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Anti-Inflammatory Mechanisms of Phenanthroindolizidine Alkaloids

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d) Abbreviations:

15d-PGJ2: 15-deoxy-12, 14-prostaglandin J2; ATF-2: activating transcription factor 2;

ERK: extracellular signal-regulated protein kinase; iNOS: inducible nitric oxide synthase;

JNK: c-Jun N-terminal kinase; LBP: LPS binding protein; MAPK: mitogen-activated protein kinase; MEK: MAPK/ERK kinase, MEKK: mitogen-activated protein/ERK

kinase kinase; p38: p38 mitogen-activated protein kinase; PDTC: pyrrolidine

dithiocarbamate; PI3K: phosphoinositide-3 kinase; TAK1: transforming growth factor

beta-activating kinase 1; TNF α : tumor necrosis factor alpha.

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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 LPS/IFN γ 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 γ 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 TNF α , iNOS, and COX-II. It also inhibited the activation of murine iNOS and COX-II promoter activity. However, out of the two common responsive elements of iNOS and COX-II promoters, NF- κ B and AP1, only AP1 activation was inhibited by tylophorine in the LPS/IFN γ 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 and therefore caused the subsequent inhibition of AP1 activity. Furthermore, the tylophorine was also able to block MEKK1 activity and its downstream signaling activation of NF- κ B and AP1. Thus, NSTP0G01 (tylophorine) exerts its anti-inflammatory effects by inhibiting expression of the pro-inflammatory factors and related signaling pathways.

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Introduction

Phenanthroindolizidine alkaloids are a small group of compounds well known for their profound cytotoxic activity (Abe et al., 1998; Pettit et al., 1984; Staerk et al., 2000; Staerk et al., 2002) and thus have been exploited as potential therapeutic leads for anti-cancer agents (Staerk et al., 2002). These alkaloids were also shown to have anti-inflammatory, anti-asthmatic, and anti-anaphylactic properties with consequences of altered immunological status *in vivo* (Ganguly and Sainis, 2001; Gopalakrishnan et al., 1979; Gopalakrishnan et al., 1980; Raina and Raina, 1980; Staerk et al., 2002). Although adenylyl cyclase was stimulated in asthmatic patients' peripheral leukocytes treated with the 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 is 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 instance, NF- κ B-dependent gene expression plays an important role in inflammatory responses and increases the expression of genes encoding cytokines and receptors involved in pro-inflammatory enzymes such as iNOS and COX-II (Giuliani et al., 2001). In addition, AP1, another early transcriptional factor, is also involved in pro-inflammatory response either alone or by coupling with NF- κ B (Adcock, 1997; Giuliani et al.,

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2001). Improper up-regulation of iNOS and/or COX-II have been associated with pathophysiology of certain types of cancers as well as inflammatory disorders (Cross and Wilson, 2003; Ristimäki, 2004; Trifan and Hla, 2003). TNF α is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, anti-tumor responses and infection.

API, NF- κ B, COX-II, TNF α , iNOS, and MAPK (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.

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Materials and Methods

Phenanthroindolizidine alkaloids - Compounds NSTP0G01 (tylophorine), NSTP0G03 (dehydrotylophorine), and NSTP0G07 (ficuseptine-A) (Fig. 1) were isolated from the leaves of *Ficus septica* as described previously and their structures were elucidated (Wu, 2002). NSTP0G08 was derived from unstable NSTP0G07 and verified by LC-Mass and LC-Mass-Mass profiling of NSTP0G08 which exhibited a peak with molecular weight 420 Da while no peak appeared at 455, the molecular mass of NSTP0G07. Moreover, the FAB-Mass of NSTP0G07 also showed the dehydroxylation fragment at m/z 438 and the dehydration fragment at m/z 420 (Data not shown). The loss of 35 mass unit 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 DMEM (HyClone, Inc.) with 4 mM glutamine, 4500 mg/L glucose, 1% non-essential amino acids (Biological industries, Israel) and 10% bovine serum (FetaClone III, HyClone Inc.) 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% CO₂. Chemicals and reagents were purchased from as follow: FuGene6TM, Roche (German); lipopolysaccharide of *E. coli* O111:B4, Chemicon International (California, USA); 15d-PGJ2, Cayman; Chemical (Michigan, USA); SB203580, SP600125, and U0126,

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Biosource (CA, USA); LBP and recombinant IFN γ , R&D systems; LY294002 and PDTC, Sigma Aldrich.

Plasmid and Transfection - pNF κ B-Luc, pFC-MEKK (encoding a. a. 360-672 of MEKK1, Accession no. L13103), and pAP1-Luc plasmids for luciferase reporter assay were obtained from Stratagene Corp. The murine iNOS promoter-Luc and murine COX-II promoter-Luc plasmid were generously provided by Drs. Charles J. Lowenstein (John Hopkins University)(Lowenstein et al., 1993) and Yu-Chih Liang (Taipei Medical University)(Liang et al., 2001) respectively. pCMV- β -gal plasmid containing the *E. coli* β -galactosidase coding sequence was used for transfection efficiency control.

Promoter or element reporter assays - RAW264.7 cells were seeded 9×10^4 cells/well in 24-well plates and grown in medium described above with antibiotics and 4-6 hr later transfected with murine iNOS or COX-II promoter-luciferase reporter plasmids (100 ng/well) and co-transfected with pCMV- β -gal (100 ng/well) using FuGene 6TM (Roche, co.) following the manufacturer's protocol. After 24 h incubation, the medium was replaced with the aforementioned medium containing stimuli of LPS (10 μ g/ml)/IFN γ (20 ng/ml) and test compounds were added at the concentrations indicated. After 18-20 h incubation, the medium was removed and 150 μ l Glo lysis buffer (Promega) was added per well and the resultant lysates were subject to luciferase and β -galactosidase assay as per manufacturer's recommendation (Tropix). Transfection efficiency was normalized by

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β -galactosidase activity.

