In Vitro Antiinflammatory Effects of Neolignan Woorenosides from the Rhizomes of *Coptis japonica*

Jae Youl Cho,*[,†,‡] Kyong Up Baik,[†] Eun Sook Yoo,[†] Kazuko Yoshikawa,[§] and Myung Hwan Park[†]

Department of Immunopharmacology, R & D Center, Daewoong Pharmaceutical Co. Ltd., Sungnam 462-120, Korea, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan, and Department of Immunology, Windeyer Institute of Medical Sciences, University College London Medical School, London W1P 6DB, United Kingdom

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Five dihydrobenzofuran neolignans, woorenosides I (**1**), II (**2**), III (**3**), IV (**4**), and V (**5**), isolated from *Coptis japonica* (Ranunculaceae), suppressed tumor necrosis factor (TNF)-α and nitric oxide (NO) production, as well as lymphocyte proliferation triggered by inflammatory signals such as various mitogens, in a dose-dependent manner. The results indicate that the woorenosides strongly inhibit the mitogenic response by activated macrophage and lymphocytes and suggest that these compounds may participate in regulating inflammatory processes.

Coptis japonica Makino (Ranunculaceae) has long been prescribed in traditional medicines for the treatment of inflammation-related diseases such as ulcerative stomatitis.[1] According to previous phytochemical studies, rhizomes of *C. japonica* are composed mainly of alkaloids, including berberine, magnofiorine, sanguinarine, norsanguinarine, oxysanguinarine, and 6-acetonyl-5,6-dihydrosanguinarine, and phenolic compounds from the polar layer.[2,3] It was demonstrated that some of the rhizome's bioactive principles possess antiinflammatory and analgesic effects.[3,4] The underlying mechanisms accounting for their antiinflammatory activities, however, remained unelucidated. Several novel neolignans and known lignans of *C. japonica* were reported to be widely distributed in the nonalkaloid fraction from the total ethanol extract of *C. japonica*,[5] although the biological activities of the neolignans and lignans have been examined only in a preliminary study of effects on tumor necrosis factor-α (TNF-α) production.[6]

It is generally accepted that activated macrophages and T lymphocytes (CD4+ and CD8+ cells) play a central role in chronic inflammatory processes.[7−9] Increasing evidence suggests not only that excessive TNF-α and nitric oxide (NO) released from activated macrophages and neutrophils are involved, but also that the proliferation and activation of T lymphocytes are related to maintaining and controlling disease conditions.[7−10,11] This led us to suppose that TNF-α and NO production and proliferation and activation of T lymphocytes may reflect the degree of inflammation in various conditions, and they provide a measurable parameter by which the effects of drugs on the inflammatory processes can be assessed. Of the many triggers that increase inflammatory processes, various mitogens such as lipopolysaccharide (LPS) and concanavalin A (Con A), as well as cytokines such as interferon-γ (IFN-γ) are known as inflammatory activators of macrophages and lymphocytes,[12,13] and therefore, generally used as in vitro and in vivo models to identify antiinflammatory agents.[14−17]

Previous in vitro and in vivo studies in our laboratory indicated that the woorenosides, or the ethanolic fraction from *C. japonica*, could inhibit LPS-induced TNF-α release in a murine macrophage cell line (RAW264.7 cells), as well as serum TNF-α production in LPS-injected mice.[6,18] In the present study, woorenosides were examined for antiinflammatory properties by studying their effects in several in vitro assays. The resultant data demonstrate that these lignans inhibit either TNF-α or NO production, as well as lymphocyte proliferation, suggesting that these compounds may participate in the reported antiinflammatory activity of *C. japonica*.

