0026-895X/06/6903-749–758$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
*Mol Pharmacol* 69:749–758, 2006

Vol. 69, No. 3
17764/3087710
*Printed in U.S.A.*



# Anti-Inflammatory Mechanisms of Phenanthroindolizidine Alkaloids

Cheng-Wei Yang, Wei-Liang Chen, Pei-Lin Wu, Huan-Yi Tseng, and Shiow-Ju Lee

*Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taipei, Taiwan, Republic of China; and Department of Chemistry, National Cheng Kung University, Tainan, Taiwan, Republic of China*

Received August 9, 2005; accepted December 5, 2005

## ABSTRACT

The molecular mechanisms for the anti-inflammatory activity of phenanthroindolizidine alkaloids were examined in an in vitro system mimicking acute inflammation by studying the suppression of lipopolysaccharide (LPS)/interferon-$\gamma$ (IFN$\gamma$)-induced nitric oxide production in RAW264.7 cells. Two of the phenanthroindolizidine alkaloids, NSTP0G01 (tylophorine) and NSTP0G07 (ficuseptine-A), exhibited potent suppression of nitric oxide production and did not show significant cytotoxicity to the LPS/IFN$\gamma$-stimulated RAW264.7 cells, in contrast to their respective cytotoxic effects on cancer cells. Tylophorine was studied further to investigate the responsible mechanisms. It was found to inhibit the induced protein levels of tumor necrosis factor-$\alpha$, inducible nitric-oxide synthase (iNOS), and cyclooxygenase (COX)-II. It also inhibited the activation of murine iNOS and COX-II promoter activity. However, of the two common responsive elements of iNOS and COX-II promoters, nuclear factor-$\kappa$B (NF-$\kappa$B) and adaptor protein (AP)1, only AP1 activation was inhibited by tylophorine in the LPS/IFN$\gamma$-stimulated RAW264.7 cells. Further studies showed that the tylophorine enhanced the phosphorylation of Akt and thus decreased the expression and phosphorylation levels of c-Jun protein, thereby causing the subsequent inhibition of AP1 activity. Furthermore, the tylophorine was able to block mitogen-activated protein/extracellular signal-regulated kinase kinase 1 activity and its downstream signaling activation of NF-$\kappa$B and AP1. Thus, NSTP0G01 exerts its anti-inflammatory effects by inhibiting expression of the proinflammatory factors and related signaling pathways.

Phenanthroindolizidine alkaloids are a small group of compounds well known for their profound cytotoxic activity (Pettit et al., 1984; Abe et al., 1998; Staerk et al., 2000, 2002) and thus have been exploited as potential therapeutic leads for anticancer agents (Staerk et al., 2002). These alkaloids were also shown to have anti-inflammatory, antiasthmatic, and antianaphylactic properties with consequences of altered immunological status in vivo (Gopalakrishnan et al., 1979, 1980; Raina and Raina, 1980; Ganguly and Sainis, 2001; Staerk et al., 2002). Although adenyl cyclase was stimulated in asthmatic patients' peripheral leukocytes treated with tylophorine (Raina and Raina, 1980), the molecular mechanisms of actions of these phenanthroindolizidine alkaloids for aforementioned functions are not clear as yet. Moreover, the analysis and knowledge of the structure-activity relationships of the phenanthroindolizidine alkaloids with their biological function are also scarce.

Inflammation is a central feature of many pathological conditions and is mediated by a variety of soluble factors and cellular signaling events. For example, NF-$\kappa$B-dependent gene expression plays an important role in inflammatory responses and increases the expression of genes encoding cytokines and receptors involved in proinflammatory enzymes such as iNOS and COX-II (Giuliani et al., 2001). In addition, AP1, another early transcriptional factor, is also involved in proinflammatory response either alone or by coupling with NF-$\kappa$B (Adcock, 1997; Giuliani et al., 2001). Improper up-regulation of iNOS and/or COX-II have been associated with pathophysiology of certain types of cancers as

This work was supported by grants BP093-PP04 and BP094-PP04 from National Health Research Institutes, Taiwan, Republic of China.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.017764.

**ABBREVIATIONS:** NF-$\kappa$B, nuclear factor-$\kappa$B; iNOS, inducible nitric-oxide synthase; COX, cyclooxygenase; AP, activator protein; TNF$\alpha$, tumor necrosis factor-$\alpha$; LC, liquid chromatography; 15d-PGJ$_2$, 15-deoxy-12,14-prostaglandin J$_2$; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; PDTC, pyrrolidine dithiocarbamate; MEKK, mitogen-activated protein/extracellular signal-regulated protein kinase kinase; LPS, lipopolysaccharide; ERK, extracellular signal-regulated protein kinase; INF$\gamma$, interferon-$\gamma$; ATF-2, activating transcription factor 2; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; GI$_{50}$, drug concentration that inhibits cell growth by 50%; JNK, c-Jun NH$_2$-terminal kinase; c-MEKK1, constitutively active mitogen-activated protein/extracellular signal-regulated protein kinase kinase.

well as inflammatory disorders (Cross and Wilson, 2003; Trifan and Hla, 2003; Ristimaki, 2004). TNF$\alpha$ is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, antitumor responses, and infection.

AP1, NF-$\kappa$B, COX-II, TNF$\alpha$, iNOS, and mitogen-activated protein kinase (p38) have been exploited as molecular targets in drug discovery and development for inflammatory-related diseases. Herein, the phenanthroindolizidine alkaloids isolated from the leaves of *Ficus septica* were investigated for their anti-inflammatory effects and mechanisms for their potential therapeutic exploitation.

## Materials and Methods

**Phenanthroindolizidine Alkaloids.** Compounds NSTP0G01 (tylophorine), NSTP0G03 (dehydrotylophorine), and NSTP0G07 (ficuseptine-A) (Fig. 1) were isolated from the leaves of *F. septica* as described previously, and their structures were elucidated (Wu et al., 2002). NSTP0G08 was derived from unstable NSTP0G07 and verified by LC-mass spectrometry and LC-tandem mass spectrometry profiling of NSTP0G08, which exhibited a peak with a molecular mass of 420 Da, whereas no peak occurred at 455 Da, the molecular mass of NSTP0G07. Moreover, the fast atom bombardment-mass spectrometry of NSTP0G07 showed the dehydroxylation fragment at *m/z* 438 and the dehydration fragment at *m/z* 420 (data not shown). The loss of 35 mass units led us to propose that NSTP0G08 was converted from NSTP0G07 through dehydroxylation and dehydration, and the deduced structure is shown in Fig. 1.

**Cell Culture and Chemicals.** RAW264.7 cells were maintained in high glucose Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) with 4 mM glutamine, 4500 mg/l glucose, 1% nonessential amino acids (Biological Industries, Ashrat, Israel), and 10% bovine serum (FetaClone III; Hyclone) without sodium pyruvate. RAW264.7 cells were scrapped off the culture plates for passage without any trypsin or EDTA treatment. All cells were grown in an incubator at 37°C and 5% $CO_2$. Chemicals and reagents were purchased from the following sources: FuGENE6 (Roche Diagnostics, Mannheim, Germany); lipopolysaccharide of *Escherichia coli* O111:B4 (Chemicon International, Temecula, CA); 15d-$PGJ_2$ (Cayman Chemical, Ann Arbor, MI); SB203580, SP600125, and U0126 (BioSource International, Camarillo, CA); lipopolysaccharide binding protein and recombinant IFN$\gamma$ (R&D Systems, Minneapolis, MN); and LY294002 and PDTC (Sigma-Aldrich, St. Louis, MO).

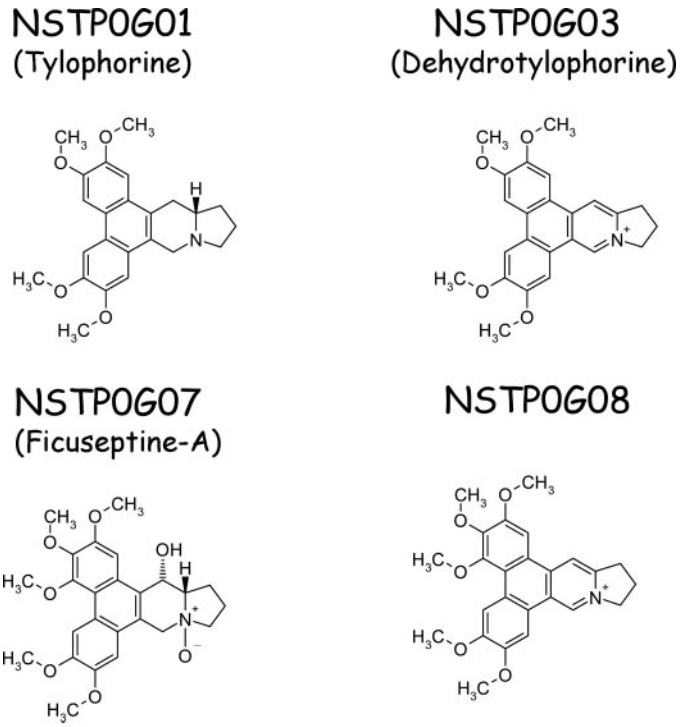


**Fig. 1.** Chemical structures of phenanthroindolizidine alkaloids.

**Plasmid and Transfection.** pNF$\kappa$B-Luc, pFC-MEKK (encoding amino acids 360–672 of MEKK1, accession no. L13103), and pAP1-Luc plasmids for luciferase reporter assay were obtained from Stratagene (La Jolla, CA). The murine iNOS promoter-Luc and murine COX-II promoter-Luc plasmid were generously provided by Drs. Charles J. Lowenstein (John Hopkins University, Baltimore, MD) (Lowenstein et al., 1993) and Yu-Chih Liang (Taipei Medical University, Taipei City, Tiawan) (Liang et al., 2001), respectively. pCMV-$\beta$-gal plasmid containing the *E. coli* $\beta$-galactosidase coding sequence was used for transfection efficiency control.