For AP1 and NF- κ B reporter assays, 10^6 RAW264.7 cells/well were seeded on 24-well plates for 4-6 h before transfection with pNF- κ B-Luc and pCMV- β -gal plasmids, 100 ng each/well, or pAP1-Luc alone 200 ng/well. In similar experiments with co-transfected MEKK1, the cells were transfected with pNF κ B-Luc (100 ng) or pAP1-Luc (100 ng) along with pFC-MEKK (50 ng) and/or pCMV- β Gal (50 ng). The following day, the transfected RAW264.7 cells were washed twice with culture medium with or without serum as experimental design. Subsequently, cells were concurrently treated with LPS (10 μ g/ml, final concentration), which was pre-incubated with LBP (100 ng in the final concentration) for 1 h at 37°C, and IFN γ (20 ng/ml) as well as the indicated phenanthroindolizidine alkaloids, 15d-PGJ2, PDTC, LY294002, or SB203580 for another 5 h and subsequently the cell extracts were assayed for luciferase and β -galactosidase activity or total amount of protein. The luciferase activity was normalized with β -galactosidase activity or total amounts of protein. For NF- κ 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 omitted NEAA.

Luciferase and β -galactosidase assays - Luciferase and β -galactosidase assays were performed respectively using a Steady-Glo luciferase assay system (Promega) and Galacto-Star (Tropix) according to the manufacturer's instructions. Luminescence was

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measured in a TopCount.NXT™ Microplate Scintillation and Luminescence Counter (Packard, Inc.).

Carcinoma Cell Growth Inhibitory Assay - HONE-1 and NUGC-3 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (Biological Industries Inc.) and were seeded 4,500 and 6,000 cells/well respectively in 96-well plates and incubated in a CO₂ incubator at 37°C for 24 h. The cells were treated with at least five different concentrations of test compounds in a CO₂ incubator for 72 h. The number of viable cells was estimated using the tetrazolium dye reduction assay (MTS assay) and the experiment was performed as manufacturer recommended (Promega, Madison, WI, USA). The results of these assays were used to obtain the dose-response curves from which GI₅₀ 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 plate. After 24 h incubation, the medium was replaced with complete medium containing stimuli of LPS (10 µg/ml)/IFNγ (20 ng/ml) and the test compounds were added at the various concentrations as indicated. After 18-24 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

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and determined spectrophotometrically with Griess reagent at OD₄₀₅.

The attached cells were subjected to cytotoxicity measurement using MTS assay (Promega). The IC₅₀ of nitric oxide production and cytotoxicity were determined from respective dose-response curves.

Cytokine Measurement –TNF α protein was detected in cell culture supernatant that was diluted to proper concentrations for assay using respective ELISA kit from R & D Systems Inc. (USA) as manufacturer recommended. The amounts of NO and relative viable cell numbers were determined by Griess and MTS assays respectively as aforementioned for inter and intra-experimental controls (data not shown).

Western Blotting - iNOS, COX-I, COX-II, and β -actin proteins were analyzed by immunoblotting with anti-iNOS (Biomol), anti-COX-I (Upstate), anti-COX-II (Upstate), and anti- β -actin (Chemicon) antibodies respectively. The antibodies against pan and phosphorylated Akt, c-Jun, and ATF-2 were purchased from Cell Signaling Technology. The antibodies against the pan protein of JNK, p38, or ERK1/2, were purchased from Biosource and antibodies for the respective phosphorylated proteins were from Promega. The cell lysates with equal amounts of total protein were subject to SDS-PAGE 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 accordingly as

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manufacturer recommended. Antigen-antibody complexes were detected using ECL detection reagents (Perkin Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instruction.

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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 nitric oxide (NO) at the concentration range of 60-100 μ M upon stimulation with LPS/IFN γ was used as a model of *in vitro* acute inflammation system. The background nitrate/nitrite generated by the control un-stimulated RAW264.7 cells ranging from ~2 to 4 μ M was excluded from the LPS/IFN γ stimulated NO production. Both NSTP0G01 (tylophorine) and NSTP0G07 (ficuseptine-A) exhibited significant inhibition in NO production in RAW264.7 cells stimulated by LPS/IFN γ with IC₅₀s of 1.8 and 2.1 μ M, respectively (Table 1A). This potency is comparable to that of 15d-PGJ2 (NO suppression IC₅₀ 2.2 μ M), a prostaglandin D2 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 IC₅₀s of 54.5 and 14.0 μ M, respectively. All four alkaloids did not exhibit significant growth inhibition toward LPS/IFN γ stimulated RAW264.7 cells at the concentration of 10 μ M with NSTP0G01 exerting a weak growth inhibition with a GI₅₀ of 8.2 μ M.

NSTP0G01 and NSTP0G07 were reported to inhibit cancer cell growth *in vitro* in a cytotoxicity study against various cancer cell lines (Wu, 2002). NSTP0G08, a novel

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compound with a planar structure derived from NSTP0G07, exerted potent growth inhibition at 4 $\mu\text{g/ml}$ ($\sim 10 \mu\text{M}$) with 72% and 82% inhibition on HONE-1 and NUGC-3 cancer cells as compared to 82% and 86% exhibited by NSTP0G07 at the same concentration, respectively (Table 1B). NSTP0G01 and NSTP0G08 displayed GI_{50}s of 0.96 μM and 1.71 μM for HONE-1 and 1.00 μM and 1.60 μM 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 μM .