Results and Discussion

The ability of LPS, IFN-γ, and Con A to stimulate TNF-α and NO production in RAW264.7 cells and T cell proliferation has been well documented.[19−23] Once we established these in vitro assays in our laboratory, we could then measure the ability of various standard components to inhibit TNF-α and NO production and T cell proliferation in vitro. The known inhibitors, pentoxifylline, dibutyryl cyclic AMP (dbcAMP), and prednisolone were shown to significantly suppress TNF-α release in a dose-dependent manner, with IC_{50} values of 247, 28.9, and 25.4 μM, respectively (Table 1). In the NO assay, nitro-L-arginine methyl ester (L-NAME) inhibited NO release in LPS- and IFN-γ-stimulated RAW264.7 cells, with IC_{50} values of 193.3 and 116.2 μM, respectively. An active metabolite of leflunomide, A77,1726 and an inhibitor of sphingolipid biosynthesis, L-cycloserine, suppressed Con A-stimulated lymphocyte proliferation, with IC_{50} values of 6.7 μM and 3.5 μM, respectively. CTLL-2 cell proliferation was attenuated in a dose-dependent manner by A77,1726 (IC_{50} = 11.2 μM) and dbcAMP (IC_{50} = 576 μM) (Table 1). The inhibitory effects of all these standard compounds were similar to the previous reports, indicating that the established model could be used for evaluation of in vitro antiinflammatory effects by woorenosides.[19−23]

Five kinds of woorenoside compounds possessing methoxy groups at C-3′ of the A ring and at C-7 of the C ring, but differing in their oxygenation patterns at other carbon positions of the A, B, and C rings (Figure 1), have been reported from *C. japonica*, although their biological activities have not yet been fully elucidated. However, based on a preliminary study in which these compounds significantly inhibited TNF-α production from mouse macrophage at 12.5 μg/mL,[6] we further evaluated in vitro biological activities related to their antiinflammatory effects. All of

* To whom correspondence should be addressed. Tel.: 44-207-679-9337. Fax: 44-207-679-9357. E-mail: jae.cho@ucl.ac.uk.
[†] Daewoong Pharmaceutical Co. Ltd..
[‡] University College London Medical School.
[§] Tokushima Bunri University.

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Table 1. Concentration of Woorenosides and Standard Compounds Producing 50% Inhibition (IC_{50}) of TNF-α and NO Production and Lymphocyte Proliferation

compound	IC_{50} values (μM)[a]				
	TNF-α	NO	splenocytes	CTLL-2	Sup-T1
pentoxifylline	247 ± 23.4				
dbcAMP	28.9 ± 5.7			576 ± 14.7	
prednisolone	25.4 ± 4.1				
L-NAME		193 ± 23.2			
A77,1726			6.7 ± 0.9	11.2 ± 1.2	
L-cycloserine			3.5 ± 0.7		
1	15.4 ± 16.9[b]	34.7 ± 1.3[b]	24.8 ± 1.6[b]	−5.4 ± 5.9[b]	32.1 ± 1.8[b]
2	75.2 ± 5.4	23.7 ± 0.7	39.7 ± 1.9	43.2 ± 0.4	42.6 ± 1.7[b]
3	56.7 ± 1.7	15.7 ± 0.3	22.9 ± 1.8	16.8 ± 0.6	67.7 ± 0.6
4	46.6 ± 0.9	14.4 ± 0.4	24.0 ± 3.7	20.2 ± 0.3	64.8 ± 0.6
5	59.8 ± 4.7	25.2 ± 0.4	19.1 ± 1.2	19.9 ± 0.3	77.9 ± 1.2

[a] Represents mean ± SEM were obtained from four separate experiments. [b] Indicates % of inhibition at 80 μM.