**Promoter or Element Reporter Assays.** RAW264.7 cells were seeded $9 \times 10^4$ cells/well in 24-well plates and grown in the medium described above with antibiotics. Four to 6 h later, cells were transfected with murine iNOS or COX-II promoter-luciferase reporter plasmids (100 ng/well) and cotransfected with pCMV-$\beta$-gal (100 ng/well) using FuGENE6 (Roche Diagnostics) following the manufacturer's protocol. After 24-h incubation, the medium was replaced with the aforementioned medium containing stimuli of LPS (10 $\mu$g/ml)/IFN$\gamma$ (20 ng/ml), and test compounds were added at the concentrations indicated. After 18- to 20-h incubation, the medium was removed, and 150 $\mu$l of Glo lysis buffer (Promega, Madison, WI) was added per well, and the resultant lysates were subject to luciferase and $\beta$-galactosidase assay per the manufacturer's recommendations (Tropix, Bedford, MA). Transfection efficiency was normalized by $\beta$-galactosidase activity.

For AP1 and NF-$\kappa$B reporter assays, $10^6$ RAW264.7 cells/well were seeded on 24-well plates for 4 to 6 h before transfection with pNF-$\kappa$B-Luc and pCMV-$\beta$-gal plasmids, 100 ng of each/well, or pAP1-Luc alone at 200 ng/well. In similar experiments with cotransfected MEKK1, the cells were transfected with pNF$\kappa$B-Luc (100 ng) or pAP1-Luc (100 ng) along with pFC-MEKK (50 ng) and/or pCMV-$\beta$-gal (50 ng). The following day, the transfected RAW264.7 cells were washed twice with culture medium with or without serum as the experimental design. Thereafter, cells were concurrently treated with LPS (10 $\mu$g/ml final concentration), which was preincubated with lipopolysaccharide binding protein (100 ng in the final concentration) for 1 h at 37°C, and IFN$\gamma$ (20 ng/ml) as well as the indicated phenanthroindolizidine alkaloids, 15d-$PGJ_2$, PDTC, LY294002, or SB203580 for another 5 h. Thereafter, the cell extracts were assayed for luciferase and $\beta$-galactosidase activity or total amount of protein. The luciferase activity was normalized with $\beta$-galactosidase activity or total amounts of protein. For NF-$\kappa$B experiments, the 5-h treatment was carried out in the serum-free medium, and for AP1 experiments, the treatment was in the complete culture medium without nonessential amino acids.

**Luciferase and $\beta$-Galactosidase Assays.** Luciferase and $\beta$-galactosidase assays were performed, respectively, using a Steady-Glo luciferase assay system (Promega) and Galacto-Star (Tropix) according to the manufacturers' instructions. Luminescence was measured in a TopCount NXT microplate scintillation and luminescence counter (Packard, Boston, MA).

**Carcinoma Cell Growth Inhibitory Assay.** HONE-1 and NUGC-3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biological Industries) and were seeded at 4500 and 6000 cells/well, respectively, in 96-well plates and incubated in a $CO_2$ incubator at 37°C for 24 h. The cells were treated with at least five different concentrations of test compounds in a $CO_2$ incubator for 72 h. The number of viable cells was estimated using the tetrazolium dye reduction assay (MTS assay), and the experiment was performed per the manufacturer's recommendations (Promega). The results of these assays were used to obtain the dose-response curves from which $GI_{50}$ values were

determined (Liou et al., 2004). The values represent averages of three independent experiments, each with duplicate samples.

**Determination of Nitric Oxide Synthesis.** RAW264.7 cells were seeded (70,000 cells/well) and cultured in 96-well plates. After 24-h incubation, the medium was replaced with complete medium containing stimuli of LPS (10 $\mu$g/ml)/IFN$\gamma$ (20 ng/ml), and the test compounds were added at the various concentrations as indicated. After 18 to 24 h, the supernatants were subject to measurement of nitric oxide production using nitrate/nitrite assay kit (Cayman Chemical). Nitric oxide was measured as the accumulation of nitrite and nitrate in the incubation medium. Nitrate was reduced to nitrite with nitrate reductase and determined spectrophotometrically with Griess reagent at optical density$_{540}$.

The attached cells were subjected to cytotoxicity measurement using MTS assay (Promega). The $IC_{50}$ values of nitric oxide production and cytotoxicity were determined from respective dose-response curves.

**Cytokine Measurement.** TNF$\alpha$ protein was detected in cell culture supernatant that was diluted to proper concentrations for assay using respective enzyme-linked immunosorbent assay kits from R&D Systems per the manufacturer's recommendations. The amounts of NO and relative viable cell numbers were determined by Griess and MTS assays, respectively, as aforementioned for inter- and intraexperimental controls (data not shown).

**Western Blotting.** iNOS, COX-I, COX-II, and $\beta$-actin proteins were analyzed by immunoblotting with anti-iNOS (BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-COX-I (Upstate Biotechnology, Lake Placid, NY), anti-COX-II (Upstate Biotechnology), and anti-$\beta$-actin (Chemicon International) antibodies, respectively. The antibodies against pan and phosphorylated Akt, c-Jun, and ATF-2 were purchased from Cell Signaling Technology Inc. (Beverly, MA). The antibodies against the pan protein of JNK, p38, or ERK1/2 were purchased from BioSource International, and antibodies for the respective phosphorylated proteins were from Promega. The cell lysates with equal amounts of total protein were subject to SDS-polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred to nitrocellulose membrane. The resultant membranes were incubated with blocking solution, primary antibody, and secondary antibody, respectively, and wash procedures were carried out according to the manufacturers' recommendations. Antigen-antibody complexes were detected using enhanced chemiluminescence detection reagents (Western Blot Chemiluminescence Reagent Plus; PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer's instructions.

## Results

**Anti-Inflammation and Cytotoxicity of the Phenanthroindolizidine Alkaloids.** Four phenanthroindolizidine alkaloids (Fig. 1) were investigated herein for their anti-inflammatory efficacy. Murine macrophage cell line RAW264.7, which produces enormous amounts of NO at the concentration range of 60 to 100 $\mu$M upon stimulation with LPS/IFN$\gamma$, was used as a model of in vitro acute inflammation system. The background nitrate/nitrite generated by the control unstimulated RAW264.7 cells ranging from ~2 to 4 $\mu$M was excluded from the LPS/IFN$\gamma$-stimulated NO production. Both NSTP0G01 (tylophorine) and NSTP0G07 (ficuseptine-A) exhibited significant inhibition in NO production in RAW264.7 cells stimulated by LPS/IFN$\gamma$ with $IC_{50}$ values of 1.8 and 2.1 $\mu$M, respectively (Table 1). This potency is comparable with that of 15d-$PGJ_2$ (NO suppression $IC_{50}$ of 2.2 $\mu$M), a prostaglandin $D_2$ metabolite reported as a potential therapeutic agent for anti-inflammation (Ricote et al., 1998). In contrast, NSTP0G03 and NSTP0G08 displayed very weak inhibition of NO production with $IC_{50}$ values of 54.5 and 14.0 $\mu$M, respectively. None of the four alkaloids exhibited significant growth inhibition toward LPS/IFN$\gamma$-stimulated RAW264.7 cells at the concentration of 10 $\mu$M, with NSTP0G01 exerting a weak growth inhibition with a $GI_{50}$ of 8.2 $\mu$M.

NSTP0G01 and NSTP0G07 were reported to inhibit cancer cell growth in vitro in a cytotoxicity study against various cancer cell lines (Wu et al., 2002). NSTP0G08, a novel compound with a planar structure derived from NSTP0G07, exerted potent growth inhibition at 4 $\mu$g/ml (~10 $\mu$M) with 72 and 82% inhibition on HONE-1 and NUGC-3 cancer cells compared with 82 and 86% exhibited by NSTP0G07 at the same concentration, respectively (Table 2). NSTP0G01 and NSTP0G08 displayed $GI_{50}$ values of 0.96 and 1.71 $\mu$M for HONE-1 and 1.00 and 1.60 $\mu$M for NUGC-3 cells, respectively. On the contrary, NSTP0G03 did not show significant growth inhibition against HONE-1 and NUGC-3 cancer cells even at the concentration of 50 $\mu$M.

