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Eudesmane-Type Sesquiterpene Lactones Inhibit Nuclear Translocation of the Nuclear Factor κB Subunit RelB in Response to a Lymphotoxin β Stimulation

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The transcription factor nuclear factor κB (NF-κB) regulates various biological processes, including inflammatory responses. We previously reported that eudesmane-type sesquiterpene lactones inhibited multiple steps in the canonical NF-κB signaling pathway induced by tumor necrosis factor-α and interleukin-1α. In contrast, the biological activities of eudesmane-type sesquiterpene lactones on the non-canonical NF-κB signaling pathway remain unclear. In the present study, we found that (11S)-2α-bromo-3-oxoecdesan-12,6α-lactone, designated santonin-related compound 2 (SRC2), inhibited NF-κB luciferase reporter activity induced by lymphotoxin β (LTβ) in human lung carcinoma A549 cells. Although SRC2 did not prevent the processing of the NF-κB subunit p100 induced by LTβ, it inhibited the nuclear translocation of RelB and p52 in response to the LTβ stimulation. In contrast to (−)-dehydromethyllepoxyquinomicin, SRC2 inhibited the LTβ-induced nuclear translocation of the RelB (C144S) mutant in a manner similar to wild-type RelB. While eudesmane derivatives possessing an α-bromoketone moiety or α,β-unsaturated carbonyl moieties inhibited LTβ-induced NF-κB luciferase reporter activity, eudesmane derivatives possessing an α-bromoketone moiety exhibited stronger inhibitory activity on the LTβ-induced nuclear translocation of RelB than those possessing a single α-methylene-γ-lactone moiety. The results of the present study revealed that SRC2 inhibits the nuclear translocation of RelB in the non-canonical NF-κB signaling pathway induced by LTβ.

Key words eudesmane; lymphotoxin β; nuclear factor κB; RelB; sesquiterpene lactone

Nuclear factor κB (NF-κB) is a family of structurally-related eukaryotic transcription factors that regulate the expression of a number of important genes, including those related to cell proliferation, cell death, and inflammation.1) The NF-κB family consists of five subunits: RelA(p65), RelB, c-Rel, p105/p50, and p100/p52.2) Various types of stimuli, including inflammatory cytokines, activate the canonical and non-canonical NF-κB signaling pathways.3) In the canonical NF-κB pathway, tumor necrosis factor (TNF) family members, such as TNF-α, induce the activation of the inhibitor of NF-κB (IκB) kinase.5) In the cytosol, IκB sequesters the NF-κB heterodimers RelA and p50 and prevents their nuclear translocation.5) Upon phosphorylation, IκB undergoes ubiquitination and proteasomal degradation, leading to the liberation and nuclear translocation of the RelA and p50 heterodimers.5) In contrast, the non-canonical NF-κB pathway is triggered by other TNF family members, such as lymphotoxin β (LTβ).6) The NF-κB subunits RelB and p100/p52 play essential roles in the non-canonical NF-κB pathway.7) In unstimulated cells, p100 constitutively associates with RelB in the cytosol and inhibits its activity.7) Upon a stimulation with LTβ, IκB kinase α is primarily activated and then directly phosphorylates p100.8) Phosphorylated p100 is ubiquitinated and processed into p52 by proteasome, leading to the nuclear translocation of the RelB and p52 heterodimers and the transcriptional activation of target genes.9)

Sesquiterpene lactones are found in many plants, and have been attracting increasing attention for drug development due to their various biological activities, including anti-cancer and anti-inflammatory activities.8) We previously reported that (11S)-2α-bromo-3-oxoecdesan-12,6α-lactone (I), initially designated as santonin-related compound 2 (SRC2; Fig. 1), inhibited cell-surface intercellular adhesion molecule-1 (ICAM-1) expression induced by interleukin-1β (IL-1β) in human lung carcinoma A549 cells.10) A structure–activity relationship study on eudesmane-type sesquiterpene lactones, including SRC2, showed that these sesquiterpene lactones inhibited multiple steps in the canonical NF-κB signaling pathway induced by TNF-α and IL-1α.11) SRC2 inhibited the nuclear translocation of RelA by targeting its cysteine 38 in the TNF-α-induced NF-κB signaling pathway.11) In contrast to the canonical NF-κB signaling pathway, the biological activity of eudesmane-type sesquiterpene lactones on the non-canonical NF-κB signaling pathway remain unclear. In the present study, we found that SRC2 inhibited the non-canonical NF-κB signaling pathway induced by LTβ. The molecular mechanisms by which SRC2 inhibits the LTβ-induced NF-κB signaling pathway were investigated in more detail.
MATERIALS AND METHODS