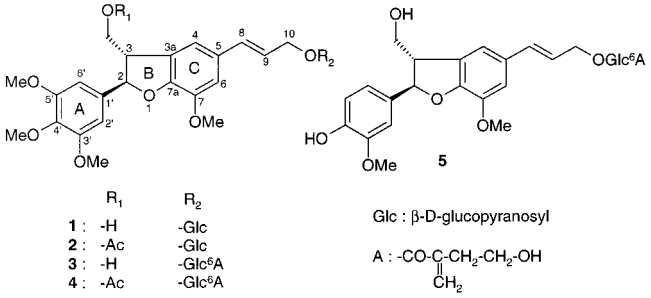

Figure 1. The chemical structures of woorenosides I (**1**), II (**2**), III (**3**), IV (**4**), and V (**5**).

the compounds tested significantly inhibited TNF-α production from RAW264.7 cells stimulated by LPS, with IC_{50} values ranging from 45 to 75 μM (Table 1 and Figure 2A, left panel). In addition to TNF-α production, we also determined whether the compounds inhibit NO production, which is another possible pathophysiological mediator produced from activated macrophages in response to a LPS signal.[10,11] As shown in Figure 2, these compounds potently attenuated LPS-induced NO production, with IC_{50} values ranging from 14 to 25 μM. Furthermore, at 20 μM the compounds also significantly inhibited NO production induced by IFN-γ (**1**, 8.5%; **2**, 20.5%; **3**, 30.8%; **4**, 42.7%; and **5**, 29.8%), the other major triggering signal of NO, suggesting that woorenosides may block a general pathway involved in NO production, regardless of triggering signals. Using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Table 1, and Figure 2, right panels), all the compounds tested, except **1**, exhibited less inhibitory effect on TNF-α and NO production and showed no significant suppressive effect on RAW264.7 cell viability in either the TNF-α or NO assay. From these data, therefore, it could be speculated that the woorenosides may interfere with a common biosynthetic pathway for TNF-α and NO production in activated macrophage cells.

It is generally known that T lymphocytes enhance chronic inflammatory conditions via activation of inflammatory cells such as mast cells, eosinophils, neutrophils, and macrophages, resulting in massive production of chemical mediators and pro-inflammatory cytokines.[9,25,26] Indeed, potent immunosuppressive agents such as cyclosporin A are reported to have a curative effect in chronic inflammatory diseases. Thus, the ability of woorenosides to block lymphocyte proliferation was investigated. To exclude the possibility that the compounds could nonselectively inhibit lymphocyte proliferation through cytotoxicity, the inhibitory activities against mitogenic proliferation and normal proliferation were compared. In normal cell proliferation assays, Sup-T1 cells, a representative CD4+ T lymphocyte cell line,[27] were utilized. As shown in Table 1 and Figure 3, results indicate that woorenosides selectively suppress mitogenic proliferation of T lymphocytes compared to normal proliferation. The inhibitory potency of compounds **3**, **4**, and **5** in the Con A-induced splenocyte proliferation assay was similar to that of IL-2-induced CTLL-2 cell proliferation, with IC_{50} values ranging from 17 to 25 μM, whereas the activities against Sup-T1 cell proliferation were 3−4-fold less. The selective inhibition observed is similar to that reported for ginkgetin,[28] which showed a 4-fold higher suppression of ovarian adenocarcinoma cell proliferation as compared to other cell lines.[28] Moreover, this selectivity of woorenosides suggests that effects on T cell proliferation are not due to nonspecific cytotoxicity, as shown in TNF-α and NO assays.

Of the mechanisms evaluated, NO production seems to be the more significant target of the woorenosides (Figure 2 and Table 1). That is, woorenosides potently suppressed NO production, with IC_{50} values ranging from 14 to 25 μM. The order of potency of the compounds differed slightly, depending on the assay; that is, the most inhibitory compounds in the TNF-α, NO, Con-A, CTLL-2, and Sup-T1 assays were compounds **4**, **4**, **5**, **3**, and **4**, respectively. In particular, it appears that compound **4** may participate in the antiinflammatory activity of *C. japonica* through multiple actions. The inhibitory effects of **4** were comparable to or greater than those of previously isolated natural products, including ginsenosides, coumarins, and flavonoids (IC_{50} = 10−100 μM),[14−16,29,30] as well as common immunosuppressants such as leflunomide and L-cycloserine,[23,24] thalidomide,[31] tyrphostin-related tyrosine kinase inhibitors,[32] and pentoxifylline.[19] In contrast, compound **2** only slightly attenuated all these inflammatory responses. A comparison of the inhibitory activities of the lignans (Figure 2, left panels, Figure 3, and Table 1) with structural features clearly reveals that the hydrophobic functional group on the C-10 position of the C ring appears to have a pivotal role in the observed inhibitory activities, regardless of the additional groups on the A ring, acetylation of R_1 of the B ring, and glycosylation on R_2 of the C ring. However, a more complete study of the structure−activity relationships requires additional woorenosides that are not yet available.