**Effects of the Phenanthroindolizidine Alkaloids on the Expression of TNF$\alpha$, iNOS, and COX-II.** Next, we examined the effects of the phenanthroindolizidine alkaloids on the induction of proinflammatory mediators TNF$\alpha$, iNOS, and COX-II in LPS/IFN$\gamma$-stimulated RAW264.7 cells to explore the extent of their anti-inflammatory effects (Fig. 2, A and B). The TNF$\alpha$ protein was induced to produce at the level of 180 to 200 ng/ml upon stimulation with LPS/IFN$\gamma$ in RAW264.7 cells. This induction was decreased ~75 to 90% by the treatment of NSTP0G01 but only moderately inhibited (~15–30%) by the treatment of NSTP0G03 at the concentrations of 3 to 10 $\mu$M, whereas 15d-$PGJ_2$ exerted no significant effect (Fig. 2A). The protein expression levels of iNOS and COX-II were enormously induced upon stimulation of LPS/IFN$\gamma$ in RAW264.7 cells and dramatically inhibited after 18-h treatment with NSTP0G01, but NSTP0G03 did not show similar effect (Fig. 2B). 15d-$PGJ_2$, a prostaglandin $D_2$ metabolite and peroxisome proliferator-activated receptor $\gamma$ agonist, was reported to be capable of exerting anti-inflammation effects in NF-$\kappa$B-dependent and -independent manners (Straus et al., 2000; Chawla et al., 2001) and inhibiting NO production and iNOS expression (Ricote et al., 1998). It also inhibits TNF$\alpha$ production at the concentrations of 25 $\mu$M in similar conditions to those used herein (Thieringer et al., 2000). Therefore, 15d-$PGJ_2$ significantly inhibited the protein expression of iNOS but not TNF$\alpha$ at 10 $\mu$M concentration as expected, and it did not significantly inhibit the COX-II protein expression. On the other hand, our results

TABLE 1
$IC_{50}$ and $GI_{50}$ values of phenanthroindolizidine alkaloids for suppression of nitric oxide production ($IC_{50}$) and growth inhibition ($GI_{50}$) against LPS/IFN$\gamma$ -stimulated RAW264.7

RAW264.7 cells were cultured in the presence of LPS/IFN$\gamma$ concurrently treated with different concentrations of indicated phenanthroindolizidine alkaloids or 15d-$PGJ_2$ for 18 to 20 h. The amounts of NO in the culture medium generated upon LPS/IFN$\gamma$ stimulation were used as 100% for comparison of the compound treatment effects and calculation for the $IC_{50}$ values, and the adherent cells were subject to MTS for $GI_{50}$ measurement.

| Compound | NO Suppression ($IC_{50}$) | Cytotoxicity ($GI_{50}$) |
|---|---|---|
| | $\mu M$ | |
| NSTP0G01 | 1.81 ± 0.51 | 8.17 ± 1.76 |
| NSTP0G03 | 54.49 ± 12.15 | >70 |
| NSTP0G07 | 2.08 ± 0.88 | >10 |
| NSTP0G08 | 13.95 ± 1.89 | >70 |
| 15d-$PGJ_2$ | 2.18 ± 0.80 | >10 |

showed that NSTP0G01 is capable of inhibiting the induced expression of TNF$\alpha$, iNOS, and COX-II protein in addition to its inhibition of induced NO production and ability to kill cancer cells. Similar effects of the phenanthroindolizidine on the expression of TNF$\alpha$, iNOS, and COX-II were obtained from murine primary peritoneal macrophages elicited by thioglycollate in vivo and stimulated with LPS/IFN$\gamma$ in vitro (data not shown).

TABLE 2

Growth inhibition of phenanthroindolizidine alkaloids against cancer cell lines HONE-1 and NUGC-3

Cells, seeded the day before use, were cultured with or without compound treatment for 3 days before being subjected to MTS assay for $GI_{50}$ or percentage of inhibition measurement. See *Materials and Methods* for details. Data from three or more experiments are expressed as means ± S.D.

| Compound | HONE-1 | | NUGC-3 | |
|---|---|---|---|---|
| | 4 $\mu$g/ml | $GI_{50}$ | 4 $\mu$g/ml | $GI_{50}$ |
| | % | $\mu M$ | % | $\mu M$ |
| NSTP0G01 | 87 | 0.96 ± 0.17 | 89 | 1.00 ± 0.46 |
| NSTP0G03 | 1 | >50 | 7 | >50 |
| NSTP0G07 | 82 | ND | 86 | ND |
| NSTP0G08 | 72 | 1.71 ± 0.08 | 82 | 1.60 ± 0.17 |
| 15d-$PGJ_2$ | N.D. | 15.86 ± 2.68 | N.D. | 21.83 ± 1.90 |

N.D., not determined.

**Effects of the Phenanthroindolizidine Alkaloids on the Promoter Activities of iNOS and COX-II.** To further explore the effective points at upstream expression regulation, we examined the effect of these compounds on the promoter activity of iNOS and COX-II for gene expression. RAW264.7 cells were transiently transfected with the respective promoter reporter plasmids of iNOS or COX-II. Both promoter activities were markedly increased after treatment with LPS/IFN$\gamma$ (Fig. 2, C and D). The activation of murine iNOS promoter activity upon stimulation of LPS/IFN$\gamma$ was significantly inhibited by NSTP0G01 (~90%) and 15d-$PGJ_2$ (~75%), but NSTP0G03 did not show any significant effect. Likewise, murine COX-II promoter activity upon stimulation with LPS/IFN$\gamma$ was significantly affected by the NSTP0G01 treatment at the concentration of 10 $\mu$M with ~80% inhibition. Moreover, both iNOS and COX-II promoter activities induced upon stimulation of LPS/IFN$\gamma$ were inhibited by NSTP0G01 in a dose-dependent manner with $IC_{50}$ values of ~1.2 and ~0.6 $\mu$M, respectively. The above-mentioned results suggest that NSTP0G01 exerts its inhibitory effect in

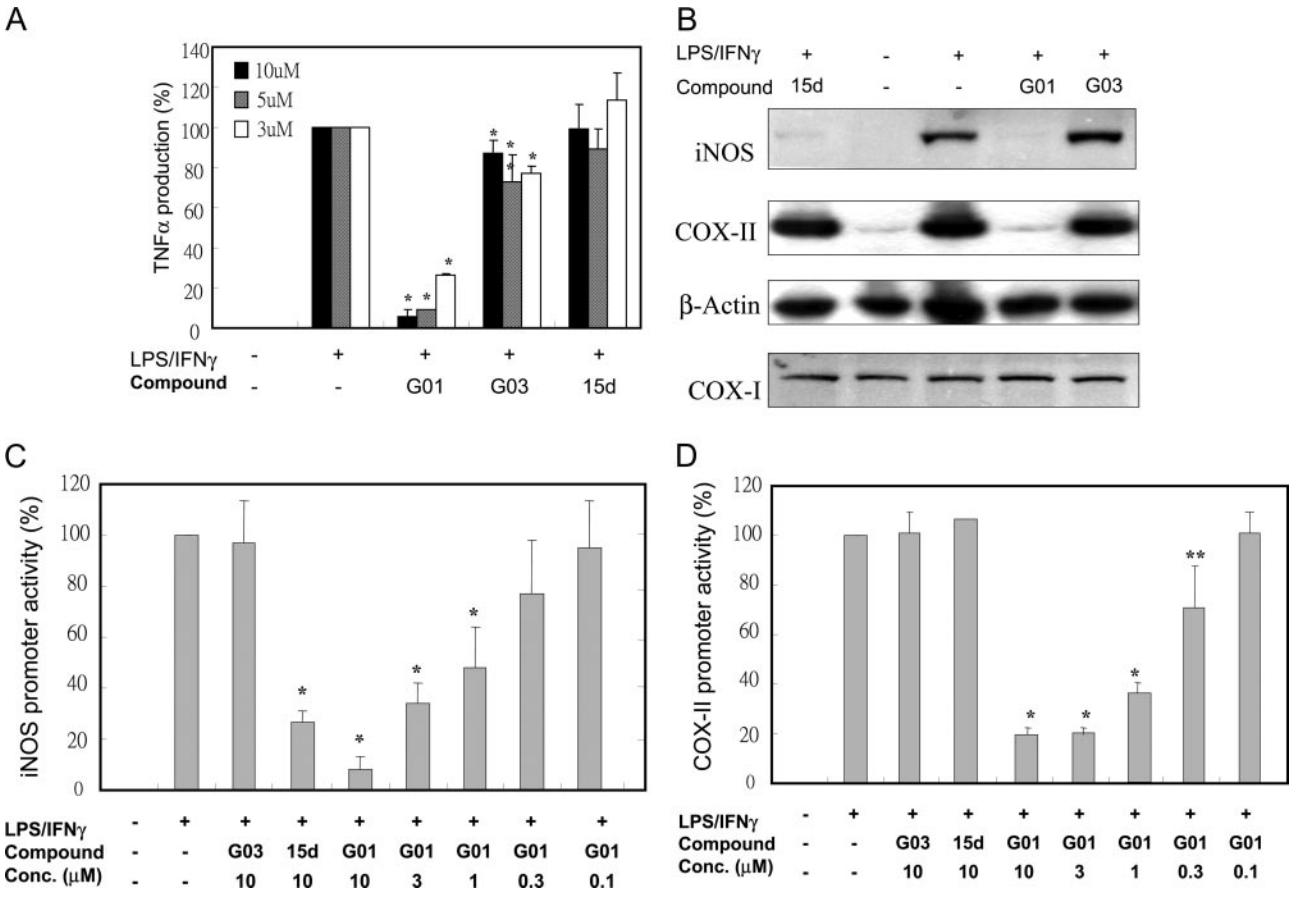


**Fig. 2.** Effects of phenanthroindolizidine alkaloids on the protein expression of TNF$\alpha$ (A), iNOS and COX-II (B), and promoter activity of iNOS (C) and COX-II (D). A, RAW264.7 cells were cultured in the presence of LPS/IFN$\gamma$ concurrently treated with 3, 5, and 10 $\mu$M concentrations of the indicated phenanthroindolizidine alkaloids or 15d-$PGJ_2$. The amounts of TNF$\alpha$ generated upon LPS/IFN$\gamma$ stimulation were used as 100% for comparison of the compound treatment effects. Data from three or more experiments were expressed as means ± S.D. B, RAW264.7 cells were cultured in the presence of concurrent treatment of LPS /IFN$\gamma$ and 10 $\mu$M concentrations of indicated phenanthroindolizidine alkaloids or 15d-$PGJ_2$. After 18 h, cell extracts were collected and subject to Western analysis for the protein expression of iNOS and COX-II as well as $\beta$-actin and COX-I. Results shown are the representatives of three independent experiments. C and D, RAW264.7 cells were transfected with murine iNOS and COX-II promoter-luciferase reporter plasmids, respectively. The promoter activity generated upon LPS/IFN$\gamma$ stimulation was used as 100% (~8000 and ~10,000 relative light units for iNOS and COX-II promoters, respectively) for comparison of compound treatment effects. The values are represented as means ± S.D. of three or more independent experiments. 15d, 15d-$PGJ_2$; G01, NSTP0G01; and G03, NSTP0G03 (*, $P < 0.005$ and **, $P < 0.05$ versus LPS/IFN$\gamma$-stimulated only).

the transcriptional events of iNOS and COX-II and thus results in the reduced protein expression levels.