Cells Human lung carcinoma A549 cells (JCRB0076) were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition JCRB Cell Bank (Osaka, Japan). A549 cells and human Burkitt’s lymphoma Raji cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Grand Island, NY, U.S.A.) supplemented with heat-inactivated fetal calf serum (Nichirei Bioscience, Tokyo, Japan) and a penicillin-streptomycin antibiotic mixture (Nacalai Tesque). Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) medium (Thermo Fisher Scientific, Grand Island, NY, U.S.A.) supplemented with heat-inactivated fetal calf serum (Nichirei Bioscience) and a penicillin-streptomycin antibiotic mixture (Nacalai Tesque).

Reagents SRC2 (1), (11S)-3-oxoeudesmano-12,6α-lactone (2), 2a-bromo-3-oxoeudesm-11(13)-eno-12,6α-lactone (3), 3-oxoeudesm-11(13)-eno-12,6α-lactone (4), (11S)-3-oxoeudesm-1-enol-12,6α-lactone (5), (11S)-3β-hydroxyeudesm-1-eno-12,6α-lactone (6), tuberiferin (7), and 3β-hydroxyeudesma-1,11(13)-dieno-12,6α-lactone (8) were synthesized as described previously,10,11 previously. Human embryonic kidney 293T cells (Clontech Laboratories, Kusatsu, Japan). A549 cells were transfected with lentiviruses together with pCAG-HIV gp and pCMV-VSV-G-RSV-Rev, produced by 293T cells transiently transfected with CSII-CSII-CMV-MCS-IRES2-Bsd. Recombinant lentiviruses were provided by Dr. Hiroyuki Miyoshi (RIKEN BioResource Center, Tsukuba, Japan). FLAG-tagged RelB mutant in which cysteine 144 was replaced by serine, designated RelB (C144S), was constructed by PCR-based site-directed mutagenesis.

Luciferase Assay A549 cells were transiently transfected with the NF-κB-responsive firefly luciferase reporter14 and cyto-megalovirus (CMV) promoter-driven Renilla luciferase reporter for 8 h by HilyMax transfection reagent (Dojindo Laboratories, Kamegumo, Tokyo, Japan) and further incubated for 16 h. Cell lysates were prepared and examined for luciferase activity as described previously.15 Relative light units were measured by Lumitester C-110 (Kikkoman Biochemica Company, Tokyo, Japan).

Western Blotting Whole cell lysates and cytoplasmic and nuclear extracts were prepared according to our previous studies.16 Proteins (30 μg) were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to ClearTran’s nitrocellulose membrane 0.2 μm (Wako). Membrane filters were analyzed by Western blotting using primary antibodies for β-actin (AC-15; Sigma-Aldrich and 2F3; Wako), FLAG (IE6; Wako), IκBα (clone 25; BD Bioscience, San Jose, CA, U.S.A.), p52 (05-361; Merck Millipore, Darmstadt, Germany), poly(ADP-ribose) polymerase (PARP) (C-2-10; Sigma-Aldrich and C2-10; Trevigen, Gaithersburg, MD, U.S.A.), phospho-IκBα (Ser32/36) (5A5; Cell Signaling Technology, Danvers, MA, U.S.A.), RelB (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), and α-tubulin (DM1A, Sigma-Aldrich). Protein bands were visualized by an ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ, U.S.A.) or ImmunoStar® Zeta (Wako) and analyzed by ImageQuant LAS 4000 Mini (GE Healthcare).