The Effects of the Phenanthroindolizidine on the Expression of $\text{TNF}\alpha$, iNOS and COX-II – Next we examined the effects of the phenanthroindolizidine alkaloids on the induction of pro-inflammatory mediators, $\text{TNF}\alpha$, iNOS, and COX-II in LPS/ $\text{IFN}\gamma$ stimulated RAW264.7 cells to explore the extent of their anti-inflammatory effects (Fig. 2A & 2B). The $\text{TNF}\alpha$ protein was induced to produce at the level of 180-200 ng/ml upon stimulation with LPS/ $\text{IFN}\gamma$ in RAW264.7 cells. This induction was decreased about $\sim 75\text{-}90\%$ by the treatment of NSTP0G01 but only moderately inhibited ($\sim 15\text{-}30\%$) by the treatment of NSTP0G03 at the concentrations of 3 to 10 μM while 15d-PGJ2 exerted no significant effect (Fig. 2A). The protein expression levels of iNOS and COX-II was enormously induced upon stimulation of LPS/ $\text{IFN}\gamma$ in RAW264.7 cells and dramatically inhibited after 18 h treatment with NSTP0G01, but NSTP0G03 did not show similar effect (Fig. 2B). 15d-PGJ2, a prostaglandin D2 metabolite and $\text{PPAR}\gamma$ agonist, was reported to be

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capable of exerting anti-inflammation effects in NF- κ B dependent and in-dependent manners (Chawla et al., 2001; Straus et al., 2000) and inhibiting NO production and iNOS expression (Ricote et al., 1998). It also inhibits TNF α production at the concentrations of 25 μ M in similar conditions to herein used (Thieringer et al., 2000). Therefore, 15d-PGJ2 significantly inhibited the protein expression of iNOS but not TNF α at 10 μ M concentration as expected and it did not significantly inhibit the COX-II protein expression. On the other hand, our results showed that NSTP0G01 is capable of inhibiting the induced expression of TNF α , 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 α , iNOS and COX-II were obtained from murine primary peritoneal macrophages elicited by thioglycollate *in vivo* and stimulated with LPS/IFN γ *in vitro* (data not shown).

The Effects of the Phenanthroindolizidine 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 γ (Fig. 2C & 2D). The activation of murine iNOS promoter activity upon stimulation of LPS/IFN γ was significantly inhibited by NSTP0G01 (~90%)

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and 15d-PGJ2 (~75%), but NSTP0G03 did not show any significant effect. Similarly, murine COX-II promoter activity upon stimulation with LPS/IFN γ was significantly affected by the NSTP0G01 treatment at the concentration of 10 μ M with ~80% inhibition. Moreover, both iNOS and COX-II promoter activities induced upon stimulation of LPS/IFN γ were inhibited by NSTP0G01 in a dose dependent manner with IC₅₀s of ~1.2 and ~0.6 μ M respectively. The above results suggest that NSTP0G01 exerts its inhibitory effect in the transcriptional events of iNOS and COX-II and thus resulting in the reduced protein expression levels.

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

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Our results showed that NSTP0G01 and NSTP0G03 did not inhibit the NF- κ B activation at the concentration of 10 μ M in contrast to 15d-PGJ2 (10 μ M) and PDTC (50 μ M)(an NF- κ B inhibitor) which exert ~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 μ M did not inhibit NF- κ B activation although 10 μ M of 15d-PGJ2 completely (~100%) blocked the activity and 50 μ M of PDTC exerted ~20 % inhibitory effect (data not shown).

On the other hand, NSTP0G01 inhibited the AP1 activation in RAW264.7 upon stimulation with LPS/IFN γ by about 86% at the concentration of 10 μ M and in a dose dependent manner with an IC₅₀ of ~2.3 μ M (Fig. 3B). However, NSTP0G03 and 15d-PGJ2 did not exhibit any inhibition under similar conditions. In addition, SB203580 (a p38 MAPK specific inhibitor) also exhibited a moderate inhibition (~35%) in the AP1 activation at the concentration of 10 μ M. Transfection did not affect the induction of TNF α , iNOS, and COX-II in RAW264.7 cells upon LPS/IFN γ stimulation and the effects of NSTP0G01, NSTP0G03, and 15d-PGJ2 on these inductions (data not shown).

The Effects of Phenanthroindolizidine on MEKK1 Specifically Triggered Activation of AP1

and NF κ B – From above 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 γ . However, in consideration of that LPS triggers multiple signaling pathways

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including TAK1, MEKK1, and PI3K for NF- κ 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 talking for signaling amplification or counteraction between these signaling cascades may mask the effect of NSTP0G01 on NF- κ B activation. Therefore, we further examined the effect of NSTP0G01 on the MEKK1 triggered activation of NF- κ B and AP1 since MEKK1 involved in upstream regulation of both NF- κ B and AP1. The effect of compound treatment on the activation of NF- κ B or AP1 by constitutively active MEKK1 (c-MEKK1) was measured by comparison of accumulated reporter luciferase activity between un-treated and treated samples in over period of 5 h of which total activity was used as 100% for data analysis. The activation of NF- κ B by transfected c-MEKK1 was inhibited by NSTP0G01 in a dose dependent manner with an IC_{50} of ~ 3.1 μ M resulting in $\sim 73\%$ inhibition at 10 μ M concentration. In similar conditions, it was $\sim 99\%$ inhibited by 50 μ M of PDTC, but NSTP0G03 and 15d-PGJ2 did not show any significant inhibition at the 10 μ M concentration (Fig. 3C). Moreover, NSTP0G01 also inhibited the AP1 activation by the over-expressed c-MEKK1 in a dose dependent manner with an IC_{50} of ~ 2.1 μ M, completely blocking it at the concentration of 10 μ M (Fig. 3D). In contrast, NSTP0G03, 15d-PGJ2, and p38 inhibitor SB203580 did not inhibit the AP1 activation by the over-expressed c-MEKK1. Thus, NSTP0G01 was suggested to inhibit MEKK1 activity consequently inhibiting induced activation of AP1

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and NF- κ B.