**Effects of the Phenanthroindolizidine Alkaloids on NF-κB and AP1 Activity.** The promoter sequences of murine iNOS and COX-II genes possess one common consensus-responsive element for NF-κB and one similar element for AP1/AP2 (Lowenstein et al., 1993; Kosaka et al., 1994; Chu et al., 1998; Yuan et al., 2000), which participate in regulating gene expression and thus their downstream protein expression. The regulatory genes by NF-κB and AP1 are also reported to be involved in inflammation signaling. Thus, NF-κB and AP1 have long been exploited as molecular targets for anti-inflammatory and immunosuppressant therapies (Chen et al., 1986; Adcock, 1997; Giuliani et al., 2001). Therefore, we examined the effect of the phenanthroindolizidine alkaloids on the activity of NF-κB and AP1 in RAW264.7 cells upon stimulation for LPS/IFNγ using reporter assays with the regulatory element sequence of NF-κB and AP1, respectively.

Our results showed that NSTP0G01 and NSTP0G03 did not inhibit NF-κB activation at the concentration of 10 μM, in contrast to 15d-$PGJ_2$ (10 μM) and PDTC (50 μM) (an NF-κB inhibitor), which exerted ~30% and ~100% inhibitory effect, respectively (Fig. 3A). In similar experiments, the RAW264.7 cells were pretreated with compounds for 30 min; NSTP0G01 and NSTP0G03 at the concentration of 10 μM did not inhibit NF-κB activation, although 10 μM 15d-$PGJ_2$ completely (~100%) blocked the activity and 50 μM PDTC exerted ~20% inhibitory effect (data not shown).

On the other hand, NSTP0G01 inhibited the AP1 activation in RAW264.7 upon stimulation with LPS/IFNγ by approximately 86% at the concentration of 10 μM and in a dose-

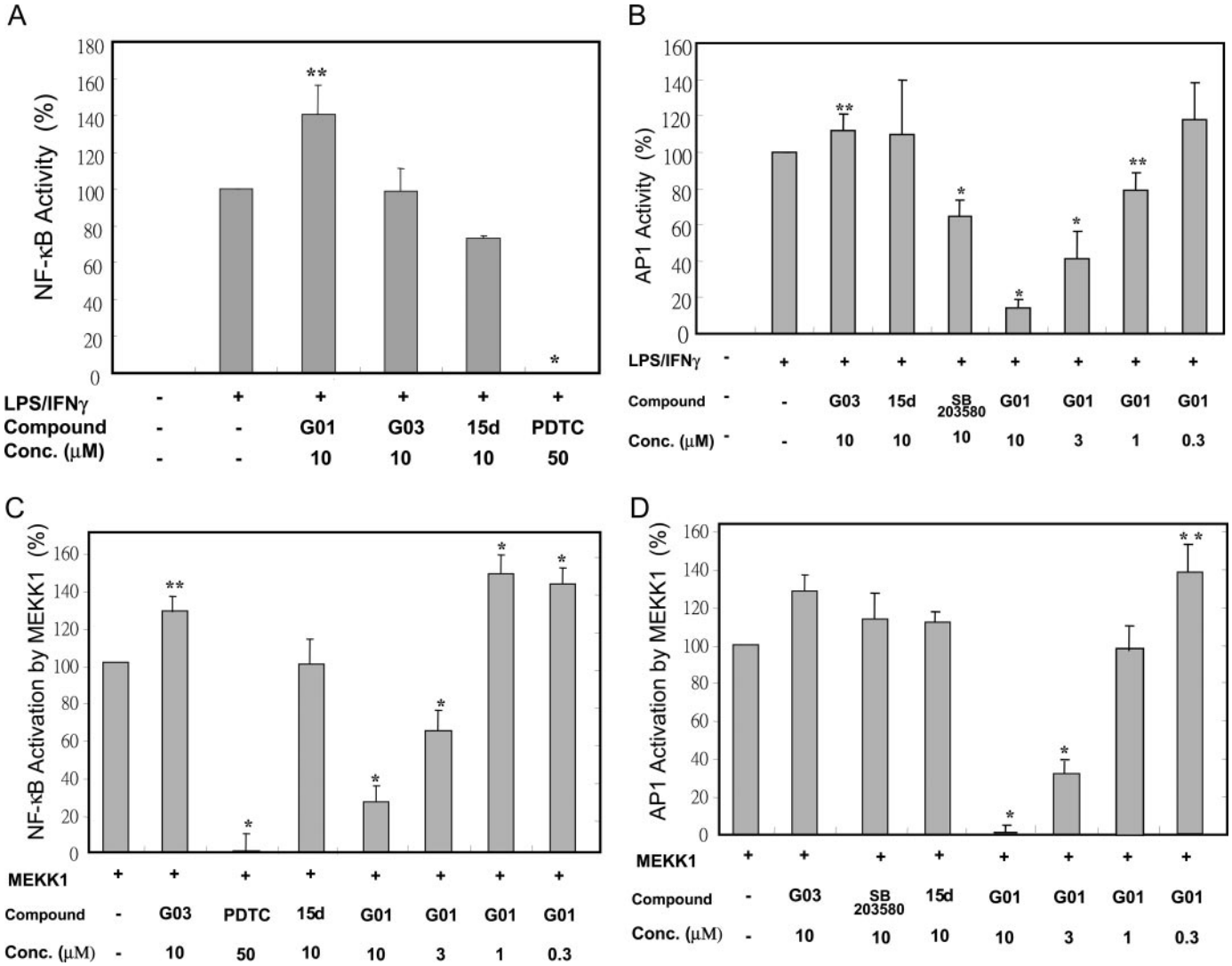


**Fig. 3.** Effects of phenanthroindolizidine alkaloids on the activation of NF-κB and AP1 in RAW264.7 cells. A and B, effects of phenanthroindolizidine alkaloids on NF-κB- (A) and AP1 (B)-dependent reporter gene expression in LPS/IFNγ-stimulated RAW264.7 cells. The NF-κB or AP1 activity generated upon LPS/IFNγ stimulation was used as 100% (~4000 and ~8000 RLU for AP1 and NF-κB, respectively) for comparison of compound treatment effects. The values were represented as means ± S.D. of three or more experiments. C and D, effects of phenanthroindolizidine alkaloids on NF-κB- (C) and AP1 (D)-dependent reporter gene expression in RAW264.7 cells overexpressing c-MEKK. RAW264.7 cells were transfected with pNF-κB-Luc (or pAP1-Luc), pCMV-β-gal, and pFC-MEKK plasmids, at 100, 50, and 50 ng/well each. The total luciferase activity without treatment generated during a period of 5 h was used as 100% (~12,000 and ~25,000 relative light units for AP1 and NF-κB, respectively). The luciferase activity was normalized with total amount of protein not β-galactosidase activity because β-galactosidase activity was evidently regulated in this experimental condition (data not shown). The values were represented as means ± S.D. of three or more experiments. 15d, 15d-$PGJ_2$; G01, NSTP0G01; and G03, NSTP0G03 (*, $P < 0.005$ and **, $P < 0.05$ versus LPS/IFNγ-stimulated only).

dependent manner with an $IC_{50}$ of ~2.3 $\mu$M (Fig. 3B). However, NSTP0G03 and 15d-$PGJ_2$ did not exhibit any inhibition under similar conditions. In addition, SB203580 (a p38 mitogen-activated protein kinase-specific inhibitor) also exhibited a moderate inhibition (~35%) in the AP1 activation at the concentration of 10 $\mu$M. Transfection did not affect the induction of TNF$\alpha$, iNOS, and COX-II in RAW264.7 cells upon LPS/INF$\gamma$ stimulation and the effects of NSTP0G01, NSTP0G03, and 15d-PGJ2 on these inductions (data not shown).