DNA Cloning and Stable Transfection Full-length human RelB cDNA was obtained from the human Burkitt’s lymphoma Raji cell cDNA library by PCR amplification. Wild-type (WT) RelB cDNA fused to the N-terminal FLAG sequence was cloned into a pC3R expression vector. The RelB mutant in which cysteine 144 was replaced by serine, designated RelB (C144S), was constructed by PCR-based site-directed mutagenesis. pCAG-human immunodeficiency virus (HIV) gp, pCMV-VSV-G-RSV-Rev, and CSII-CMV-MCS-ires2-Bsd were provided by Dr. Hiroyuki Miyoshi (RIKEN BioResource Center, Tsukuba, Japan). FLAG-tagged RelB WT and RelB (C144S) were inserted into the lentivirus vector CSII-CMV-MCS-ires2-Bsd. Recombinant lentiviruses were produced by 293T cells transiently transfected with CSII-CMV-MCS-ires2-Bsd encoding RelB WT or RelB (C144S), together with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev, and concentrated by a Lenti-X™ Concentrator (Takara Bio, Kusatsu, Japan). A549 cells were transfected with lentiviruses in the presence of Polybran (8 μg/mL) for 24 h, incubated for an additional 48 h, and then incubated with blasticidin S hydrochloride (10 μg/mL) for at least 5 d. A549 cell lines resistant to blasticidin S were confirmed to express FLAG-RelB WT or FLAG-RelB (C144S) by Western blotting.

mRNA Expression Assay ICAM-1 mRNA expression was evaluated by Real-Time PCR as described previously,7 except for primers: 5′-GGCTGGGAAACACCGGAAAGGT G-3′ and 5′-GGTGGCCAGTTCCACCCCGTT-3′ for the 148-bp fragment of ICAM-115 and 5′-GGACATCGCAAGAACCCT GTA-3′ and 5′-GCTCAGGAGAGCAAATGACTCTC-3′ for the 143-bp fragment of β-actin18 and PCR conditions: 94°C for 3 min, followed by 45 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s.

Proliferation Assay Proliferation was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was performed as described previously,20 except that A549 cells were incubated with MTT (500 μg/mL) for the last 4 h of the incubation and that absorbance at 570 nm was measured by iMark™ microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Statistical Analysis The significance of differences was calculated by a one-way ANOVA followed by Tukey’s test for
RESULTS

SRC2 Inhibited NF-κB Luciferase Reporter Activity Induced by LTβ We investigated the biological activity of SRC2 on the non-canonical NF-κB signaling pathway induced by LTβ. A549 cells were preincubated with various concentrations of SRC2 for 1 h and then stimulated with LTβ for 4 h. Decreases in NF-κB luciferase activities correlated with increases in the concentrations of SRC2 (Fig. 2A). We then investigated the inhibitory effects of SRC2 at different time points after the LTβ stimulation. SRC2 at 25 µM effectively suppressed the increase in NF-κB luciferase activity during the 4-h incubation (Fig. 2B). These results suggested that SRC2 prevented the non-canonical NF-κB signaling pathway induced by LTβ.

SRC2 Inhibited the Nuclear Translocation of RelB and p52 in Response to the LTβ Stimulation The RelB and p100 heterodimers are present in unstimulated cells, and p100 is processed into p52 by proteasome. Upon the LTβ stimulation, the amount of p100 gradually decreased during the 4-h incubation, and this was accompanied by a slight increase in p52 in control A549 cells (Fig. 3). LTβ also caused a decrease in p100 and increase in p52 in SRC2-treated A549 cells (Fig. 3).

We investigated the effects of SRC2 on the LTβ-induced phosphorylation of IκBα. LTβ increased the amount of phospho-IκBα, but did not decrease that of total IκBα in the 2-h incubation (Fig. 4). SRC2 decreased the amounts of phospho-IκB and total IκBα in the presence of LTβ (Fig. 4).

We then investigated whether SRC2 inhibited the nuclear translocation of the RelB and p52 heterodimers. A549 cells were preincubated with various concentrations of SRC2 for 1 h and then incubated with LTβ for 2 h. The nuclear levels of RelB and p52 were diminished by SRC2 in a dose-dependent manner (Fig. 5A). In addition, the basal levels of RelB and p52 in the nucleus appeared to be decreased by SRC2 (Fig. 5A). SRC2 at 25 µM inhibited the nuclear translocation of RelB during the 4-h incubation upon the LTβ stimulation (Fig. 5B).