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- κ B in RAW264.7 cells stimulated by LPS/IFN γ . The phosphorylation of Akt, JNK, ERK1/2, and p38 in RAW264.7 cells upon stimulation of LPS/IFN γ were 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- κ B (PDTC) in activation of these signaling molecules. NSTP0G01, compared to NSTP0G03, SB203580, SP600125, U0126, and PDTC, significantly enhanced the phosphorylation of Akt in LPS/IFN γ stimulated RAW264.7 cells, even at the lower concentrations of 1 and 3 μ M (Fig. 4B). However, NSTP0G01 and NSTP0G03 did not exert any significant inhibitory effect on the phosphorylation of p38, ERK1/2, and JNK while 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 effect on the phosphorylation of Akt, p38, JNK, and ERK1/2 at the concentrations of 3 and 10 μ M (data not shown).

In conjunction treatment of NSTP0G01 and LY294002 inhibited NF- κ B activation in

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LPS/IFN γ stimulated RAW264.7 cells- Phosphorylation of Akt leads to the activation of NF- κ B (Guha and Mackman, 2001). LY294002, a PI3K/Akt inhibitor, dramatically decreased the amounts of phosphorylated Akt in LPS/IFN γ 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- κ B activation of LPS/IFN γ stimulated RAW264.7 with or without conjunction of over expressed c-MEKK1. In LPS/IFN γ stimulated RAW264.7 cells, LY294002 did not significantly inhibit NF- κ B activation (~18% inhibition) while NSTP0G01 moderately augmented (~35%) NF- κ B activation (Fig. 3A & 5A). However, NF- κ B activation was inhibited to the extent of 53-38% when the cells were treated with NSTP0G01 and LY294002 in conjunction (Fig. 5A). The LY294002 was suggested to decrease the phosphorylation of Akt enhanced by NSTP0G01 in NSTP0G01 and LY294002 treated LPS/IFN γ stimulated RAW264.7 cells and thus resulted in the inhibition of NF- κ B activation while NSTP0G01 also simultaneously inhibited the MEKK1 activity.

In contrast, in LPS/IFN γ stimulated and c-MEKK1 over-expressed RAW264.7 cells, NSTP0G01 alone was able to inhibit about ~70% of NF- κ B activation (Fig. 5B). This extent of inhibition was comparable to that observed in un-stimulated RAW264.7 cells with over expressed c-MEKK1 only (Fig. 3C). As expected, no significant inhibition in NF- κ B activation was obtained by LY294002 treatment alone since NF- κ B activation was mainly

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driven by the over expressed 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- κ B activation between the respective LPS/IFN γ stimulated and c-MEKK1 over-expressed RAW264.7 cells. Therefore, the dual effects of NSTP0G01, inhibiting MEKK1 and enhancing Akt phosphorylation, resulted in it not inhibiting NF- κ B activation in LPS/IFN γ stimulated RAW264.7 cells (Fig.3A&C, 5A, 7A).

LY294002 restored the NSTP0G01 inhibited AP1 activity - The expression and phosphorylation of c-Jun, 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/IFN γ stimulated RAW264.7 cells and were examined within 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 while it increased the phosphorylation of ATF-2 compared to those of the stimulated control. LY294002 treatment alone had no significant effect on the ATF-2 expression and phosphorylation and slightly increased those of c-Jun. Interestingly, the expression and phosphorylation of c-Jun

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by the treatment of the LPS/IFN γ 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 can 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 co-treatment with LY294002.

The restored c-Jun expression and phosphorylation in the co-treatment 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 co-treatment with LY294002 from ~12% back to ~95% (Fig. 6B). Thus, the enhanced phosphorylation of Akt by NSTP0G01 in LPS/IFN γ 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*.

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Discussions

The phenanthroindolizidine alkaloids have been subjected to clinical trials either in the form of pure compounds, such as tylocrebrine for anti-cancer, or of alkaloids extracts or leave powders etc. for anti-bronchial asthmatics (Huntley and Ernst, 2000; Staerk et al., 2000; Staerk et al., 2002). The trials using tylocrebrine for anti-cancers were withdrawn due to nervous side effects in 1960s (Staerk et al., 2000). Recently tylophorine analogues have again attracted attention for drug development and were proposed to exert anti-tumor effects in a novel mode of action (Gao et al., 2004). Tylophorine analogues were found to inhibit the activity of CRE, AP1, and NF- κ B in HepG2 lung carcinoma cells treated with forskolin, TPA, and TNF α respectively. However, more evidences may be needed to bolster the relationship between anti-tumor activity and cytotoxicity and the inhibitions in the activation of CRE, AP1, and NF- κ B by these tylophorine analogues. Additionally, 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 anti-cancer activity (Rao et al., 1997; Rao and Venkatachalam, 2000).

On the other hand, the mechanisms responsible for anti-inflammation or anti-asthmatics of the phenanthroindolizidine alkaloids are not clear as yet. “*Tylophora indica*” has been used for decades in India in connection with the inflammatory related

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conditions, e.g. asthma, bronchitis, bronchial asthma, hay fever, and rheumatism. The major alkaloid of *Tylophora 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 in order to elucidate its efficacies in a variety of anti-inflammatory related therapies.”

Up to date, all effective phenanthroindolizidine alkaloids reported for anti-carcinoma activity are angular molecules (Gao et al., 2004; Komatsu et al., 2001; Lee et al., 2003; Rao and Venkatachalam, 2000; Staerk et al., 2000; Staerk et al., 2002). As far as we are aware, this is the first report describing a planar compound of phenanthroindolizidine alkaloids could exert significant cytotoxicity to carcinoma cells. The planar structure of NSTP0G08 as compared to the angular counterpart of the NSTP0G07 may account for reduction in its anti-inflammatory property. Interestingly, a similar difference between NSTP0G03 and NSTP0G01 caused a significant decrease in both anti-inflammatory and anti-cancer 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

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also exhibit similar relative activity (unpublished data, will publish elsewhere). However, more phenanthroindolizidine alkaloids with similar planar and angular structures need to be studied to validate this postulate.

