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Stereospecific Induction of Nuclear Factor-κB Activation by Isochamaejasmin

Qinghai Tian, Jing Li, Xin Xie, Meiling Sun, Hairong Sang, Caihong Zhou, Tianying An, Lihong Hu, Richard D. Ye, and Ming-Wei Wang

The National Center for Drug Screening, Shanghai Institute of Materia Medica (X.X., M.S., C.Z., T.A., L.H., M.-W.W.) and the Graduate School (Q.T.), Chinese Academy of Sciences, Shanghai, China; and Department of Pharmacology, College of Medicine, University of Illinois, Chicago, Illinois (J.L., H.S., R.D.Y.)

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ABSTRACT

The root of *Stellera chamaejasme* L. is a traditional Chinese herb termed Rui Xiang Lang Du and has been used to treat solid tumors, tuberculosis and psoriasis. Exactly how *S. chamaejasme* L. regulates cellular responses remains unclear. We examined four biflavonoids isolated from *S. chamaejasme* L., including isochamaejasmin, two of its stereo-isomers and a methyl derivative, in functional assays originally designed to screen ligands for the G protein-coupled formyl peptide receptor-like 1 (FPRL1). Isochamaejasmin was found to induce the expression of a nuclear factor (NF)-κB-directed reporter gene in transfected HeLa cells with an EC_{50} of 3.23 μM, independently of FPRL1. The isochamaejasmin-stimulated NF-κB reporter activity was accompanied by nuclear translocation of NF-κB proteins and was blocked by a dominant-negative construct of IκBα. Isochamaejasmin also induced time-dependent phosphorylation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 and p38, and a novel protein kinase C (PKCδ). Likewise, inhibition of these kinases with the respective pharmacological inhibitors significantly reduced the isochamaejasmin-stimulated NF-κB activation. It is noteworthy that the two stereoisomers and the methyl derivative did not induce detectable activation of NF-κB and were more cytotoxic than isochamaejasmin, which could partially rescue cycloheximide-induced apoptosis. Inhibition of NF-κB activation reversed the anti-apoptotic effect of isochamaejasmin. These results provide the first evidence for a potential mechanism of action by *S. chamaejasme* L., and indicate that structurally similar compounds derived from *S. chamaejasme* L. may have different pharmacological properties.

In ancient China, medicines were called 'Ben Cao' (Chinese materia medica). The root of *Stellera chamaejasme* L. (Thymelaeaceae) was first described, among 365 types of herbs, in the oldest herbal medicine book ('Shen Nong Ben Cao') written in the late Han Dynasty (25–220 CE) (Lin and Zhu, 1992). Named Rui Xiang Lang Du in traditional Chinese medicine, this herb was shown to possess both toxic and therapeutic effects (Zhou et al., 1997) and has been used for many years to treat solid tumors (e.g., liver and lung cancer) (Yang and Lu, 1989), tuberculosis (Yang, 1956), and certain skin diseases (e.g., psoriasis and acariasis) (Guo, 1986; Si, 1989). The clinical applications of this plant were supported by its pharmacological properties, such as tumor suppression and bactericidal action as demonstrated by a large number of experimental studies conducted in the past decade (Jian et al., 2003). Despite a long history of clinical use in China, very little is known about the mechanisms underlying its therapeutic effects. There have been continued efforts in isolating individual compounds from *S. chamaejasme* L. and its water and methanol extracts include biflavonoids, lignans, and diterpenes (Pei et al., 2001). Although similar biflavonoids were found in *Euphorbia fischeriana* and *Euphorbia ebracteolata* (Euphorbiaceae), the contents varied considerably among the three different plant families (Gong, 1982; Xing et al., 1992). Whether these chemical constituents (biflavonoids

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ABBREVIATIONS: FPRL1, formyl peptide receptor-like 1; NF-κB, nuclear factor-κB; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; IKKβ, IκB kinase β; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; WKYMVm, Trp-Lys-Tyr-Met-Val-D-Met-NH_2; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; GF109203X, 3-[1-[3-(dimethylaminopropyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene.

in particular) alone or in combination are responsible for the bioactivities of *S. chamaejasme* L. remains to be determined.

Formyl peptide receptor-like 1 (FPRL1) is a G protein-coupled receptor that binds natural and synthetic peptides as well as lipoxin A4 and mediates important biological functions such as inflammation (Chiang et al., 2000). Among more than 10 ligands identified for FPRL1, WKYMVm (Baek et al., 1996; Le et al., 1999) has been widely used in in vitro and ex vivo studies of this G protein-coupled receptor. In an attempt to discover novel small molecule ligands for FPRL1, a high throughput, highly sensitive, cell-based, NF-κB-dependent luciferase reporter system was developed recently to screen both natural and synthetic compound libraries (Nanamori et al., 2004). Isochamaejasmin isolated from *S. chamaejasme* L. was one of the compounds initially discovered to display a potent NF-κB activation activity using this system.

In the present study, we examined four biflavonoids derived from ethanol extracts of *S. chamaejasme* L. for their biological effects at the cellular level. We found that stereoisomers have drastically different functional properties, such that only isochamaejasmin (S/R/R/S at C2/C3/C2″/C3″) possesses the ability to stimulate NF-κB activation. The all-S as well as the S/S/R/S configurations were ineffective. Functional characterization suggests that isochamaejasmin-induced NF-κB stimulation in HeLa cells is accompanied by the activation of several protein kinases including extracellular signal-related kinase (ERK) 1/2 and p38, and the novel protein kinase C isoform, PKCδ. The activation of these kinases may contribute to the IκB kinase β (IKKβ)-mediated NF-κB activation and nuclear translocation of the NF-κB proteins.

Materials and Methods

Materials. W-peptide (WKYMVm) was synthesized at GL Biochem (Shanghai) Ltd. (Shanghai, China). Phorbol 12-myristate 13-acetate was purchased from Sigma-Aldrich (St. Louis, MO). Steady-Glo luciferase assay solutions were obtained from Promega Corp. (Madison, WI). Other reagents were bought from Sigma-Aldrich.

