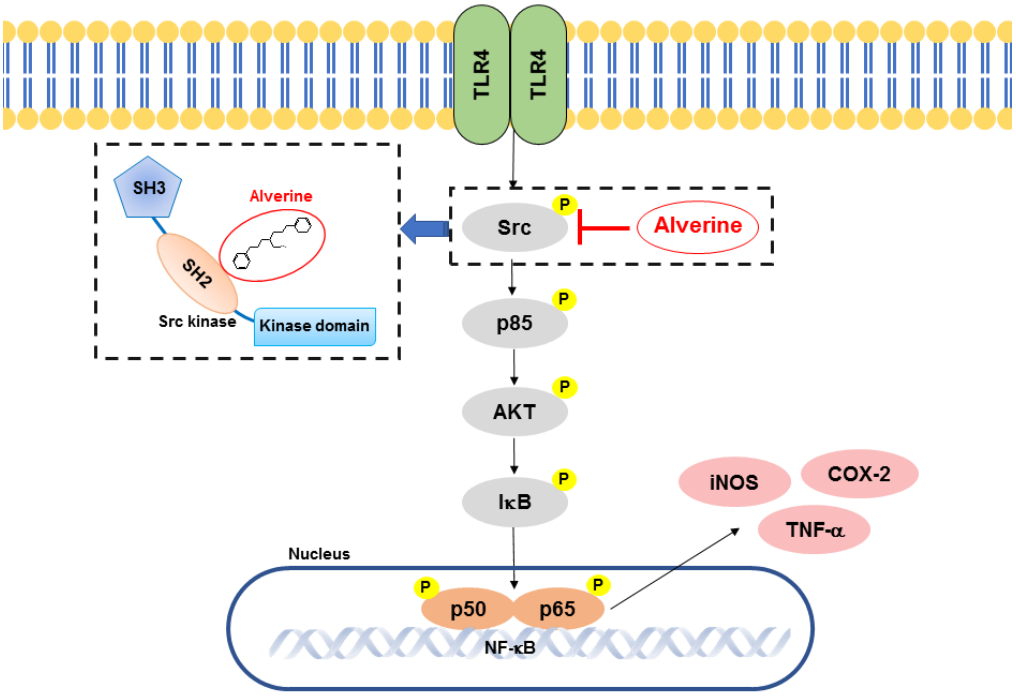

Figure Putative inhibitory pathway for the anti-inflammatory action of alverine.

Abbreviations

Pathogen-associated molecular patterns (PAMPs)
pattern recognition receptors (PRRs)
Toll-like receptors (TLRs)
myeloid differentiation primary response 88 (MYD88)
TIR-domain-containing adapter-inducing interferon-β (TRIF)
cyclooxygenase-2 (COX-2)
interleukin (IL)
nitric oxide (NO)
Prednisolone (Pred)
tumor necrosis factor-alpha (TNF-α)
Interleukin (IL)
activator protein 1 (AP-1)
intraperitoneal (IP)
NO synthases (NOS)

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