NSTP0G01 (tylophorine) in contrast to NSTP0G03 significantly inhibits several induced expression of pro-inflammatory factors, e.g. iNOS, COX-II, and TNF α . NF- κ B 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- κ B and AP1 (Fig. 3C&D), only AP1 activation was inhibited by NSTP0G01 in the LPS/IFN γ stimulated RAW264.7 cells (Fig. 3A&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 talks between overlapping signaling of Akt, MEKK1, c-Jun/AP1, and NF κ B, could compromise some cellular events (Aikin et al., 2004; Cerezo et al., 1998; Funakoshi-Tago et al., 2003; Go et al., 2001; Guha and Mackman, 2001; Levresse et al., 2000; Li et al., 2004; Shimoke et al., 1999). Therefore, the NSTP0G01 exerts dual functions of enhancing Akt activation and inhibiting MEKK1 explaining the opposite results of AP1 and NF κ B activation affected by NSTP0G01 in the LPS/IFN γ stimulated RAW264.7 cells (Fig. 3A&B, 7A). Thus, in the presence of LY294002 and NSTP0G01, when the Akt activation is

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counteracted between these two compounds, the NF κ B activation is inhibited (Fig. &7B &7C). Thus, we have provided novel insights 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 interplays between Akt and JNK or c-Jun in apoptosis or stress-induced inflammation are diverse. Change in Akt activity increases or decreases the JNK activation or the subsequent effect in c-Jun phosphorylation in different cell contents (Aikin et al., 2004; Cerezo et al., 1998; Funakoshi-Tago et al., 2003; Go et al., 2001; Levresse et al., 2000; Li et al., 2004; Shimoke et al., 1999). Despite the relevant relation between the JNK activation and c-Jun phosphorylation that cross talks with Akt pathway or not, the c-Jun activation 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 the LPS/IFN γ stimulated macrophage cells (Fig. 4B, Fig. 6, & Fig.7). It is also the first time revealing that this interplay induced by NSTP0G01 (tylophorine) plays an important role in the anti-inflammation process.

Taking together the differential between NSTP0G01 and NSTP0G03 in their cytotoxicity toward cancer cells and anti-inflammatory effect, our results also demonstrate

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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. More understanding of the effective cellular mechanisms of NSTP0G01 and other similar effective compounds compared to in-effective ones e.g. NSTP0G03 will lead to identification of more effective analogues of phenanthroindolizidine alkaloids with less un-favored 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 insights for selecting more proper assays for structure-activity relationship analysis of phenanthroindolizidine alkaloids and its biological functions

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Acknowledgement

We sincerely acknowledge Dr. Weir-Torn Jianng for his LC-Mass spectrum analysis of NSTP0G08, Mr. Ta-Hsien Chuang in preparation of part of NSTP0G01, Dr. Hwan-You Chang and Dr. Neeraj Mahindroo's help in proofreading and valued suggestion in preparation of this manuscript.

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Footnotes

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Legends of Figures:

Figure 1. Chemical structures of phenanthroindolizidine alkaloids.

Figure 2. Effects of phenanthroindolizidine alkaloids on the protein expression of

TNF α (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 γ concurrently treated

with 3, 5 and 10 μ M of indicated phenanthroindolizidine alkaloids or 15d-PGJ2. The

amounts of TNF α generated upon LPS/IFN γ stimulation were used as 100% for

comparison of the compound treatment effects. Data from three or more experiments

were expressed as means \pm S.D..

B: RAW264.7 cells were cultured in the presence of concurrent treatment of LPS /IFN γ

and 10 μ M of indicated phenanthroindolizidine alkaloids or 15d-PGJ2. After 18 h, cell

extracts were collected and subject to Western analysis for the protein expression of iNOS

and COX-II as well as β -actin and COX-I. Results shown were the representatives of

three independent experiments.

C & D: RAW264.7 cells were transfected with murine iNOS and COX-II

promoter-luciferase reporter plasmids respectively. The promoter activity generated upon

LPS/IFN γ stimulation was used as 100% (~8,000 and ~10,000 RLU for iNOS and

COX-II promoters respectively) for comparison of compound treatment effects. The

values were represented as means \pm S.D. of three or more independent experiments. 15d:

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15d-PGJ2, G01: NSTP0G01, G03: NSTP0G03. (*P<0.005 & **P<0.05 *versus* LPS/IFN γ stimulated only)

Figure 3. Effects of phenanthroindolizidine alkaloids on the activation of NF- κ B and AP1 in RAW264.7 cells.

A & B. Effects of phenanthroindolizidine alkaloids on NF- κ B (A) and AP1(B)–dependent reporter gene expression in LPS/IFN γ stimulated RAW264.7 cells. The NF- κ B or AP1 activity generated upon LPS/IFN γ stimulation were used as 100% (~4,000 and ~8,000 RLU for AP1 and NF κ B respectively) for comparison of compound treatment effects. The values were represented as means \pm S.D. of three or more experiments.

C & D. Effects of phenanthroindolizidine alkaloids on NF- κ B (C) and AP1 (D)–dependent reporter gene expression in RAW264.7 cells over-expressing c-MEKK. RAW264.7 cells were transfected with pNF- κ B –Luc (or pAP1-Luc), pCMV- β Gal and pFC-MEKK plasmids, 100, 50,50ng/well each. The total luciferase activity without treatment generated during the period of 5 h was used as 100% (~12,000 and ~25,000 RLU for AP1 and NF κ B respectively). The luciferase activity was normalized with total amount of protein not β -galactosidase activity since β -galactosidase activity was evidently regulated in this experimental condition (Data not shown). The values were represented as means \pm S.D. of three or more experiments. 15d: 15d-PGJ2, G01: NSTP0G01, G03: NSTP0G03. (*P<0.005 & **P<0.05 *vs* LPS/IFN γ stimulated only)

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Figure 4. Effects of phenanthroindolizidine alkaloids on the expression and

phosphorylation of proteins involved in NF- κ B and AP1 activation. A: The

phosphorylation of Akt , p38, ERK1/2, and JNK upon the LPS/IFN γ stimulation in

RAW264.7 cells exhibited dynamic response within 60 min's treatment. LPS /IFN γ

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 γ

stimulated RAW264.7 cells. The effect of indicated compound treatment in the

phosphorylated and pan proteins of Akt, p38, ERK1/2, and JNK were examined at the

time point of 30 min 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 were the representatives of two or three independent

experiments.