Isochamaejasmin and Analogs. Dried and powdered root of *S. chamaejasme* L. (2 kg), collected from Sichuan Province, China, was extracted with ethanol (8 liters × 3) at room temperature. The concentrated extract obtained under reduced pressure was partitioned with ethyl acetate and H_2O. The ethyl acetate layer (41 g) was subjected to D-101 macroporous resin (Shanghai Chemical Reagent Factory, Shanghai, China), and eluted with ethanol/H_2O (50:50, 70:30, and 95:5). After sequential chromatography on silica gel ($CHCl_3$/MeOH/H_2O, 7:3:0.5), Mitsubishi Chemical Corporation gel CHP 20P (40% MeOH), Chromatorex ODS (50% MeOH), and Sephadex LH-20 (100% MeOH), four biflavonoids were isolated by liquid chromatography on a Waters 2690/996 high-performance liquid chromatography-photodiode array system, with a solvent gradient of 50% methanol/50% water (0.5% HCOOH) to 100% methanol at 25°C, at a flow rate of 1 ml/min for 15 min. They were identified based on spectral properties: Isochamaejasmin (Niwa et al., 1984; 1986), neochamaejasmin A (Niwa et al., 1984), neochamaejasmin B (Niwa et al., 1984), and chamaejasmin B (Liu et al., 1984). The chromatograms (with analysis wavelength set at 300 nm) are shown in Fig. 1, and the purity of each biflavonoid is summarized in Table 1. These compounds were deposited into the natural compound library at the National Center for Drug Screening for random screening with different bioassay systems.

Cell Culture. The human cervical carcinoma cell line HeLa was transfected with pNF-κB-Luc reporter plasmid, which contains five copies of NF-κB binding sequence (Stratagene, La Jolla, CA), with or without a human FPRL1 cDNA expression vector in pSFFV.neo vector. The transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Rat basophilic leukemia cell line RBL-2H3 expressing the human FPRL1 was described previously and maintained in DMEM supplemented with 20% fetal bovine serum (He et al., 2000).

Whole Cell-Based Compound Screening. HeLa cells expressing NF-κB-Luc/FPRL1 (HeLa/NF-κB/FPRL1) were seeded in 96-well plates at a density of 1.5×10^4 cells per well. After cells became adherent, they were serum-starved in DMEM without phenol red for 16 h before screening assay. A total of 320 pure compounds derived from natural products at a concentration of approximately 10 μg/ml were screened. After incubation with the compounds for 5 h, the expressed luciferase activity was determined in a Wallac 1420 multilabel counter (VICTOR^2; PerkinElmer Life and Analytical Sciences, Boston, MA) using the Steady-Glo luciferase assay solutions. Seven hits, that produced more than 5-fold induction of luciferase activity, were initially discovered. Secondary screening with the same assay confirmed that one compound (NPLC0169), namely isochamaejasmin, has an EC_{50} of 3.23 μM. It was further evaluated with HeLa cells expressing pNF-κB-Luc but not FPRL1 (HeLa/NF-κB) along with the three analogs of isochamaejasmin.

Chemotaxis. WKYMVm (W-peptide)-induced migration of cells was assessed in a 48-well micro-chemotaxis chamber (Neuro Probe, Cabin John, MA) and compared with that of isochamaejasmin. In brief, different concentrations of isochamaejasmin (between 0 and 100 μM) were placed in the lower chamber (30 μl) and RBL-FPRL1 cells (50 μl at 1×10^6 cells/ml) were loaded in the upper chamber, which was separated from the lower chamber by a polycarbonate filter (pore size 10 μm). After incubation at 37°C for 4 h, the filter was removed, fixed, and stained with Diff-Quick staining solutions (IMEB Inc., San Marcos, CA). The numbers of migrated cells were determined by counting in a high power field (400×). Results were presented as chemotaxis index that represents the ratio of the density of the area where cells migrated toward W-peptide or isochamaejasmin over the density of the area where cells migrated toward medium.

Cytotoxicity Measurement. HeLa cells (American Type Culture Collection, Manassas, VA) were seeded in a 96-well plate at a density of 3×10^3 per well in the presence of serial dilutions of isochamaejasmin and its analogs. After incubation at 37°C for 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the cells and incubated for an additional 2 h at 37°C. The reaction product, formazan, was extracted with DMSO and absorbance at 560 nm was determined in a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA) (Liu et al., 2002). For rescue experiment, cycloheximide (5 μg/ml; Supelco, Bellefonte, PA) and various concentrations of isochamaejasmin were introduced to the cells 24 h before the MTT assay. NF-κB SN50, a cell-permeable inhibitory peptide (Lin et al., 1995) (Calbiochem, San Diego, CA), or a negative control peptide (SN50M), were added 30 min before the above reagents to determine whether the rescue effect is associated with NF-κB activation.

NF-κB Nuclear Translocation. The NF-κB translocation assay was conducted according to the manufacturer's instructions using a NF-κB activation kit for high content screening (Cellomics Inc., Pittsburgh, PA). In brief, HeLa cells were seeded in the μ Clear-plates (Kaminform; Greiner Bio-One, Kremsmuenster, Austria) at a density of 5×10^3 cells per well and incubated for 24 h at 37°C. After stimulation with various concentrations of interlekin-1α, phorbol 12-myristate 13-acetate, isochamaejasmin, or neochamaejasmin A at 37°C for 40 min, cells were fixed with prewarmed 3.7% formalin in PBS for 10 min at room temperature. After washing, the cells were permeabilized and incubated with the rabbit anti-NF-κB antibody supplied with the kit for 1 h, followed by a 15-min wash with detergent buffer (once) and PBS (twice). The cells were then stained with the staining solution containing goat anti-rabbit IgG conjugated to Alexa Fluor 488 and Hoechst dye for 1 h before a 10-min wash with the detergent buffer and two additional washes with PBS. The cells

were soaked in PBS at 4°C until imaging procedure using the ArrayScan HCS system (Cellomics).