**Effects of Phenanthroindolizidine on MEKK1 Specifically Triggered Activation of AP1 and NF-κB.** From the above-mentioned results, the inhibition of AP1 activation by NSTP0G01 was thus suggested to account for its anti-inflammatory effect in RAW 264.7 cells stimulated with LPS/IFN$\gamma$. However, considering that LPS triggers multiple signaling pathways, including transforming growth factor $\beta$-activating kinase 1, MEKK1, and phosphoinositide-3 kinase for NF-$\kappa$B activation as well as Ras/ERK, MEKK1/JNK, and PKR/p38 for AP1 activation in RAW264.7 cells (Guha and Mackman, 2001; Monick and Hunninghake, 2003), cross-talk for signaling amplification or counteraction between these signaling cascades may mask the effect of NSTP0G01 on NF-$\kappa$B activation. Therefore, we further examined the effect of NSTP0G01 on the MEKK1-triggered activation of NF-$\kappa$B and AP1 because MEKK1 is involved in upstream regulation of both NF-$\kappa$B and AP1. The effect of compound treatment on the activation of NF-$\kappa$B or AP1 by constitutively active MEKK1 (c-MEKK1) was measured by comparison of accumulated reporter luciferase activity between untreated and treated samples in a period of 5 h. Total activity was used as 100% for data analysis. The activation of NF-$\kappa$B by transfected c-MEKK1 was inhibited by NSTP0G01 in a dose-dependent manner with an $IC_{50}$ of ~3.1 $\mu$M, resulting in ~73% inhibition at 10 $\mu$M concentration. In similar conditions, it was ~99% inhibited by 50 $\mu$M PDTC, but NSTP0G03 and 15d-$PGJ_2$ did not show any significant inhibition at the 10 $\mu$M concentration (Fig. 3C). Moreover, NSTP0G01 also inhibited the AP1 activation by the overexpressed c-MEKK1 in a dose-dependent manner with an $IC_{50}$ of

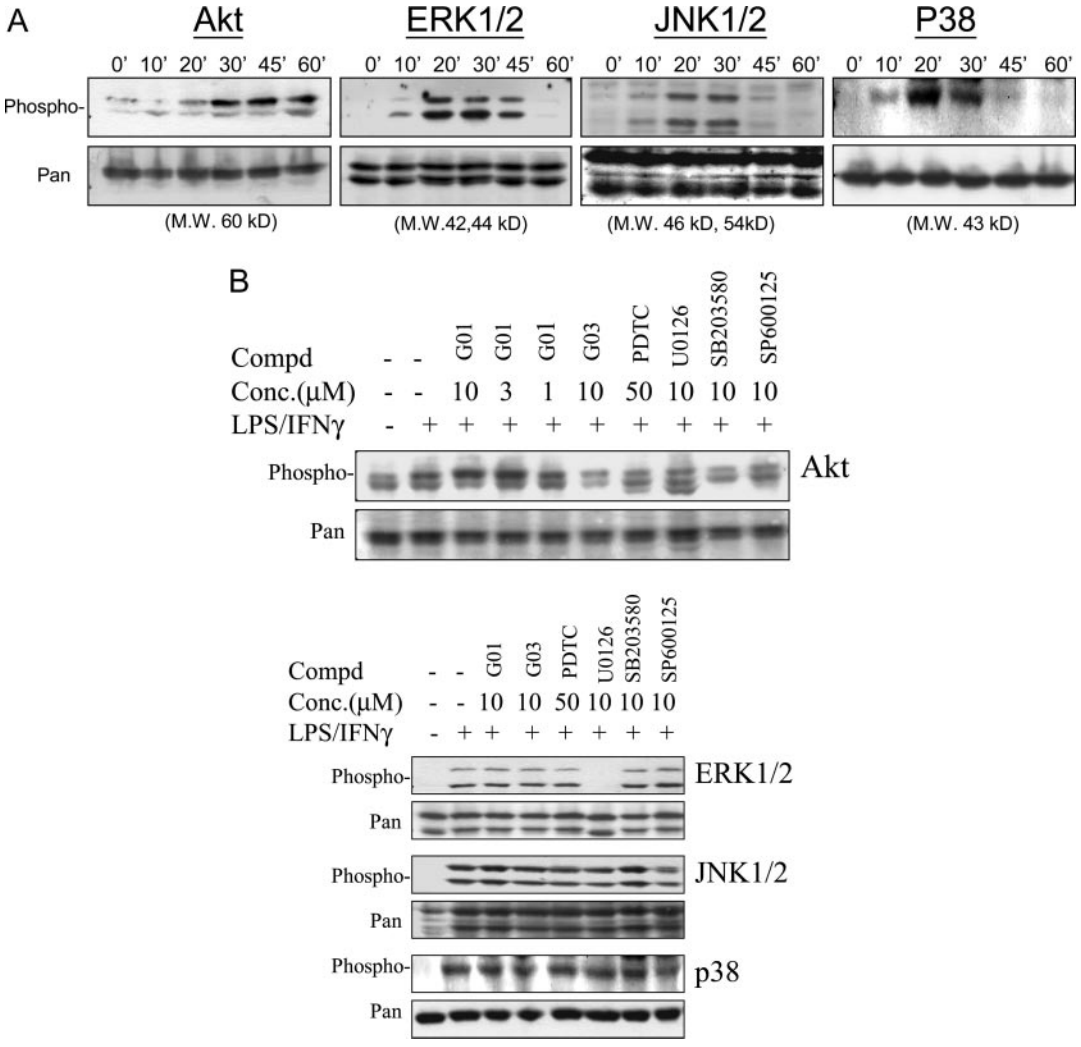


**Fig. 4.** Effects of phenanthroindolizidine alkaloids on the expression and phosphorylation of proteins involved in NF-$\kappa$B and AP1 activation. A, phosphorylation of Akt, p38, ERK1/2, and JNK upon the LPS/IFN$\gamma$ stimulation in RAW264.7 cells exhibited dynamic response within 60 min of treatment. LPS /IFN$\gamma$-stimulated cells were concurrently treated with the indicated compound and concentration, respectively. Cell lysates were harvested in the lysis buffer containing phosphatase inhibitors at the indicated time points and subjected for Western analysis. B, phosphorylation of Akt was increased by the treatment of NSTP0G01 in LPS/IFN$\gamma$-stimulated RAW264.7 cells. The effect of indicated compound treatment in the phosphorylated and pan proteins of Akt, p38, ERK1/2, and JNK was examined at the 30-min time point and at the indicated concentrations. At the indicated time points, cell extracts were collected in lysis buffer containing the phosphatase inhibitors and subjected to Western analysis. Results shown are the representatives of two or three independent experiments.

~2.1 μM, completely blocking it at the concentration of 10 μM (Fig. 3D). In contrast, NSTP0G03, 15d-PGJ2, and p38 inhibitor SB203580 did not inhibit the AP1 activation by the overexpressed c-MEKK1. Thus, NSTP0G01 was suggested to inhibit MEKK1 activity consequently inhibiting induced activation of AP1 and NF-κB.

**NSTP0G01 Increased the Akt Phosphorylation.** We further explored the effective mechanism of NSTP0G01 to account for its selective inhibition of the AP1 activation over NF-κB in RAW264.7 cells stimulated by LPS/IFNγ. The phosphorylation of Akt, JNK, ERK1/2, and p38 in RAW264.7 cells upon stimulation of LPS/IFNγ was examined at time points of 10, 20, 30, 45, and 60 min (Fig. 4A). Phosphorylation of each protein exhibited a course of dynamic response within 60 min. The time point of 30 min, at which each protein was phosphorylated most significantly, was chosen for further investigation of the effects of NSTP0G01, NSTP0G03, and inhibitors of JNK (SP600125), ERK1/2 (U0126), p38 (SB203580), and NF-κB (PDTC) in activation of these signaling molecules. NSTP0G01, compared with NSTP0G03, SB203580, SP600125, U0126, and PDTC, significantly enhanced the phosphorylation of Akt in LPS/IFNγ-stimulated RAW264.7 cells, even at the lower concentrations of 1 and 3 μM (Fig. 4B). However, NSTP0G01 and NSTP0G03 did not exert any significant inhibitory effect on the phosphorylation of p38, ERK1/2, and JNK, whereas each specific inhibitor of JNK, ERK1/2, p38, and PDTC significantly inhibited their respective target's phosphorylation at varied range (Fig. 4B). Each inhibitor exhibited similar extent of effect on the phosphorylation of Akt, p38, JNK, and ERK1/2 at the concentrations of 3 and 10 μM (data not shown).

**In Conjunction Treatment of NSTP0G01 and LY294002 Inhibited NF-κB Activation in LPS/IFNγ-Stimulated RAW264.7 Cells.** Phosphorylation of Akt leads to the activation of NF-κB (Guha and Mackman, 2001). LY294002, a phosphoinositide-3 kinase/Akt inhibitor, dramatically decreased the amounts of phosphorylated Akt in LPS/IFNγ-stimulated RAW264.7 cells in conjunction with or without NSTP0G01 (data not shown). Therefore, LY294002 was used to investigate the effect of NSTP0G01 in NF-κB activation of LPS/IFNγ-stimulated RAW264.7 with or without conjunction of overexpressed c-MEKK1. In LPS/IFNγ-stimulated RAW264.7 cells, LY294002 did not significantly inhibit NF-κB activation (~18% inhibition), whereas NSTP0G01 moderately augmented (~35%) NF-κB activation (Figs. 3A and 5A). However, NF-κB activation was inhibited by 53 to 38% when the cells were treated with NSTP0G01 and LY294002 in conjunction (Fig. 5A). LY294002 was suggested to decrease the phosphorylation of Akt enhanced by NSTP0G01 in NSTP0G01- and LY294002-treated LPS/IFNγ-stimulated RAW264.7 cells and thus resulted in the inhibition of NF-κB activation, whereas NSTP0G01 also simultaneously inhibited the MEKK1 activity.

In contrast, in LPS/IFNγ-stimulated and c-MEKK1-overexpressed RAW264.7 cells, NSTP0G01 alone was able to inhibit approximately 70% of NF-κB activation (Fig. 5B). This extent of inhibition was comparable with that observed in unstimulated RAW264.7 cells with overexpressed c-MEKK1 only (Fig. 3C). As expected, no significant inhibition in NF-κB activation was obtained by LY294002 treatment alone because NF-κB activation was mainly driven by the overexpressed c-MEKK1 under this condition. Only a little further inhibition (~10%) was obtained when the cells were treated with LY294002 in conjunction with NSTP0G01 (Fig. 5B).