SRC2 Inhibited the Nuclear Translocation of RelB (C144S) in Response to the LTβ Stimulation We previously showed that SRC2 inhibited the TNF-α-induced nuclear translocation of RelA by targeting its cysteine 38 in the Rel homology domain; SRC2 did not inhibit the nuclear translocation of the RelA (C38S) mutant. RelB possesses cysteine 144 in the Rel homology domain, which corresponds...
to cysteine 38 in RelA.\textsuperscript{19} \((–)-\text{DHMEQ}\) has been shown to bind to cysteine 38 of RelA and cysteine 144 of RelB.\textsuperscript{19} We established A549 cells stably expressing FLAG-tagged RelB WT and the RelB (C144S) mutant (Fig. 6A). Transfected RelB WT and RelB (C144S) of established cell lines were mainly expressed in the cytoplasm, and not in the nucleus without the LTβ stimulation (Fig. 6B). SRC2 inhibited the nuclear translocation of FLAG-RelB WT in response to the LTβ stimulation (Fig. 6C). Moreover, the LTβ-induced nuclear translocation of RelB (C144S) was inhibited by SRC2 in a similar manner to RelB WT (Fig. 6D). In order to validate our assay system, \((–)-\text{DHMEQ}\) was used as an NF-κB inhibitor. \((–)-\text{DHMEQ}\) at concentrations greater than 25 μM inhibited the LTβ-induced nuclear translocation of FLAG-RelB WT (Fig. 7A), whereas the inhibitory effect of \((–)-\text{DHMEQ}\) on the nuclear translocation of FLAG-RelB (C144S) became weaker at the same concentrations (Fig. 7B).

**Eudesmane Derivatives Inhibited the Non-canonical NF-κB Signaling Pathway** Eudesmane-type sesquiterpene lactones, including SRC2, were shown to inhibit multiple steps in the canonical NF-κB signaling pathway induced by TNF-α and IL-1α.\textsuperscript{10} We investigated whether eudesmane derivatives, \textit{i.e.}, SRC2 (1), 2–6, tuberiferin (7), and 8, inhibited the non-canonical NF-κB signaling pathway induced by LTβ. A549 cells were preincubated with 1–8 at 50 μM for 1 h and then incubated with LTβ for 4 h. NF-κB luciferase activity was inhibited by 1, 3, 4, 5, 7, and 8, but not by 2 or 6 (Figs. 8A, B). We further investigated the effects of 1–8 on the LTβ-induced expression of ICAM-1 mRNA. ICAM-1 mRNA expression was inhibited by 1, 3, 4, 5, 7, and 8, but not by 2 or 6 (Figs. 8C, D). These results suggested that an α-bromoketone moiety and α,β-unsaturated carbonyl moieties, including an α-methylene γ-lactone moiety, were required to inhibit LTβ-induced NF-κB luciferase activity and ICAM-1 mRNA expression. Moreover, the nuclear translocation of RelB induced by LTβ was inhibited by 1, 3, 5, and 7, and not or only weakly by 2, 4, 6, and 8 at a concentration of 50 μM (Figs. 8E, F). Among these derivatives, 3 appeared to decrease the amount of RelB in the cytoplasm (Fig. 8E). These results indicated that eudesmane derivatives SRC2 (1) possessing an α-bromoketone moiety, 3 possessing an α-bromoketone moiety and an α-methylene γ-lactone moiety, and tuberiferin (7) possessing two α,β-unsaturated carbonyl moieties including an α-methylene γ-lactone exhibited stronger inhibitory activity on the LTβ-induced nuclear translocation of RelB than that of 4 and 8 possessing a single α-methylene γ-lactone moiety.

**SRC2 Inhibited the Proliferation of A549 Cells** A549 cells were treated with serial dilutions of SRC2 for 8, 24, and 48 h, and the MTT assay was performed to evaluate the antiproliferative effects of SRC2. SRC2 decreased the reduction of proliferative effects of SRC2. SRC2 decreased the reduction of RelB WT (Fig. 7A), whereas the inhibitory effect of \((–)-\text{DHMEQ}\) on the LTβ-induced nuclear translocation of RelB and p52 into the nucleus in response to the LTβ stimulation. In addition, the basal amounts of RelB and p52 in the nucleus appeared to be decreased by SRC2 in the absence of LTβ. These results suggest that the non-canonical NF-κB pathway is activated at low levels, possibly by an autocrine stimulation with certain TNF superfamily ligands in A549 cells, and SRC2 may block common steps in the non-canonical NF-κB signaling pathway.