Western Blot Analysis. Cells grown in six-well plates were stimulated with selected compounds for indicated times. The reaction was stopped by addition of 1 ml ice-cold PBS, and the cells were collected. After centrifugation, the cell pellet was resuspended in 1 ml ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na_3VO_4, 5 mM NaF, and protease inhibitors). The contents were incubated on ice for 15 min, centrifuged, and the supernatant was transferred to fresh Eppendorf tubes. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with antibodies against either phospho-ERK1/2 or p38 (Cell Signaling Technologies, Beverly, MA) at 1:1000 dilution for 18 h. A similar experiment was performed with an anti-phospho-PKCδ antibody (Thr505), from the same vendor. The membrane was washed and incubated with 1:5000 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h. Excess antibody was removed by washing, and immunocomplexes

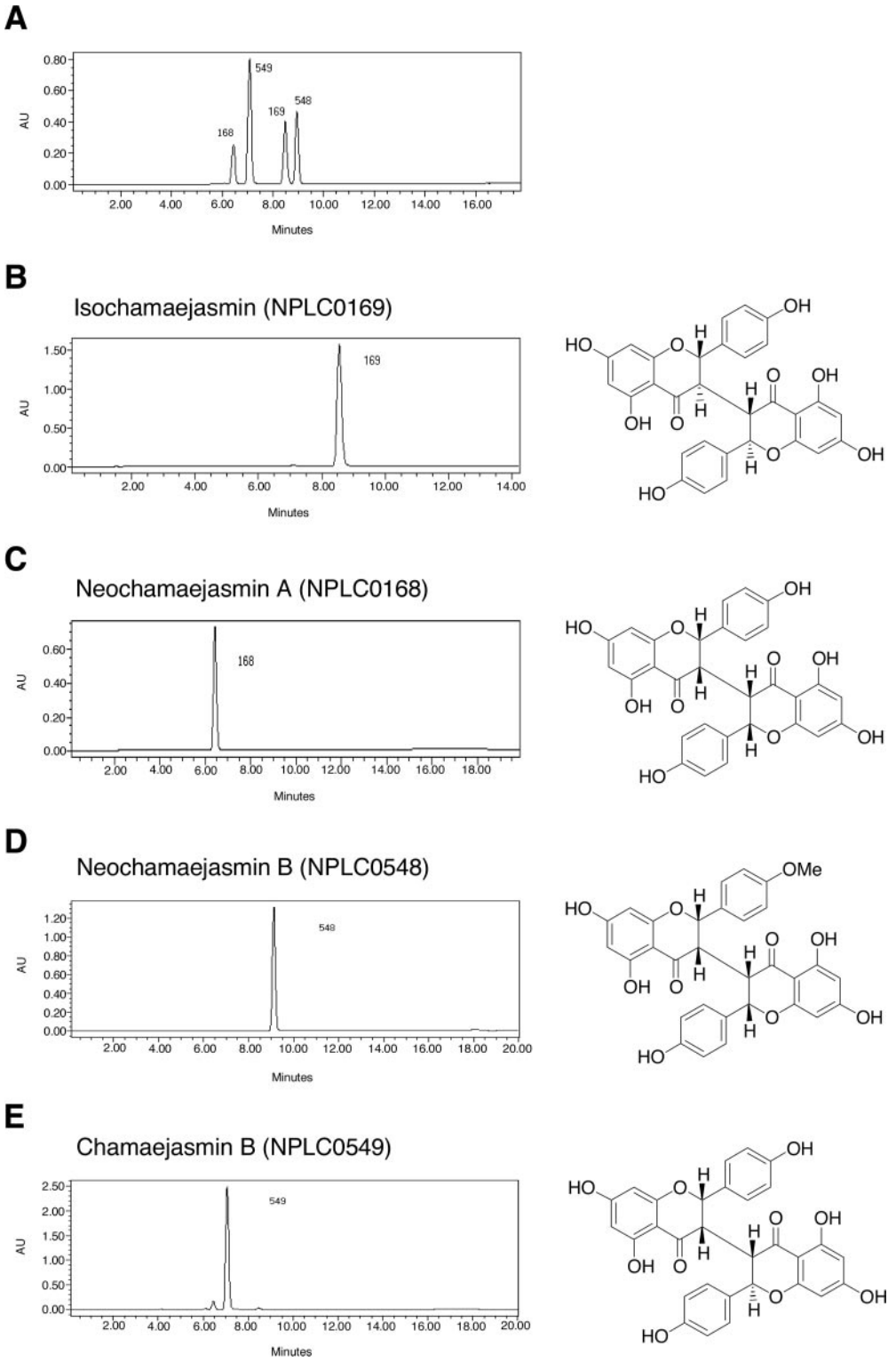

Fig. 1. Chromatograms and chemical structures of the four biflavonoids extracted from *S. chamaejasme* L. High-performance liquid chromatography chromatograms showing the fraction of *S. chamaejasme* L containing the four biflavonoids (A), and separation of isochamaejasmin (B), neochamaejasmin A (C), neochamaejasmin B (D), and chamaejasmin B (E). The chemical structures of the individual biflavonoids are shown next to the respective chromatograms. The purity of each biflavonoid is given in Table 1.

were visualized using enhanced chemiluminescence detection (Pierce, Rockford, IL) according to the manufacturer's instructions.

Calcium Mobilization Assay. RBL-FPRL1 cells were harvested with the enzyme-free cell dissociation buffer (Invitrogen, Carlsbad, CA). The cells (5×10^5) were incubated with 4 μM Fluo-3/acetoxymethyl ester in Hanks' buffered saline solution/bovine serum albumin at 37°C for 1 h, and examined for calcium mobilization in response to the compounds using a PTI Spectrofluorometer (Photon Technology International, Lawrenceville, NJ) with excitation wavelength at 488 nm and emission wavelength at 525 nm.

Results

Isochamaejasmin Induces NF-κB Activation in Transfected HeLa Cells. The human cervical carcinoma cell line HeLa has been used extensively in studies of the transcription factor NF-κB. The HeLa/NF-κB/FPRL1 cell line, which contains a 5× NF-κB binding sequence and expresses the human FPRL1, was initially generated for screening of small molecular weight ligands for FPRL1 (Nanamori et al., 2004). Isochamaejasmin (Fig. 1) was identified as one of the hits that stimulated NF-κB activation in this screening assay and was further analyzed in the current study. Quantitative analysis demonstrated that stimulation of HeLa/NF-κB/FPRL1 with isochamaejasmin resulted in a dose-dependent increase in NF-κB-directed luciferase reporter expression that peaked at 10 to 15 μM with an EC_{50} of 3.23 μM (Fig. 2). At higher concentrations of isochamaejasmin ($\geq$10 μM), there was a progressive decrease in the NF-κB luciferase activities, probably because of its cytotoxicity (Lin and Zhu, 1992).