The enhanced phosphorylation of Akt through NSTP0G01 thus was suggested to account for the distinct effects of NSTP0G01 in NF-κB activation between the respective LPS/IFNγ-stimulated and c-MEKK1-overexpressed RAW264.7 cells. Therefore, the dual effects of NSTP0G01, inhibiting MEKK1 and enhancing Akt phosphorylation, resulted in it not inhibiting NF-κB activation in LPS/IFNγ-stimulated RAW264.7 cells (Figs. 3, A and C, 5A, and 7A).

**LY294002 Restored the NSTP0G01-Inhibited AP1 Activity.** The expression and phosphorylation of c-Jun, the main component of AP1, and another component, ATF-2, were further examined to account for the inhibition of AP1 activation by NSTP0G01. The dynamic responses of the expression and phosphorylation of c-Jun and ATF-2 were induced in LPS/IFNγ-stimulated RAW264.7 cells and were examined within a 180-min time course after stimulation (Fig. 6A). NSTP0G01 was able to significantly decrease the induced expression of c-Jun and thus the amount of phosphorylated c-Jun, whereas it increased the phosphorylation of ATF-2 compared with those of the stimulated control. LY294002 treatment alone had no sig-

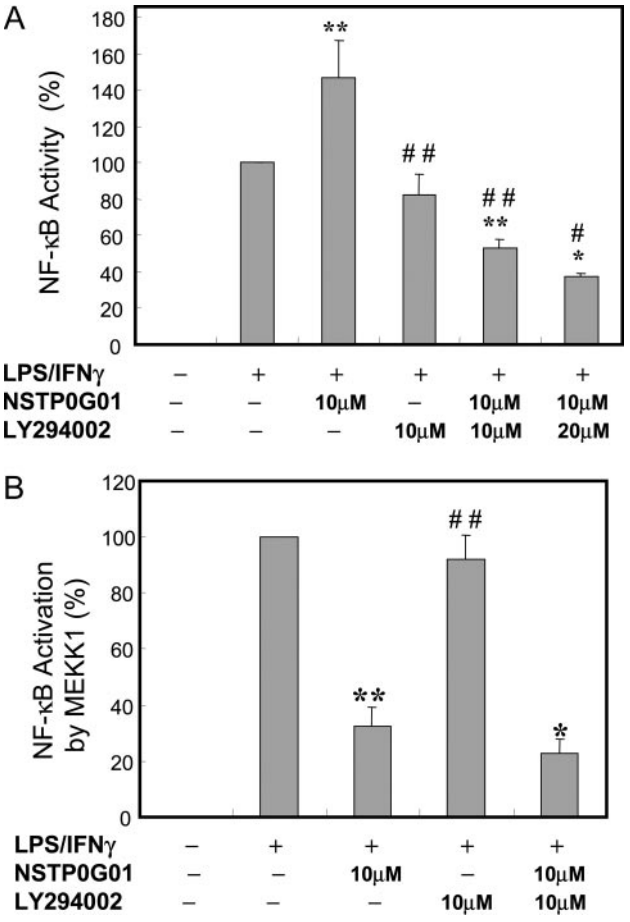


**Fig. 5.** LY294002 inhibited the increased NF-κB activation by NSTO0G01 in LPS/IFNγ-stimulated RAW264.7 cells. A, NSTP0G01 significantly inhibited the NF-κB activation only when treated in conjunction with LY294002 in LPS/IFNγ-stimulated RAW264.7 cells. B, NSTP0G01 but not LY294002 inhibited MEKK1 signaling NF-κB activation in LPS/IFNγ-stimulated RAW264.7 cells. LPS/IFNγ-stimulated cells were concurrently treated with the indicated compounds and concentrations. See *Materials and Methods* for the experimental procedure. The values were represented as means ± S.E. of two independent experiments, each in triplicate (*, $P < 0.005$ and **, $P < 0.05$ versus LPS/IFNγ-stimulated only; #, $P < 0.05$ and ##, $P < 0.01$ versus LPS/IFNγ-stimulated and NSTP0G01-treated).

nificant effect on the ATF-2 expression and phosphorylation and slightly increased that of c-Jun. It is noteworthy that the expression and phosphorylation of c-Jun by the treatment of the LPS/IFNγ-stimulated RAW264.7 cells with NSTP0G01 in conjunction with LY294002 showed similar extents to those with LY294002 treatment alone. Thus, LY294002 was suggested to restore the decreased expression and phosphorylated c-Jun caused by NSTP0G01 treatment. Another interpretation could be that the inhibitory effects of NSTP0G01 in c-Jun expression and phosphorylation were counteracted by LY294002 decreasing Akt phosphorylation (data not shown). In contrast, the increased ATF-2 phosphorylation on treatment with NSTP0G01 was not affected by the cotreatment with LY294002.

The restored c-Jun expression and phosphorylation in the cotreatment with NSTP0G01 and LY294002 was further validated by the AP1 activation (Fig. 6B). Results showed that the inhibited AP1 activation by NSTP0G01 was restored by the cotreatment with LY294002 from ~12% back to ~95% (Fig. 6B). Thus, the enhanced phosphorylation of Akt by NSTP0G01 in LPS/INFγ-stimulated RAW264.7 cells was suggested to be mainly responsible for its inhibition in AP1 activation and thus conceivably its suppression in NO production and anti-inflammatory effects in vitro.

## Discussion

The phenanthroindolizidine alkaloids have been subjected to clinical trials either in the form of pure compounds, such as tylocrebrine for anticancer, or of, for example, alkaloid ex-

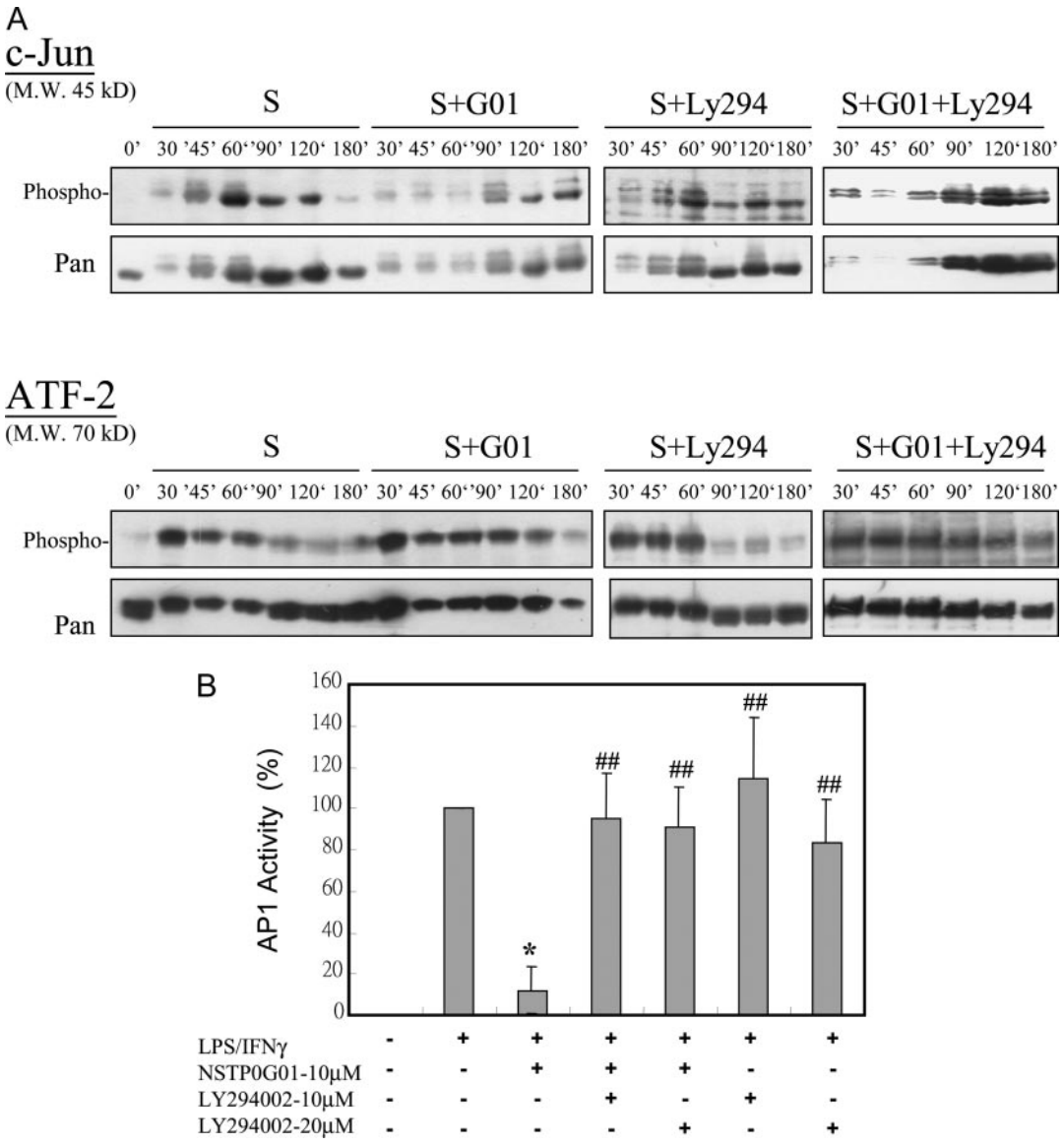


**Fig. 6.** LY294002 restored the inhibited AP1 activation by NSTP0G01. A, effects of NSTP0G01 and LY294002 on the c-Jun and ATF-2 expression and phosphorylation in LPS/IFNγ-stimulated RAW264.7 cells. LPS/IFNγ-stimulated (S) cells were concurrently treated with the indicated compound at the concentration of 10 μM. Cell lysates were harvested in the lysis buffer containing phosphatase inhibitors at the indicated time points and subjected for Western analysis. Results shown were the representatives of two to three independent experiments. B, LY294002 restored the AP1 activation that inhibited by NSTP0G01 in LPS/IFNγ-stimulated RAW264.7 cells. See *Materials and Methods* for the experimental procedure. LPS/IFNγ-stimulated cells were concurrently treated with the indicated compounds and concentrations. The values are represented as means ± S.D. of three independent experiments, each in triplicate (*, $P < 0.005$ versus LPS/IFNγ-stimulated only; #, $P < 0.05$ versus LPS/IFNγ-stimulated and NSTP0G01-treated).