Sequence homology between NF-κB subunits revealed that the cysteine residues equivalent to cysteine 38 of RelA are conserved in five NF-κB subunits.\textsuperscript{21} \((–)-\text{DHMEQ}\) binds to cysteine 38 of RelA, cysteine 144 of RelB, cysteine 27 of c-Rel, and cysteine 62 of p50, but not to cysteine 57 of p52.\textsuperscript{21} \((–)-\text{DHMEQ}\) was previously reported to inhibit the LTβ-induced nuclear translocation of RelB and p52 in human breast carcinoma MCF-7 cells.\textsuperscript{22} We confirmed that \((–)-\text{DHMEQ}\) inhibited the LTβ-induced nuclear translocation of RelB in A549 cells. Moreover, we showed that the inhibitory effect of \((–)-\text{DHMEQ}\) on the LTβ-induced nuclear translocation of the

**DISCUSSION**

SRC2 was initially synthesized from the starting material \((–)-\text{o-santonin}, a eudesmane-type sesquiterpene lactone, and found to inhibit the canonical NF-κB signaling pathway induced by IL-1β.\textsuperscript{10} SRC2 inhibited multiple steps in the canonical NF-κB pathway induced by IL-1α and TNF-α.\textsuperscript{31} In the present study, we found that SRC2 inhibited the non-canonical NF-κB signaling pathway induced by LTβ. We further showed that SRC2 did not inhibit the processing of p100, but inhibited the translocation of RelB and p52 into the nucleus in response to the LTβ stimulation. In addition, the basal amounts of RelB and p52 in the nucleus appeared to be decreased by SRC2 in the absence of LTβ. These results suggest that the non-canonical NF-κB pathway is activated at low levels, possibly by an autocrine stimulation with certain TNF superfamily ligands in A549 cells, and SRC2 may block common steps in the non-canonical NF-κB signaling pathway.
Fig. 5. SRC2 Inhibited the Nuclear Translocation of RelB and p52 in Response to the LTβ Stimulation

(A, B) A549 cells were preincubated with various concentrations of SRC2 for 1 h, and then incubated with 25 ng/mL of LTβ (LTβ) or without LTβ (Control) for 2 h in the presence or absence of SRC2 (A). A549 cells were preincubated with or without SRC2 (25 µM) for 1 h, and then incubated with LTβ (25 ng/mL) for different time points in the presence or absence of SRC2 (B). Cytoplasmic and nuclear extracts were analyzed by Western blotting. Data are representative of three independent experiments. The amounts of RelB and p52 relative to PARP in the nucleus and β-actin in the cytoplasm are shown as the mean±S.E. of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001, significantly different from no SRC2 in the presence or absence of LTβ.
Fig. 6. SRC2 Inhibited the Nuclear Translocation of RelB WT and RelB (C144S) in Response to the LTβ Stimulation

(A, B) Whole cell lysates (A) and cytoplasmic and nuclear extracts (B) were prepared from non-transfected A549 cells (Control) and stable A549 transfectants expressing FLAG-RelB WT and FLAG-RelB (C144S), and analyzed by Western blotting. Data are representative of three independent experiments. The amounts of RelB and p52 relative to β-actin are shown as the mean±S.E. of three independent experiments (A). The amounts of FLAG (RelB), RelB, PARP, and β-tubulin are shown as the mean±S.E. of three independent experiments (B).

(C, D) A549 transfectants stably expressing RelB WT (C) and RelB (C144S) (D) were preincubated with various concentrations of SRC2 for 1 h and then incubated with or without LTβ (25 ng/mL) for 2 h in the presence or absence of SRC2. Cytoplasmic and nuclear extracts were analyzed by Western blotting. Data are representative of three (C) and four (D) independent experiments. The amount of RelB relative to PARP in the nucleus and β-actin in the cytoplasm is shown as the mean±S.E. of three (C) and four (D) independent experiments. *p<0.05 and **p<0.01, significantly different from no SRC2 in the presence of LTβ.
RelB (C144S) mutant became weaker than that of RelB WT, indicating that cysteine 144 of RelB is a target of (−)-DHMEQ in A549 cells. However, in contrast to (−)-DHMEQ, SRC2 still inhibited the LTβ-induced nuclear translocation of the RelB (C144S) mutant as effectively as RelB WT. Although we cannot exclude the possibility that SRC2 does not bind to cysteine 144 of RelB, our results suggest that SRC2 targets RelB at amino acid residue(s) other than cysteine 144 and/or possibly additional protein(s) other than RelB, which are required for the nuclear translocation of RelB and p52.