Isochamaejasmin Stereospecifically Induces NF-κB Activation. We examined the two isochamaejasmin stereoisoforms, neochamaejasmin A and chamaejasmin B, as well as a methyl derivative of neochamaejasmin A (Fig. 1 and Table 1), for their abilities to stimulate NF-κB activation in HeLa/NF-κB/FPRL1 cells. It is noteworthy that none of these compounds was able to induce NF-κB luciferase reporter at concentrations up to 100 μM (Fig. 2 and data not shown). Given the purity of the compounds used in this study (Table 1), this result suggests that the isochamaejasmin-induced NF-κB activation is a stereospecific effect not shared by the isomers and the structurally similar derivative.

The Effect of Isochamaejasmin Is Independent of FPRL1. Because the cell line used in the initial study expresses FPRL1, it was necessary to determine whether isochamaejasmin is an agonist of this chemoattractant receptor (Su et al., 1999). HeLa cells were transfected to express the NF-κB luciferase reporter with or without FPRL1. The cells were stimulated with isochamaejasmin (1 μM), and induced NF-κB luciferase activity over a 5-h period was determined. As shown in Fig. 3A, the presence of FPRL1 had no effect on the induced NF-κB luciferase reporter activity. Independent experiments were conducted with an RBL-2H3 cell line expressing FPRL1 (RBL-FPRL1), which functionally responds to FPRL1 agonists (Nanamori et al., 2004). Isochamaejasmin was unable to stimulate calcium mobilization in the RBL-FPRL1 cell line (Fig. 3B), a response typically induced by FPRL1 agonists such as WKYMVm. Likewise, isochamaejasmin failed to induce chemotaxis at concentrations up to 100 μM; in contrast, the control peptide (WKYMVm) induced a 13.4-fold increase in cell migration with a typical bell-shaped chemotaxis dose curve (Fig. 3C). Based on these results, we concluded that isochamaejasmin induces NF-κB activation independently of FPRL1.

Isochamaejasmin-Stimulated NF-κB Activation Involves IKKβ and Requires Degradation of IκBα. The NF-κB activation pathway has been well established through studies in recent years. The majority of NF-κB stimuli induce activation of IKKβ, whereas others use an alternative pathway involving IKKα. Phosphorylation of IκBα by IKKβ causes subsequent ubiquitination and degradation of this inhibitory subunit, leading to nuclear translocation of the NF-κB proteins (Karin et al., 2004). We examined whether isochamaejasmin could induce nuclear translocation of the NF-κB protein p65/RelA. As shown in Fig. 4A, isochamaejasmin (169), but not buffer (Ctl) or neochamaejasmin A (168), caused enrichment of p65/RelA in the nucleus, suggesting nuclear translocation of this NF-κB protein. We next examined the potential involvement of IKKs and IκBα in isochamaejasmin-induced NF-κB activation by cotransfection of dominant-negative constructs of IKKα and IKKβ (Lys44→Met44 mutation) (Mercurio et al., 1997). Expression of the IKKβ dominant-negative construct, but not that of IKKα, resulted in a significant ($p \leq 0.01$) reduction of the

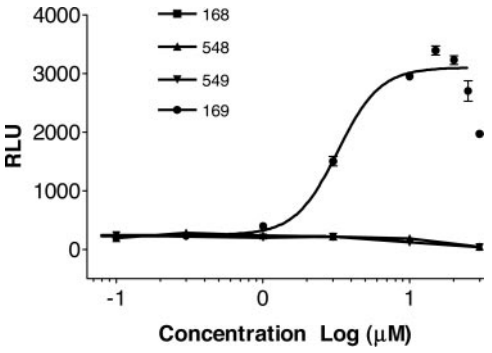

Fig. 2. Induction of NF-κB luciferase reporter expression by isochamaejasmin. HeLa/NF-κB/FPRL1 cells (1.5×10^4 per well) were stimulated with either isochamaejasmin (169) or one of the other biflavonoids tested, at different concentrations. Five hours after stimulation, the produced luciferase activity was assayed as described under *Materials and Methods*. Data shown are means ± S.E.M. of relative luminescence units (RLU) based on triplicate measurements from one of the representative experiments ($n = 3$).

TABLE 1
Stereoconfiguration and bioactivity of the four biflavonoids isolated from *S. chamaejasme* L.

Compound	Configuration at				Purity (HPLC)	NF-κB Activation
	C2	C3	C2"	C3"		
Isochamaejasmin (169)	S	R	R	S	98.67%	+
Neochamaejasmin A (168)	S	S	S	S	99.83%	−
Neochamaejasmin B (548)	S	S	S	S	100.00%	−
Chamaejasmin B (549)	S	S	R	S	90.72%	−

HPLC, high-performance liquid chromatography; +, activation; −, no activation.

isochamaejasmin-induced NF-κB luciferase reporter activity (Fig. 4B). Likewise, expression of a dominant-negative IκBα with mutated serines at positions 32 and 36 (S32/36A) (Van Antwerp et al., 1996) completely blocked the isochamaejasmin-induced NF-κB activation in luciferase reporter assays. Taken together, these results indicate that isochamaejasmin stimulates the canonical NF-κB activation pathway.

Potential Involvement of Selected Protein Kinases in Isochamaejasmin-Stimulated NF-κB Activation. To identify the signaling pathways leading to isochamaejasmin-induced NF-κB activation, we used pharmacological inhibitors for selected protein kinases in addition to the IKKs. Three different PKC inhibitors were used: Gö6976, an inhibitor for conventional PKC; GF109203X, an inhibitor for both conventional and novel PKCs; and rottlerin, an inhibitor that selectively blocks the novel PKC isoform, PKCδ. Although all three inhibitors produced statistically significant ($p \leq 0.01$) inhibition of the isochamaejasmin-stimulated NF-κB luciferase reporter activity, the effects of GF109203X and rottlerin were more prominent than those of Gö6976 (Fig. 5A). To extend this finding, we examined isochamaejasmin-stimulated PKCδ activation using an antibody against phosphorylated PKCδ (Fig. 5B). Isochamaejasmin (1 μM) induced a time-dependent phosphorylation that symbolizes activation of the kinase (Stahelin et al., 2004). Together, these results indicate that isochamaejasmin stimulates both the conventional PKCs and PKCδ, which are involved in the induced NF-κB activation.