tracts or leaf powders for antibronchial asthmatics (Huntley and Ernst, 2000; Staerk et al., 2000, 2002). The trials using tylocrebrine for anticancer treatment were withdrawn because of nervous side effects in 1960s (Staerk et al., 2000). Tylophorine analogs have again attracted attention for drug development and have been proposed to exert antitumor effects in a novel mode of action (Gao et al., 2004). Tylophorine analogs were found to inhibit the activity of cAMP response element, AP1, and NF-κB in HepG2 lung carcinoma cells treated with forskolin, TPA, and TNFα, respectively. However, more evidence may be needed to bolster the relationship between antitumor activity and cytotoxicity and the inhibitions in the activation of cAMP response element, AP1, and NF-κB by these tylophorine analogs. In addition, another two phenanthroindolizidine alkaloids, pergularine and tylophorinidine, were found to inhibit the activity of dihydrofolate reductase and thymidylate synthase, which may account for their underlying mechanisms for anticancer activity (Rao et al., 1997; Rao and Venkatachalam, 2000).

On the other hand, the mechanisms responsible for anti-inflammation or antiasthmatics of the phenanthroindolizidine alkaloids are not clear as yet. *Tylophora indica* has been used for decades in India in connection with the inflammatory related conditions, e.g., asthma, bronchitis, bronchial asthma, hay fever, and rheumatism. The major alkaloid of *T. indica* is tylophorine that is conceivable to account for the therapeutic efficacies. Thus, it is important to delineate the underlying mechanisms for the anti-inflammatory effects of tylophorine to elucidate its efficacies in a variety of anti-inflammatory-related therapies.

Thus far, all effective phenanthroindolizidine alkaloids reported for anticarcinoma activity are angular molecules (Rao and Venkatachalam, 2000; Staerk et al., 2000, 2002; Komatsu et al., 2001; Lee et al., 2003; Gao et al., 2004). As far as we are aware, this is the first report describing a planar compound of phenanthroindolizidine alkaloids that could exert significant cytotoxicity to carcinoma cells. The planar structure of NSTP0G08 compared with the angular counterpart of the NSTP0G07 may account for reduction in its anti-inflammatory property. It is noteworthy that a similar difference between NSTP0G03 and NSTP0G01 caused a significant decrease in both anti-inflammatory and anticancer properties of NSTP0G03. NSTP0G01 and NSTP0G07 are angular molecules with a reactive group at the indolizidine moiety, such as a nitrogen atom with a lone pair of electrons or a hydroxyl group for potential hydrogen bonding. On the other hand, NSTP0G03 and NSTP0G08 are planar molecules with no reactive atom at the indolizidine moiety. This structure-activity relationship may account for their anti-inflammation property. The planar and angular prhenanthroindolizidines without any methoxyl group also exhibit similar relative activity (S.-J. Lee, P.-L. Wu, C.-W. Yang, T.-H. Chuang, unpublished data). However, more phenanthroindolizidine alkaloids with similar planar and angular structures need to be studied to validate this postulate.

NSTP0G01 in contrast to NSTP0G03 significantly inhibits induced expression of proinflammatory several factors (e.g., iNOS, COX-II, and TNFα). NF-κB and AP1 are the two common and important transcriptional factors for gene induction of iNOS and COX-II. On the other hand, NSTP0G01 enhances Akt activation and decreases c-Jun expression. Although NSTP0G01 was also able to specifically block the MEKK1 activity and the subsequently triggered NF-κB and AP1 (Fig. 3, C and D), only AP1 activation was inhibited by NSTP0G01 in the LPS/IFNγ-stimulated RAW264.7 cells (Fig. 3, A and B). Thus, in addition to MEKK1, the interplay between c-Jun/AP1 and Akt induced by NSTP0G01 treatment in different cell contents plays a vital role in the AP1 activity. The cross-talk between overlapping signaling of Akt, MEKK1, c-Jun/AP1, and NF-κB could compromise some cellular events (Cerezo et al., 1998; Shimoke et al., 1999; Levresse et al., 2000; Go et al., 2001; Guha and Mackman, 2001; Funakoshi-Tago et al., 2003; Aikin et al., 2004; Li et al., 2004). Therefore, the NSTP0G01 exerts dual functions of enhancing Akt activation and inhibiting MEKK1, explaining the opposite results of AP1 and NF-κB activation affected by NSTP0G01 in the LPS/IFNγ-stimulated RAW264.7 cells (Figs. 3, A and B, and 7A). Thus, in the presence of LY294002 and NSTP0G01, when the Akt activation is counteracted between these two compounds, the NFκB activation is inhibited (Fig. 7, B and C). Thus, we have provided novel insight into the understanding of the underlying molecular mechanisms of the nature products, phenanthroindolizidine alkaloids, for the treatment of inflammation and possible therapeutic potential for the related disorders (e.g., asthma and arthritis).

The interplay between Akt and JNK or c-Jun in apoptosis or stress-induced inflammation is diverse. Change in Akt activity increases or decreases the JNK activation or the subsequent effect in c-Jun phosphorylation in different cell contents (Cerezo et al., 1998; Shimoke et al., 1999; Levresse et al., 2000; Go et al., 2001; Funakoshi-Tago et al., 2003; Aikin et al., 2004; Li et al., 2004). Despite the relevant relation between the JNK activation and c-Jun phosphorylation that cross-talk with Akt pathway or not, the c-Jun acti-

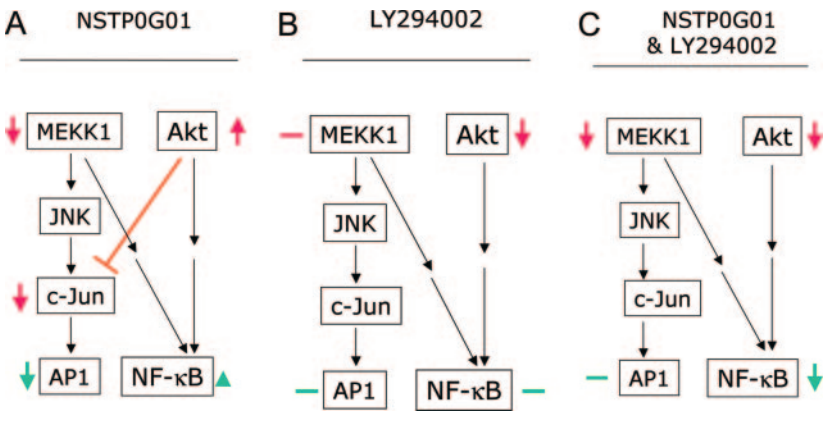


**Fig. 7.** Diagrams for illustrating the effective points and outcomes by the treatment of NSTP0G01 or LY294002 in LPS/IFNγ-stimulated RAW264.7 cells. All the effective points and outcomes are compared with those in LPS/IFNγ-stimulated RAW264.7 cells with no compound treatment. In the LPS/IFNγ-stimulated RAW264.7 cells, AP1 and NF-κB are activated (Fig. 3), and moderate Akt phosphorylation is observed (Fig. 4) compared with those in RAW264.7 cells with no LPS/IFNγ stimulation. Highlighted in red are the effective points and green shows the outcomes. ↑, significant enhancement or increase in activation or signaling; ↓, significant decrease; ⊥, blocking or decrease; ▲, moderate increase; and −, no significant change. The pathways in black are mainly referenced from Guha and Mackman (2001) and incorporated with results reported herein in red and green.

vation independent of JNK has also been reported in neuron apoptosis (Watson et al., 1998). Herein, we reported the decreased c-Jun expression and phosphorylation correlated with enhanced phosphorylation of Akt by the treatment with NSTP0G01 in LPS/IFNγ-stimulated macrophage cells (Figs. 4B, 6, and 7). It is also the first time that this interplay induced by NSTP0G01 (tylophorine) has been revealed to play an important role in the anti-inflammation process.

Together, the differential between NSTP0G01 and NSTP0G03 in their cytotoxicity toward cancer cells and anti-inflammatory effect demonstrates the subtle change in structure of phenanthroindolizidine alkaloids could account for their biological functions and thus warrant further investigation for structure-activity relationships of this group of compounds. A better understanding of the effective cellular mechanisms of NSTP0G01 and other similar effective compounds compared with ineffective mechanisms (e.g., NSTP0G03) will lead to identification of more effective analogs of phenanthroindolizidine alkaloids with fewer unfavored functions, thus facilitating development of this class of compounds into successful therapeutic drugs. The further identification of the direct target cellular events of effective phenanthroindolizidine alkaloids will also provide additional insight for selecting more proper assays for structure-activity relationship analysis of phenanthroindolizidine alkaloids and its biological functions

**Acknowledgments**

We acknowledge Dr. Weir-Torn Jianng for LC-mass spectrometry analysis of NSTP0G08, Ta-Hsien Chuang for preparation of part of NSTP0G01, and Drs. Hwan-You Chang and Neeraj Mahindroo for help in proofreading and valued suggestions in preparation of this manuscript.