Although the mechanism by which the woorenosides inhibit the mitogenic response for TNF-α and NO production and lymphocyte proliferation has yet to be established, these compounds may inhibit a common pathway of the mitogenic responses. At present, several important common biological pathways have been identified. For example, it is known that the transcription factor, nuclear factor-kappa

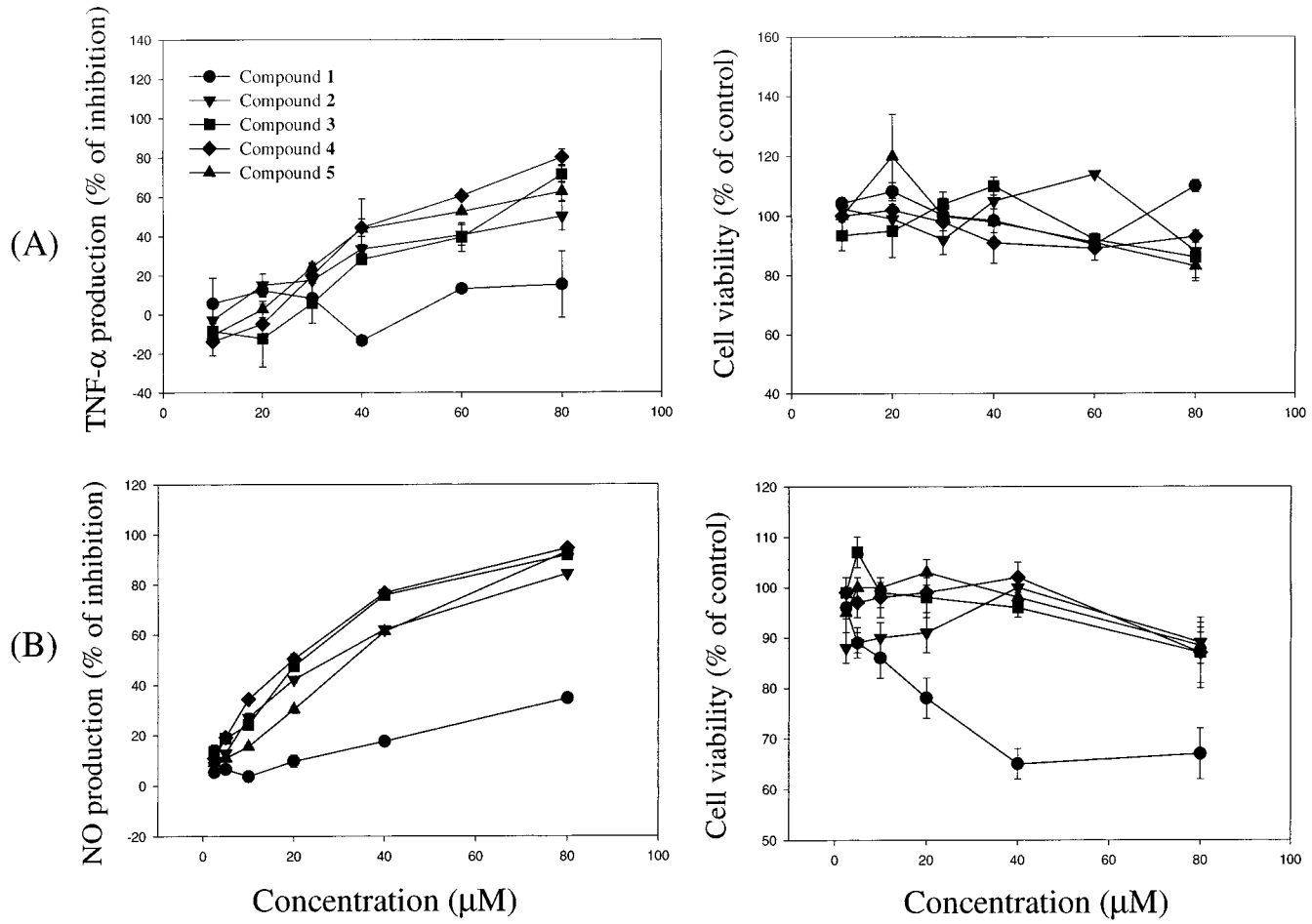

Figure 2. The inhibitory effect of woorenosides on TNF-α (A) and NO production (B) (left panel) and cell viability (right panel) in stimulated macrophages. Woorenosides were added to LPS-activated RAW264.7 (TNF-α) and LPS-activated RAW264.7 (NO) cells. Supernatants were collected after 4 h (TNF-α) and 24 h (NO), and determined TNF-α or NO level as described in Experimental Section. Data represent mean ± SEM of four separate observations.

B (NF-κB), is one of the key regulators of the immune and inflammatory responses, as it controls gene expression of cytokines and adhesion molecules as well as acute-phase proteins and immunoreceptors stimulated by various inflammatory cytokines, mitogens, and stress signals.[33] Indeed, some natural NF-κB inhibitors such as sesquiterpene lactones (SL) may exhibit multiple antiinflammatory effects.[17,34] The second common biological pathway is protein tyrosine phosphorylation, which is important in the activation or regulation of cell functions and is triggered by various activators, including mitogens (such as Con A, phytohemaglutinin, and LPS) and cytokines (such as IL-2 and IL-4) which results in induction of the expression of cyclooxygenase-2, TNF-α, and inducible NO synthase.[32,35−37] As a consequence, protein tyrosine kinase inhibitors are being developed as therapeutic agents to treat acute or chronic inflammatory diseases such as rheumatoid arthritis and septic shock.[14,36] Although woorenosides may suppress the activation of NF-κB or protein tyrosine phosphorylation, this has yet to be established. Moreover, the possibility that woorenosides may act as either antioxidants or other enzyme inhibitors cannot be excluded from the present data (Figure 2 and Table 1).