We next examined several other protein kinases that have been implicated as upstream stimuli of NF-κB activation. Using anti-phosphospecific antibodies, we found that isochamaejasmin could induce phosphorylation of ERK1/2 (p44 and p42 mitogen-activated protein kinase; Fig. 6A) and p38 (Fig. 6B). The phosphorylation of these kinases increased with time up to 20 min, suggesting persistent activation by isochamaejasmin. The roles played by these kinases in isochamaejasmin-induced NF-κB activation were evidenced by a partial blockade of the induction of NF-κB luciferase reporter in cells pretreated with the p38 inhibitor SB203580 or with the mitogen-activated protein kinase kinase inhibitor U0126. When the cells were treated with both SB203580 and U0126 before isochamaejasmin stimulation, a more pronounced inhibitory effect was observed (Fig. 6C), suggesting that the two kinase pathways work together in the isochamaejasmin-induced response.

Comparison of the Different Compounds for Cytotoxicity. Because *S. chamaejasme* L. is known for its cytotoxicity at higher concentrations (Zhang et al., 2000), we examined whether the four compounds could all exhibit this property. It is noteworthy that at low micromolar concentrations, all compounds exhibited a stimulatory effect in cell proliferation. At concentrations above 25 μM, there was a

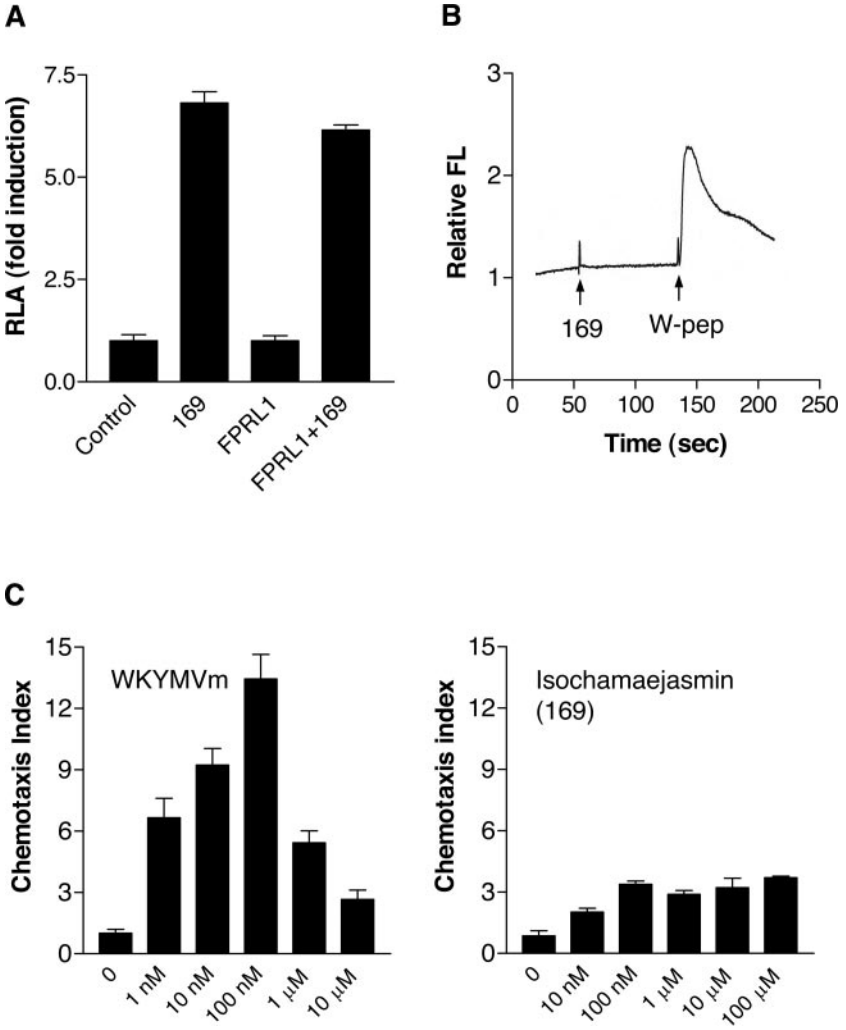

Fig. 3. Isochamaejasmin induces NF-κB activation independently of FPRL1. A, HeLa cells (without FPRL1) were transiently transfected with vector (pcDNA3.1, control) or FPRL1 in pcDNA3.1, together with the 5×NF-κB luciferase reporter plasmid and a constitutive β-galactosidase expression construct. Forty-three hours after transfection, the cells were stimulated with or without isochamaejasmin (169; 1 μM) for 5 h. The expressed luciferase activity was normalized against the activity of β-galactosidase and shown as relative luciferase activity (RLA; means± S.D. based on duplicate measurements; $n = 3$). Functional expression of FPRL1 was confirmed by its response to WKYMVm in NF-κB luciferase reporter assay (data not shown). B, calcium mobilization in RBL-FPRL1 cells in response to isochamaejasmin (169; 1 μM), and to WKYMVm (W-pep, 100 nM), which was used as a positive control. C, chemotaxis of RBL-FPRL1 in response to WKYMVm (left) and isochamaejasmin (169; right) at various concentrations. Data shown are means ± S.E.M. from quadruplicate measurements.

dramatic increase in cytotoxicity for all compounds except isochamaejasmin, which is much less toxic for the HeLa cell line (Fig. 7). At 100 μM, all compounds except isochamaejasmin reduced cell viability to less than 25%. In isochamaejasmin-treated samples, however, ~90% of the cells were still viable. This finding provides additional evidence that isochamaejasmin differs from the other structurally similar compounds. To determine whether the reduced cytotoxicity of isochamaejasmin was related to its ability to activate NF-κB, a rescue study was conducted using cells treated with cycloheximide. As shown in Fig. 8A, the cycloheximide-induced apoptosis was partially reversed by isochamaejasmin in concentrations ranging from 10 to 40 μM. Although the reversal effect was small (an increase of cell viability from 42 to 48%), the difference was statistically significant. We further determined whether this effect was related to NF-κB activation by using a cell-permeable peptide that inhibits NF-κB. Our results showed that the NF-κB-inhibitory peptide (SN50), but not the control peptide (SN50M), ablated the reversal effect of isochamaejasmin (Fig. 8B).