Figure 5. LY294002 inhibited the increased NF- κ B activation by NSTO0G01 in

LPS/IFN γ stimulated RAW264.7 cells. A: NSTP0G01 significantly inhibited the NF- κ B

activation only when treated in conjunction with LY294002 in LPS/IFN γ stimulated

RAW264.7 cells. B. NSTP0G01 but not LY294002 inhibited MEKK1 signaling NF- κ B

activation in LPS/IFN γ stimulated RAW264.7 cells. LPS/IFN γ stimulated cells were

concurrently treated with the indicated compounds and concentrations. See "Materials

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and Methods” for the experimental procedure. The values were represented as means \pm S.E. of two independent experiments, each in triplicate. (*P<0.005 & **P<0.05 vs LPS/IFN γ stimulated only, #P<0.05 & ##P<0.01 vs LPS/IFN γ stimulated and NSTP0G01 treated)

Figure 6. LY294002 restored the inhibited AP1 activation by NSTP0G01. A: The effects of NSTP0G01, and LY294002 on the c-Jun and ATF-2 expression and phosphorylation in LPS/IFN γ stimulated RAW264.7 cells. LPS/IFN γ stimulated cells were concurrently treated with the indicated compound at the concentration of 10 μ M. 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/IFN γ stimulated RAW264.7 cells. See “Materials and Methods” for the experimental procedure. LPS/IFN γ stimulated cells were concurrently treated with the indicated compounds and concentrations. The values were represented as means \pm S.D. of three independent experiments, each in triplicate. (*P<0.005 vs LPS/IFN γ stimulated only, #P<0.05 vs LPS/IFN γ stimulated and NSTP0G01 treated)

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Figure 7. Diagrams for illustrating the effective points and outcomes by the treatment of NSTP0G01 or LY294002 in LPS/IFN γ stimulated RAW264.7 cells. All the effective points and outcomes are compared to those in LPS/IFN γ stimulated RAW264.7 cells with no compound treatment. In the LPS/IFN γ stimulated RAW264.7 cells, the AP1 and NF- κ B are activated (Fig. 3) and moderate Akt phosphorylation is observed (Fig.4) as compared to those in RAW264.7 cells with no LPS/IFN γ stimulation. Highlighted in red indicate the effective points and green show the outcomes. “ \uparrow ” indicates significant enhancement or increase in activation or signaling, “ \downarrow ” for significant decrease, “ \perp ” for blocking or decrease, \blacktriangle for moderate increase, and “—” for no significant change. The pathways in black are mainly referenced from Guha & Mackman, 2001 (Guha and Mackman, 2001) and incorporated with results reported herein in red and green.

Table 1A

| Compound | NO suppression IC ₅₀ (μM) | Cytotoxicity GI ₅₀ (μM) |
|----------|---|---------------------------------------|
| 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-PGJ2 | 2.18 +/- 0.80 | >10 |

Table 1B

| | HONE-1 | | NUGC-3 | |
|----------|------------|-----------------------|------------|-----------------------|
| Compound | 4μg/ml (%) | GI ₅₀ (μM) | 4μg/ml (%) | GI ₅₀ (μM) |
| 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-PGJ2 | ND | 15.86+/-2.68 | ND | 21.83+/-1.90 |

*ND: Not determined.

Table 1. Biological activities of phenanthroindolizidine alkaloids. A: IC₅₀s and GI₅₀s

(μM) of phenanthroindolizidine alkaloids for suppression of nitric oxide production

(IC₅₀s) and growth inhibition (GI₅₀s) against LPS/IFNγ stimulated RAW264.7.

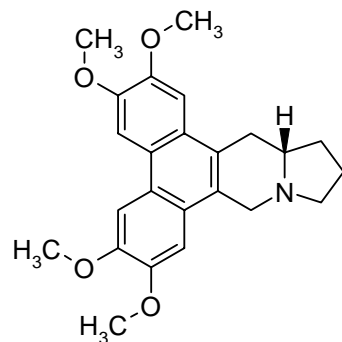
RAW264.7 cells were cultured in the presence of LPS/IFNγ concurrently treated with

different concentrations of indicated phenanthroindolizidine alkaloids or 15d-PGJ2 for 18-20 hours. The amounts of NO in the culture medium generated upon LPS/IFN γ stimulation were used as 100% for comparison of the compound treatment effects and calculation for the IC₅₀s and the adherent cells were subject to MTS for GI₅₀ measurement.

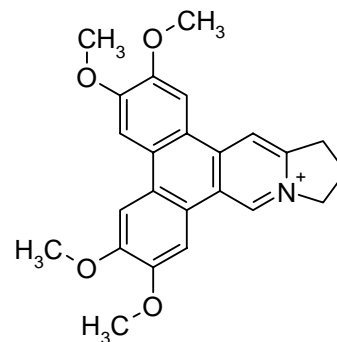
B: Growth inhibition of phenanthroindolizidine alkaloids against cancer cell lines, HONE-1 and NUGC-3. Cells, seeded a day before, were cultured with or without compound treatment for three days before subjected to MTS assay for GI₅₀ or % inhibition measurement. See Materials and Methods for the details. Data from three experiments or more were expressed as means \pm S.D..