In conclusion, our observations that the dibenzofuran neolignan woorenosides exert a suppressive effect on TNF-α and NO production and lymphocyte proliferation stimulated by Con A and IL-2 suggest the possibility that the inhibition of the acute and chronic inflammatory mediators may represent an important aspect of the antiinflammatory effects of the compounds. These results also indicate that the pharmacology of woorenosides may participate in the antiinflammatory effect of *C. japonica*. However, the underlying mechanism by which woorenosides selectively inhibit mitogenic responses remains to be determined in further studies.

Experimental Section

Animals. Eight-week-old BALB/c male mice were purchased from B & K Universal (Fremont, CA). The BALB/c mice were maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied ad libitum.

Materials. Woorenosides I (**1**), II (**2**), III (**3**), IV (**4**), and V (**5**) were purified by methods described previously.[5,6] Standard woorenosides were a gift from Prof. K. Yoshikawa (Tokushima Bunri Universtiy, Japan). A77,1726 (an active metabolite of leflunomide) was synthesized in the Chemistry Department, Daewoong R & D Center (Sungnam, Korea). Sulfanilamide, dbcAMP, *N*-[1-naphthyl]-ethylenediamine dihydrochloride, L-NAME, MTT, Con A, LPS (*E. coli* 0111:B4), and phorbol 12-myristate 13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human IFN-γ and IL-2 were obtained from Boehringer Mannheim (Ottweiler, Germany). Fetal bovine serum (FBS), penicillin, streptomycin, and RPMI 1640 were obtained from GIBCO (Grand Island, NY). RAW-264.7, Sup-T1, and CTLL-2 cells were purchased from ATCC (Rockville, MD). All other chemicals were of Sigma grade.