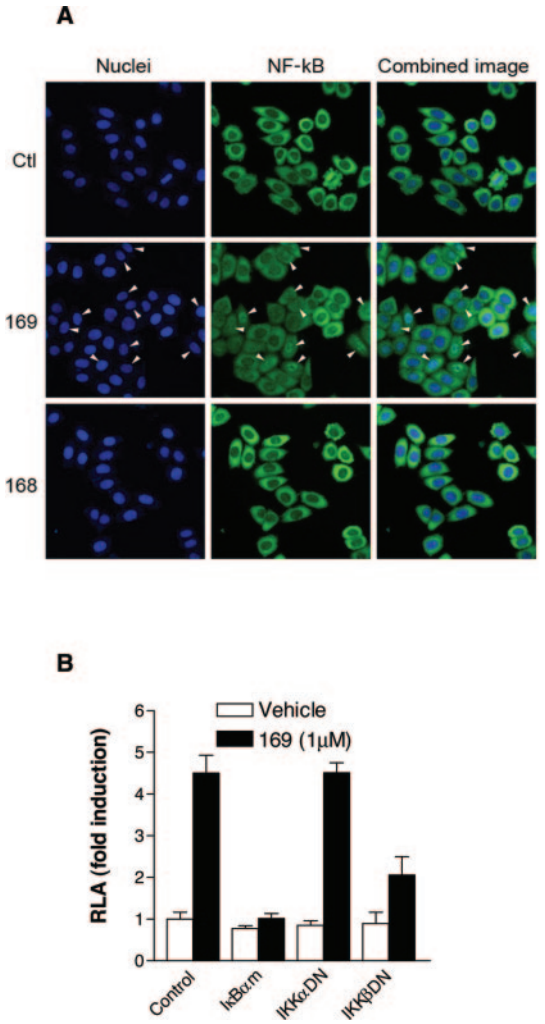

Fig. 4. Nuclear translocation of NF-κB and involvement of IκBα. A, HeLa cells were stimulated with vehicle (buffer with 1% DMSO; Ctl, control; top), isochamaejasmin (169; 20 μM; middle), or neochamaejasmin A (168; 20 μM; bottom). After 40 min, the cells were fixed and stained with an antibody against p65/RelA (see *Materials and Methods*) for visualization of NF-κB translocation (middle, arrows). The left column was stained with Hoechst for nuclei, the center column represents NF-κB staining, and the right column is combined images of both. Approximately 35% of the cells in isochamaejasmin (169)-stimulated samples showed nuclear staining of the NF-κB protein compared with less than 10% in the unstimulated or neochamaejasmin A (168)-stimulated samples. B, the isochamaejasmin-induced NF-κB activation involves IκBα. HeLa cells were transiently transfected with the 5×NF-κB luciferase reporter construct together with one of the following: a pcDNA3.1 vector (control), a dominant-negative (DN) construct of IκBα (IκBαm), a dominant-negative IKKα construct, and a dominant-negative IKKβ construct. Forty-three hours after transfection, the cells were stimulated with isochamaejasmin (169, 1 μM) or buffer (vehicle) for 5 h. Measurement of the luciferase activity and normalization were made as described in the legend for Fig. 3A. RLA, relative luciferase activity (as defined in *Materials and Methods*). A representative set of data from three experiments was shown as mean ± S.D., based on duplicate measurements.

Discussion

The therapeutic values of herbal medicine have drawn increasing attention in recent years. However, the widespread use of herbal medicine is often hampered by a lack of understanding of the effective ingredients. Although clinical efficacy of *S. chamaejasme* L. has been long documented, it was not until the early 1980s that major chemistry efforts were made to isolate bioactive constituents. Apart from pharmacological analyses of the water or methanol extracts in vitro and in vivo, potential molecular targets for diterpenes purified from *S. chamaejasme* L. were identified near the end of the last century. For instance, daphnetin, a coumarin derivative, was implicated as an inhibitor of several protein kinases, including EGF receptor (Yang et al., 1999), and

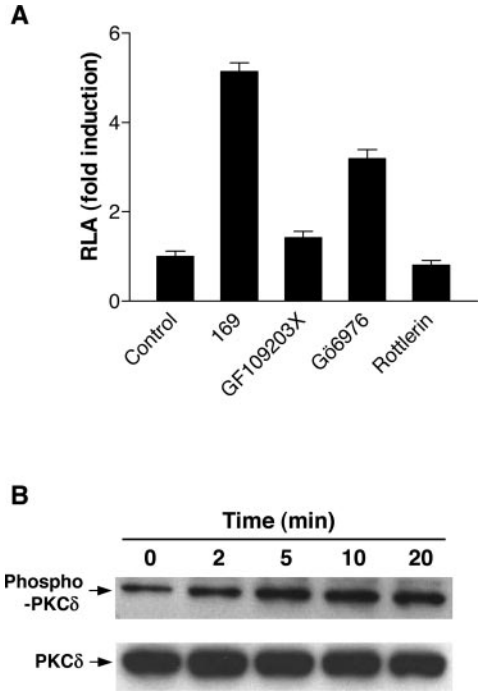

Fig. 5. A role of PKC in isochamaejasmin-induced NF-κB activation. A, HeLa cells were transiently transfected with the 5×NF-κB luciferase reporter as described above. After 43 h, the cells were treated with buffer (control) or with isochamaejasmin (169, 1 μM). Some samples were pretreated for 10 min with the PKC inhibitors GF109203X (0.1 μM), Gö6976 (0.1 μM), or rottlerin (20 μM) as indicated. The normalized luciferase reporter activities were shown as mean ± S.D. based on two independent experiments, each with duplicate samples. B, phosphorylation of PKCδ as detected in total cell lysate prepared from isochamaejasmin (1 μM)-stimulated HeLa cells. The samples were blotted with an anti-phospho-PKCδ antibody recognizing the phosphorylated Thr505. Loading control was shown in the lower panel with an antibody against total PKCδ.

gnidimacrin was shown to suppress cancer cell proliferation via an inhibition of cdk2 mediated by protein kinase C (particularly βII subtype) (Yoshida et al., 1996, 1998). However, information pertaining to the biological activities of biflavonoids from *S. chamaejasme* L. is very limited. In this study, we compared four of these biflavonoids for their cellular effects and found that these structurally similar compounds have different pharmacological properties.