**References**

Abe F, Hirokawa M, Yamauchi T, Honda K, Hayashi N, Ishii M, Imagawa S, and Iwahana M (1998) Further investigation of phenanthroindolizidine alkaloids from *Tylophora tanakae*. *Chem Pharm Bull (Tokyo)* **46:**767–769.

Adcock IM (1997) Transcription factors as activators of gene transcription: AP-1 and NF-kappa B. *Monaldi Arch Chest Dis* **52:**178–186.

Aikin R, Maysinger D, and Rosenberg L (2004) Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology* **145:**4522–4531.

Cerezo A, Martinez AC, Lanzarot D, Fischer S, Franke TF, and Rebollo A (1998) Role of Akt and c-Jun N-terminal kinase 2 in apoptosis induced by interleukin-4 deprivation. *Mol Biol Cell* **9:**3107–3118.

Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, and Evans RM (2001) PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med* **7:**48–52.

Chen KX, Gresh N, and Pullman B (1986) A theoretical study of anthracene and phenanthrene derivatives acting as A-T specific intercalators. *Nucleic Acids Res* **14:**9103–9115.

Chu SC, Marks-Konczalik J, Wu HP, Banks TC, and Moss J (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. *Biochem Biophys Res Commun* **248:**871–878.

Cross RK and Wilson KT (2003) Nitric oxide in inflammatory bowel disease. *Inflamm Bowel Dis* **9:**179–189.

Funakoshi-Tago M, Tago K, Sonoda Y, Tominaga S, and Kasahara T (2003) TRAF6 and C-SRC induce synergistic AP-1 activation via PI3-kinase-AKT-JNK pathway. *Eur J Biochem* **270:**1257–1268.

Ganguly T and Sainis KB (2001) Inhibition of cellular immune responses by *Tylophora indica* in experimental models. *Phytomedicine* **8:**348–355.

Gao W, Lam W, Zhong S, Kaczmarek C, Baker DC, and Cheng YC (2004) Novel mode of action of tylophorine analogs as antitumor compounds. *Cancer Res* **64:**678–688.

Giuliani C, Napolitano G, Bucci I, Montani V, and Monaco F (2001) NF-κB transcription factor: role in the pathogenesis of inflammatory, autoimmune and neoplastic diseases and therapy implications. *Clin Ter* **152:**249–253.

Go YM, Boo YC, Park H, Maland MC, Patel R, Pritchard KA Jr, Fujio Y, Walsh K, Darley-Usmar V, and Jo H (2001) Protein kinase B/Akt activates c-Jun NH(2)-terminal kinase by increasing NO production in response to shear stress. *J Appl Physiol* **91:**1574–1581.

Gopalakrishnan C, Shankaranarayan D, Kameswaran L, and Natarajan S (1979) Pharmacological investigations of tylophorine, the major alkaloid of *Tylophora indica*. Indian *J Med Res* **69:**513–520.

Gopalakrishnan C, Shankaranarayanan D, Nazimudeen SK, and Kameswaran L (1980) Effect of tylophorine, a major alkaloid of *Tylophora indica*, on immunopathological and inflammatory reactions. *Indian J Med Res* **71:**940–948.

Guha M and Mackman N (2001) LPS induction of gene expression in human monocytes. *Cell Signal* **13:**85–94.

Huntley A and Ernst E (2000) Herbal medicines for asthma: a systematic review. *Thorax* **55:**925–929.

Komatsu H, Watanabe M, Ohyama M, Enya T, Koyama K, Kanazawa T, Kawahara N, Sugimura T, and Wakabayashi K (2001) Phenanthroindolizidine alkaloids as cytotoxic substances in a danaid butterfly, *Ideopsis similis*, against human cancer cells. *J Med Chem* **44:**1833–1836.

Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, Takahashi E, and Tanabe T (1994) Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. *Eur J Biochem* **221:**889–897.

Lee SK, Nam KA, and Heo YH (2003) Cytotoxic activity and $G_2$/M cell cycle arrest mediated by antofine, a phenanthroindolizidine alkaloid isolated from *Cynanchum paniculatum*. *Planta Med* **69:**21–25.

Levresse V, Butterfield L, Zentrich E, and Heasley LE (2000) Akt negatively regulates the cJun N-terminal kinase pathway in PC12 cells. *J Neurosci Res* **62:**799–808.

Li J, Chen H, Tang MS, Shi X, Amin S, Desai D, Costa M, and Huang C (2004) PI-3K and Akt are mediators of AP-1 induction by 5-MCDE in mouse epidermal Cl41 cells. *J Cell Biol* **165:**77–86.

Liang YC, Tsai SH, Tsai DC, Lin-Shiau SY, and Lin JK (2001) Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages. *FEBS Lett* **496:**12–18.

Liou JP, Chang YL, Kuo FM, Chang CW, Tseng HY, Wang CC, Yang YN, Chang JY, Lee SJ, and Hsieh HP (2004) Concise synthesis and structure-activity relationships of combretastatin A-4 analogues, 1-aroylindoles and 3-aroylindoles, as novel classes of potent antitubulin agents. *J Med Chem* **47:**4247–4257.

Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, and Murphy WJ (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc Natl Acad Sci USA* **90:**9730–9734.

Monick MM and Hunninghake GW (2003) Second messenger pathways in pulmonary host defense. *Annu Rev Physiol* **65:**643–667.

Pettit GR, Goswami A, Cragg GM, Schmidt JM, and Zou JC (1984) Antineoplastic agents, 103. The isolation and structure of hypoestestatins 1 and 2 from the East African *Hypoestes verticillaris*. *J Nat Prod* **47:**913–919.

Raina V and Raina S (1980) The responsiveness of leukocyte adenyl cyclase to tylophorine in asthmatic subjects. *Biochem Biophys Res Commun* **94:**1074–1077.

Rao KN, Bhattacharya RK, and Venkatachalam SR (1997) Inhibition of thymidylate synthase and cell growth by the phenanthroindolizidine alkaloids pergularinine and tylophorinidine. *Chem Biol Interact* **106:**201–212.

Rao KN and Venkatachalam SR (2000) Inhibition of dihydrofolate reductase and cell growth activity by the phenanthroindolizidine alkaloids pergularinine and tylophorinidine: the in vitro cytotoxicity of these plant alkaloids and their potential as antimicrobial and anticancer agents. *Toxicol In Vitro* **14:**53–59.

Ricote M, Li AC, Willson TM, Kelly CJ, and Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature (Lond)* **391:**79–82.

Ristimaki A (2004) Cyclooxygenase 2: from inflammation to carcinogenesis. *Novartis Found Symp* **256:**215–226, 259–269.

Shimoke K, Yamagishi S, Yamada M, Ikeuchi T, and Hatanaka H (1999) Inhibition of phosphatidylinositol 3-kinase activity elevates c-Jun N-terminal kinase activity in apoptosis of cultured cerebellar granule neurons. *Brain Res Dev Brain Res* **112:**245–253.

Staerk D, Christensen J, Lemmich E, Duus JO, Olsen CE, and Jaroszewski JW (2000) Cytotoxic activity of some phenanthroindolizidine N-oxide alkaloids from *Cynanchum vincetoxicum*. *J Nat Prod* **63:**1584–1586.

Staerk D, Lykkeberg AK, Christensen J, Budnik BA, Abe F, and Jaroszewski JW (2002) In vitro cytotoxic activity of phenanthroindolizidine alkaloids from *Cynanchum vincetoxicum* and *Tylophora tanakae* against drug-sensitive and multidrug-resistant cancer cells. *J Nat Prod* **65:**1299–1302.

Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, and Glass CK (2000) 15-Deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc Natl Acad Sci USA* **97:**4844–4849.

Thieringer R, Fenyk-Melody JE, Le Grand CB, Shelton BA, Detmers PA, Somers EP, Carbin L, Moller DE, Wright SD, and Berger J (2000) Activation of peroxisome proliferator-activated receptor gamma does not inhibit IL-6 or TNF-alpha responses of macrophages to lipopolysaccharide in vitro or in vivo. *J Immunol* **164:**1046–1054.

Trifan OC and Hla T (2003) Cyclooxygenase-2 modulates cellular growth and promotes tumorigenesis. *J Cell Mol Med* **7:**207–222.

Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, and Ham J (1998) Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* **18:**751–762.

Wu P-L, Rao KV, Su C-H, Kuoh C-S, and Wu T-S (2002) Phenanthroindolizidine alkaloids and their cytotoxicity from the leaves of *Ficus septica*. *Heterocycles* **57:**2401–2408.

Yuan CJ, Mandal AK, Zhang Z, and Mukherjee AB (2000) Transcriptional regulation of cyclooxygenase-2 gene expression: novel effects of nonsteroidal anti-inflammatory drugs. *Cancer Res* **60:**1084–1091.

**Address correspondence to:** Dr. S.-J. Lee, Division of Biotechnology and Pharmaceutical Research, 35, Keyan Rd., Zhunan Town, Miaoli County 350, Taiwan, Republic of China. E-mail: slee@nhri.org.tw