Cell Culture. RAW264.7 and Sup-T1 cells were maintained in RPMI 1640 supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin and 10% FBS. In the case of CTLL-2 cell maintenance, the cells were cultured with the

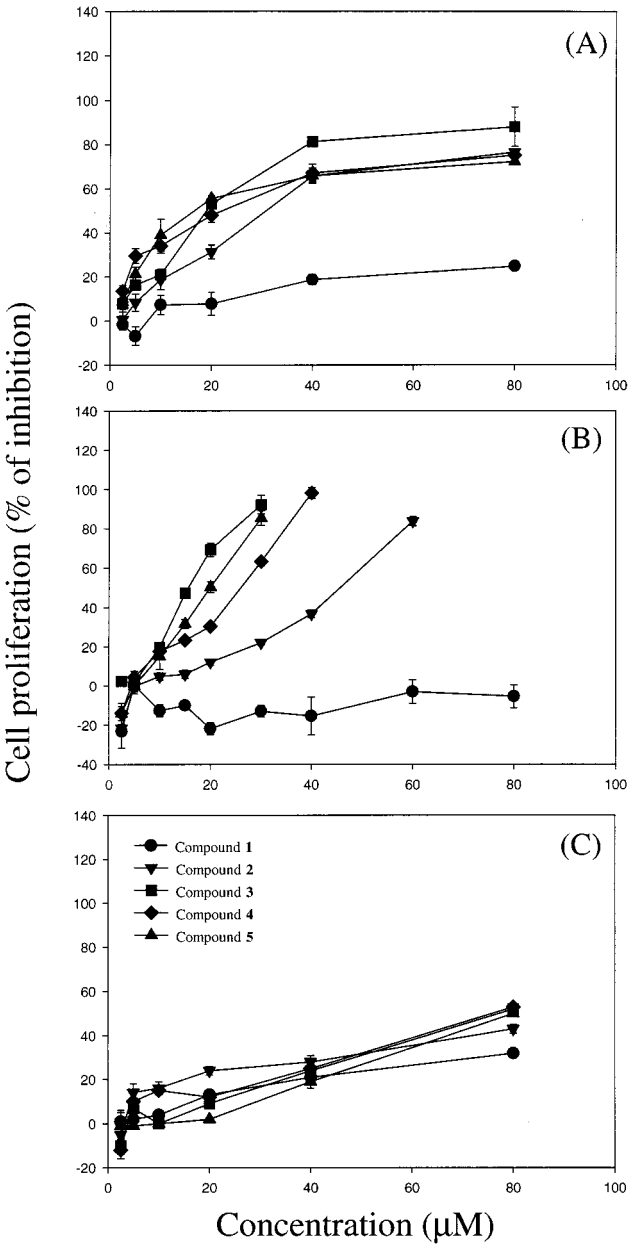

Figure 3. The inhibitory effect of woorenosides on T lymphocyte proliferation from mouse splenocytes in response to Con A (A), and CTLL-2 proliferation stimulated by IL-2 (B), and Sup-T1 cell proliferation (C). Splenocytes (5×10^6 cells/mL), CTLL-2 (5×10^5 cells/mL), and Sup-T1 (5×10^5 cells/mL) cells were incubated with various concentrations of woorenosides in the presence or absence of 1 μg/mL of Con A or 50 U/mL of IL-2 for 48 h. Cell proliferation was assayed by conventional MTT method. Data represent mean ± SEM of four separate observations.

same medium containing 25 U/mL of IL-2. Cells were grown at 37 °C and 5% CO_2 in humidified air.