NF-κB proteins are nuclear transcription factors that regulate different physiological actions. Malfunction in related signal transduction pathways may result in inflammatory and autoimmune diseases, as well as different types of cancer (Caramori et al., 2004; Karin et al., 2004). To search for small molecules that may modulate this pivotal mechanism, an assay system was developed for our initial assessment of the functional properties of isochamaejasmin and the three analogs. The HeLa cell-based NF-κB luciferase reporter system is highly sensitive in detecting chemical signals that stimulate pathways leading to NF-κB activation. The same approach was successfully employed in the recent identification of a novel nonpeptide FPRL1 agonist (Nanamori et al., 2004). In this study, we showed that isochamaejasmin was able to stimulate NF-κB activation when used at high nanomolar to low micromolar concentrations. This is a previously unreported property of compounds isolated from the root of *S. chamaejasme* L. The finding may partially explain the immunomodulatory functions of this plant (Zhang et al., 2000), but the linkage between the reported antitumor and bactericidal effects and isochamaejasmin remains to be further investigated.

It is known through clinical practices and laboratory research that *S. chamaejasme* L. is toxic and has to be handled with care (Lin and Zhu, 1992). The present study confirmed this historical experience and found that isochamaejasmin, along with neochamaejasmin A, neochamaejasmin B, and chamaejasmin B, are cytotoxic when used at high micromolar concentrations. However, isochamaejasmin was much less toxic than the other three biflavonoids, and it could partially rescue apoptosis of cells treated with cycloheximide (Fig. 8A). We speculated that the reduced cytotoxicity and the anti-apoptotic effect of isochamaejasmin might be caused by its ability to stimulate NF-κB, because NF-κB is generally considered antiapoptotic (Van Antwerp et al., 1996; Sonenshein, 1997; Karin and Yinon, 2000). Indeed, treatment of a cell-permeable NF-κB inhibitory peptide abolished the antiapoptotic effect of isochamaejasmin (Fig. 8B). Therefore, isochamaejasmin joins a list of anticancer compounds that lower their cytotoxic potential through induction of NF-κB (Arlt and Schafer, 2002). In other clinical applications, isochamaejasmin has been shown to have antimicrobial functions. The reduced cytotoxicity offers a clinical advantage and allows for the use of higher doses in treating relevant conditions. Although NF-κB activation is critical to the induced expression of numerous pro-inflammatory cytokines and to cell survival, this transcription factor also plays dual

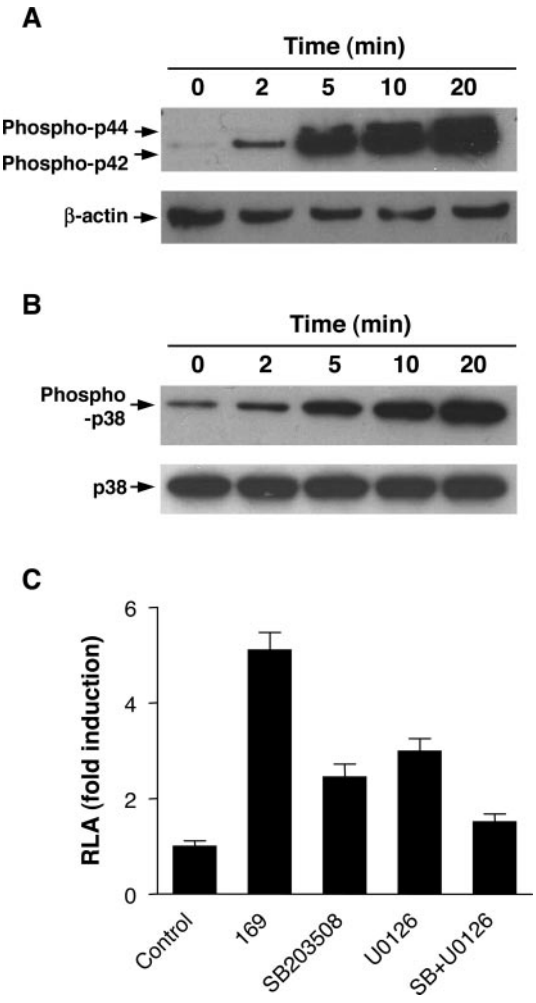

Fig. 6. Isochamaejasmin-induced activation of ERK1/2 and p38 and their involvement in the induction of NF-κB activity. HeLa cells were stimulated with isochamaejasmin (1 μM) for the indicated time. Cell lysate was prepared, electrophoresed, transferred to membrane, and blotted with an anti-phospho-ERK1/2 antibody (A) or anti-phospho-p38 antibody (B). These experiments were repeated at least three times, and one representative blot from each is presented. C, transiently transfected HeLa cells expressing the NF-κB luciferase reporter were pretreated with the p38 inhibitor SB203580 (10 μM), the mitogen-activated protein kinase kinase inhibitor U0126 (5 μM), or both for 10 min. Buffer treatment served as control (first two samples). The cells were then stimulated with isochamaejasmin (169, 1 μM) or buffer (control) for 5 h. The expressed luciferase reporter activity was normalized against β-galactosidase activity (relative luciferase activity, RLA) and shown as means ± S.D. from duplicate samples, based on two separate experiments.