(−)-DHMEQ blocks the nuclear translocation of the NF-κB subunits in the canonical and non-canonical NF-κB pathways. (−)-DHMEQ has also been shown to inhibit the growth of human cancer cells, including carcinoma and leukaemia, without any toxicity in animal models. We previously reported that SRC2 inhibited the canonical NF-κB pathway at the steps of the TNF-α-induced RelA nuclear translocation and the IL-1α-induced IκBα phosphorylation. The present results suggested that SRC2 inhibited the non-canonical NF-κB pathway by targeting RelB and/or possibly additional protein(s). The multiple targets of SRC2 in the NF-κB pathway may be responsible for stronger anti-cancer and anti-inflammatory activities as an advantage, but may also result in cell toxicity due to weaker selectivity.

A structure–activity relationship study for compounds 1–8 showed that eudesmane derivatives possessing an α-bromoketone moiety or α,β-unsaturated carbonyl moieties inhibited LTβ-induced NF-κB reporter activity. These results...
Fig. 8. Eudesmane Derivatives Inhibited NF-κB-Responsive Luciferase Activity and the Nuclear Translocation of RelB in Response to the LTβ Stimulation

(A, B) A549 cells were transiently transfected with reporter plasmids encoding NF-κB-responsive firefly luciferase and CMV promoter-driven Renilla luciferase for 8 h and further incubated for 16 h. A549 cells were preincubated with or without 1–4 (each 50 µM) (A) or 5–8 (each 50 µM) (B) for 1 h and then incubated with (+) or without (−) LTβ (25 ng/mL) for 4 h in the presence or absence of the derivatives. Luciferase activity (fold) in the presence of LTβ was calculated relative to that in its absence for each derivative. Data are shown as the mean ± S.E. of six (A) and five (B) independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

(C, D) A549 cells were preincubated with (+) or without (−) 1–4 (50 µM each) (C) or 5–8 (50 µM each) (D) for 1 h and then incubated with (+) or without (−) LTβ (25 ng/mL) for 2 h in the presence or absence of the derivatives. ICAM-1 mRNA expression was analyzed by quantitative PCR. Data are shown as the mean ± S.E. of three (C) and four (D) independent experiments. ***p < 0.001.

(E, F) A549 cells were preincubated with (+) or without (−) 1–4 (each 50 µM) (E) or 5–8 (each 50 µM) (F) for 1 h and then incubated with (+) or without (−) LTβ (25 ng/mL) for 2 h in the presence or absence of the derivatives. Cytoplasmic and nuclear extracts were analyzed by Western blotting. Data were representative of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.
are consistent with our previous findings obtained for the expression of ICAM-1 induced by IL-1α and TNF-α.11) We previously reported that these eudesmane-type sesquiterpene lactones inhibited multiple steps in the canonical NF-κB pathway induced by TNF-α and IL-1α.11) In the LTβ-induced NF-κB signaling pathway, α-bromoketones SRC2 (1) and 3 exhibited stronger inhibitory activity on the nuclear translocation of RelB than 4 and 8, possessing a single α-methylene-γ-lactone moiety in a molecule. In contrast, tuberiferin (7) exhibited stronger inhibitory activities in a similar manner to SRC2 (1), and this may have been due to the presence of two active functional α,β-unsaturated carbonyl moieties in a molecule.

In conclusion, we herein demonstrated that eudesmane-type sesquiterpene lactones inhibited the nuclear translocation of RelB in the non-canonical NF-κB signaling pathway induced by LTβ. In contrast to the canonical NF-κB pathway, only a limited number of compounds are known to inhibit the non-canonical NF-κB pathway. The non-canonical NF-κB pathway plays an important role in immunity as well as diverse pathologies, such as cancer and autoimmune disease. Therefore, small-molecule inhibitors are useful for elucidating the regulatory mechanisms and physiological roles of the non-canonical NF-κB pathway.

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Conflict of Interest The authors declare no conflict of interest.

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