Determination of TNF-α Production. The inhibitory effects of woorenosides on TNF-α production were determined as previously described.[6] Woorenosides solubilized with vehicle (89.9% propylene glycol, 10% ethanol, and 0.1% dimethyl sulfoxide) were diluted with RPMI 1640. The final concentration of vehicle never exceeded 0.1% in the culture medium. Under these conditions, none of the solubilization solvents altered TNF-α production in RAW264.7 cells. Before stimulation with LPS (1 μg/mL) and testing samples, RAW264.7 cells (2×10^6 cells/mL) were incubated under the same conditions for 18 h in 24-well plates. Stimuli and the various concentrations of testing samples were added to the wells for 4 h. Supernatants were then collected and assayed for TNF-α content using mouse TNF-α ELISA kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Determination of NO Production. RAW 264.7 cells were incubated in 96-well plates at a density of 1×10^6 cells/mL for 18 h. The various concentrations of woorenosides or positive control drug with LPS (1 μg/mL) or IFN-γ (50 U/mL) were added and then incubated for 24 h. Nitrite in culture supernatants was measured, as previously described by Ding et al.,[12] by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μL samples of medium, respectively, for 10 min at room temperature. The OD at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). The nitrite concentration (μM) was calculated from sodium nitrite standard curve. The detection limit of the assay is 0.5 μM.

Splenocyte Proliferation Assay. Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions, as described previously.[16] Briefly, splenocytes were released by teasing into RPMI 1640 medium supplemented with 20 mM HEPES buffer. After removing red blood cells using 0.83% NH_4Cl−Tris buffer (pH 7.4), splenocytes were washed three times in Ca^{2+}-Mg^{2+} free Hank's balanced salt solution and resuspended to 5×10^6 cells/mL in RPMI 1640 with 100 U/mL of penicillin and 100 μg/mL of streptomycin, and 10% FBS. Splenocytes (5×10^6 cells/mL) were cultured in flat-bottom 96-well microtiter plates (Corning Glass, Corning, NY) in the presence or absence of T lymphocyte mitogen (1 μg/mL of Con A) with woorensides and positive control drugs, in a total volume of 200 μL/well at the same conditions for 48 h.[16] The proliferation assay was performed by MTT assay (colorimetric assay).[15] At 4 h prior to culture termination, 10 μL of MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added to culture in each well and cells continuously cultured until termination. The culture was stopped by addition of 15% sodium dodecyl sulfate (SDS) into each well for solubilization of formazan. The optical density (OD) at 570 nm ($OD_{570-630}$) was measured by a microplate Spectramax 250 microplate reader.

IL-2-Dependent Cell Proliferation Assay. CTLL-2 cells were harvested from IL-2 containing growth medium and washed twice with RPMI 1640 without FBS and IL-2. They were resuspended in growth medium without IL-2 to 5×10^5 cells/mL. Of the cell suspension, 50 μL were placed into each well of a 96-well tissue culture plate and incubated in the presence of testing compounds and 50 U/mL of IL-2 for 48 h.[38] The proliferation of the cell was measure by MTT assay.[15]

Cytotoxicity Assay. Cytotoxicity of woorenosides in TNF-α and NO production assay was evaluated by MTT assay with minor modification as reported previously.[15] The cell suspension, having a concentration of 1×10^6 cells/mL, was plated in a 96-well plate. After 18 h culture, varying concentrations of test compounds and stimuli were added to each well and cultured for a further 4 h (TNF-α assay) and 24 h (NO assay). The mean value of OD content of four wells was used for calculating the viability (% of control).

Statistical Analysis. All values expressed as mean ± SEM were obtained from four separate observations performed in triplicate. Statistical analyses were carried out using Student's *t*-test for unpaired observation between control and experimental samples, with $p \leq 0.05$ considered statistically significant.

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