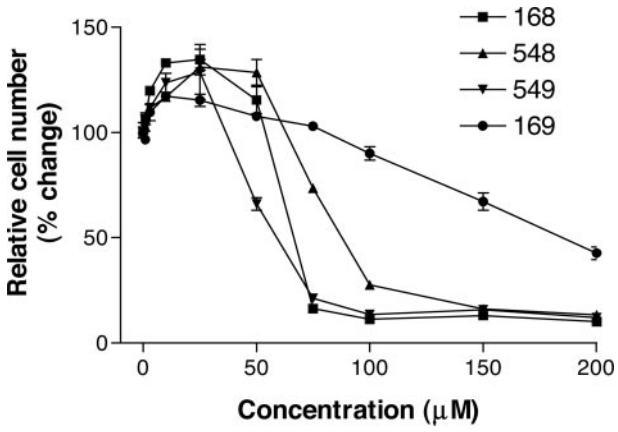

Fig. 7. Comparison of the cytotoxic effects of isochamaejasmin and other biflavonoids. HeLa cells (3×10^3 per well in 96-well plate) were cultured in the presence of serial dilutions of the biflavonoids for 48 h at 37°C. MTT was added for 2 h and formazan was extracted with DMSO for absorbance measurement at 560 nm. The cell viability is shown as percentage change in cell numbers (means ± S.E.M. from triplicate samples), based on two separate experiments.

roles in the regulation of the pathological processes of inflammation and cancer development. Factors such as the stages of tumor progression (early versus late) and inflammation (acute versus chronic) can affect the outcome of treatment (Perkins, 2004; Philip et al., 2004). These mechanisms may also be applicable to the mode of action of isochamaejasmin.

Our experimental data indicate that isochamaejasmin does not require FPRL1 for its NF-κB-stimulating effect, although the cell line used for the initial screening contains this receptor. Based on its structure, isochamaejasmin is probably cell-permeable and able to directly activate signaling molecules inside the cell. However, it cannot be ruled out that isochamaejasmin exerts its effect through a cell surface receptor. Based on the finding that isochamaejasmin, but not the three structurally similar compounds, was able to induce NF-κB activation, it is speculated that the binding mechanism for this biflavonoid is highly specific. High-performance liquid chromatograms show that the individual compounds are more than 98.5% pure except for chamaejasmin B, which has a purity of 90.72% but did not elicit NF-κB activation in our functional assays. Moreover, the action of isochamaejasmin was not abrogated by the other three biflavonoids introduced to the assay system at micromolar concentrations (data not shown). Thus, the observed biological activity of isochamaejasmin is specific and unlikely to have been caused by contaminants from the other biflavonoids in the preparation. We also have shown that multiple signaling pathways, including the PKC and mitogen-activated protein kinase pathways, are activated by isochamaejasmin. Isochamaejasmin apparently activates more than one type of PKCs, in that its biological activity is sensitive to multiple PKC inhibitors. Although there is considerable overlap in the inhibitory spectrum of these pharmacological agents, the combined use of the three inhibitors provides a preliminary assessment of the PKC isoforms that may be involved in its functions. It is unclear whether isochamaejasmin can directly interact with these kinases or activate them through an upstream signaling molecule. However, these signaling mechanisms seem to work together as suggested by the additional inhibition of NF-κB activation when both SB203580 and U0126 were applied together. There remains the possibility that isochamaejasmin binds to more than one target protein to elicit its biological functions.

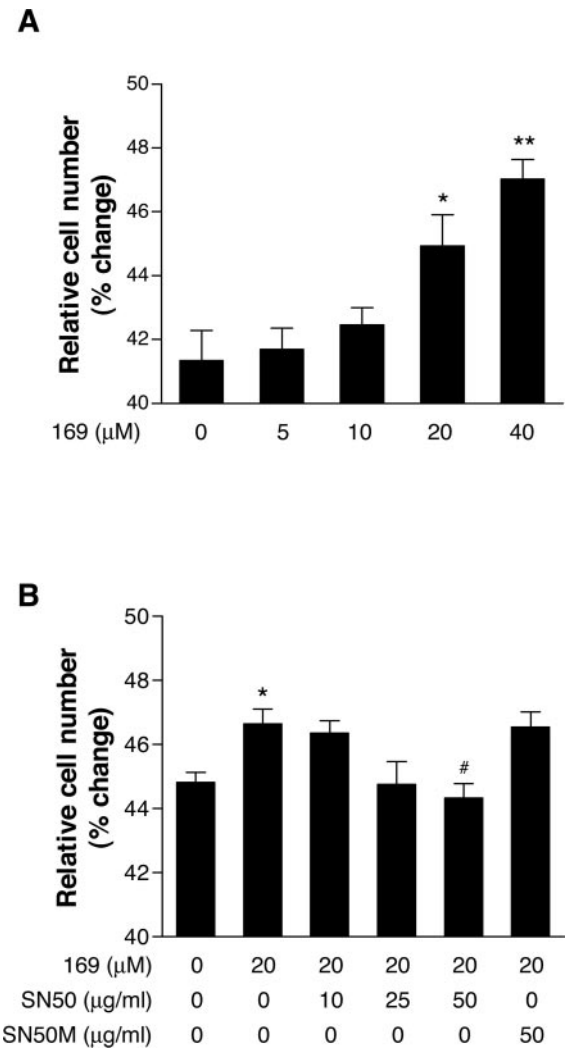

Fig. 8. Rescue of cycloheximide-induced apoptosis by isochamaejasmin and its association with NF-κB activation. HeLa cells (3×10^3 per well in 96-well plate) were cultured in the presence of cycloheximide (CHX, 5 μg/ml) alone, or cycloheximide plus various concentrations of isochamaejasmin, for 24 h at 37°C (A). A peptide inhibitor of NF-κB (SN50) or the negative control peptide (SN50M) was added 30 min before the above reagents (B). MTT assay was performed thereafter. The cell viability is shown as percentage change in cell numbers (means ± S.E.M. from triplicate samples), based on three separate experiments. *, $p < 0.05$; **, $p < 0.01$ compared with cycloheximide-treated panel; #, $p < 0.05$ compared with the cycloheximide plus 169-treated panel (Student's t test).

In summary, the present study represents a first step in the study of *S. chamaejasme* L.-derived biflavonoids for their potential cellular signaling mechanisms. Our finding that isochamaejasmin could induce NF-κB activation suggests that this compound is a unique ingredient of the plant. The observation that isochamaejasmin, but not other isomers, has this functionality indicates that cells have highly selective mechanisms in the interaction with and response to constituents from natural sources. A better understanding of the structural basis of isochamaejasmin action is expected to contribute to the development of novel therapeutics based on this class of natural compounds.

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Address correspondence to: Ming-Wei Wang, the National Center for Drug Screening, 189 Guo Shou Jing Road, Shanghai 201203, China. E-mail: center@mail.shcnc.ac.cn