Figure 1

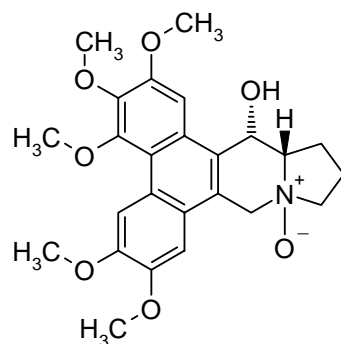
NSTPOG01
(Tylophorine)



NSTPOG03
(Dehydrotylophorine)



NSTPOG07
(Ficuseptine-A)



NSTPOG08

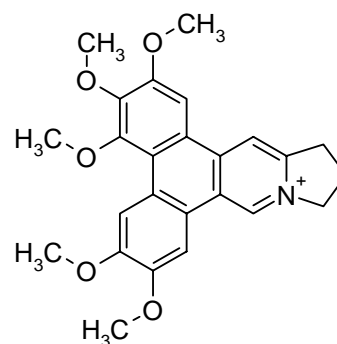


Figure 2A

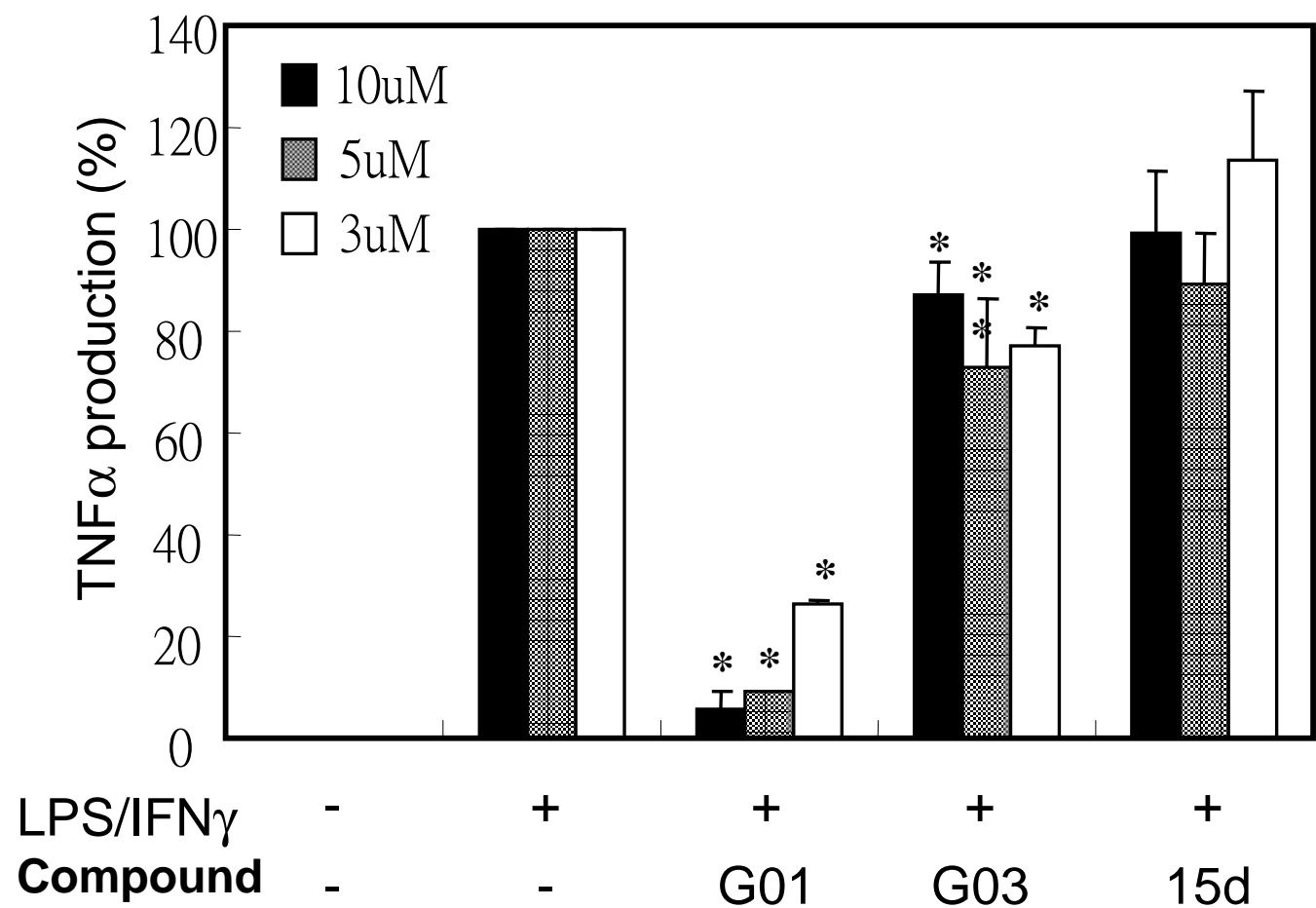


Figure 2B

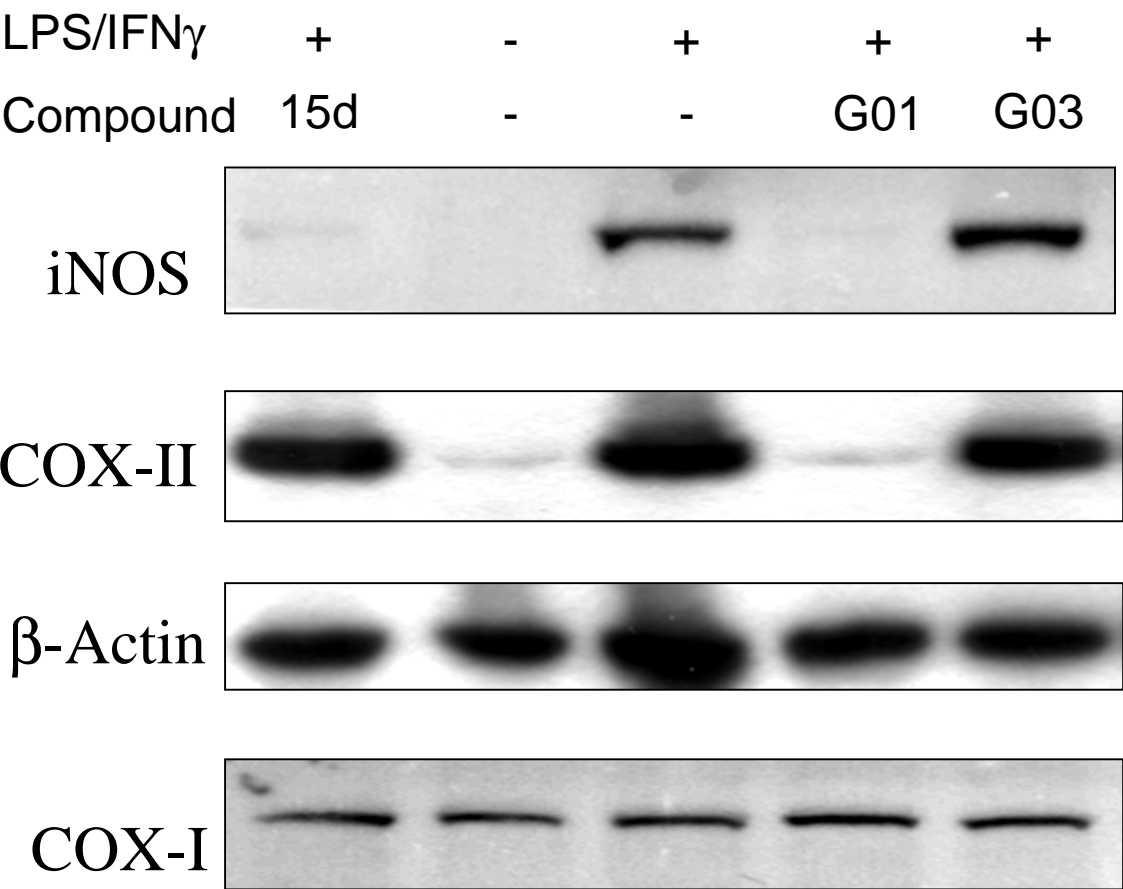


Figure 2C

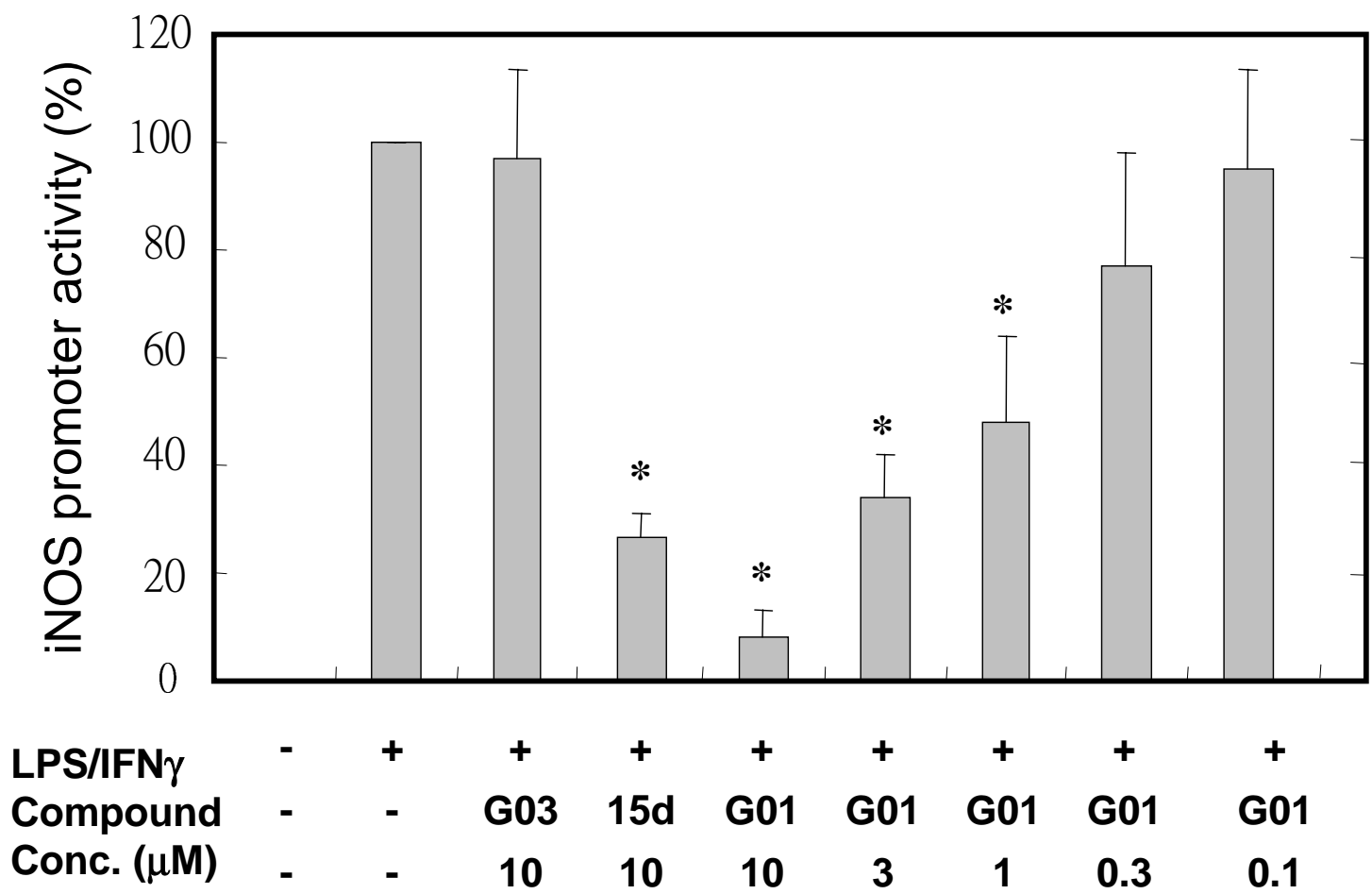


Figure 2D.

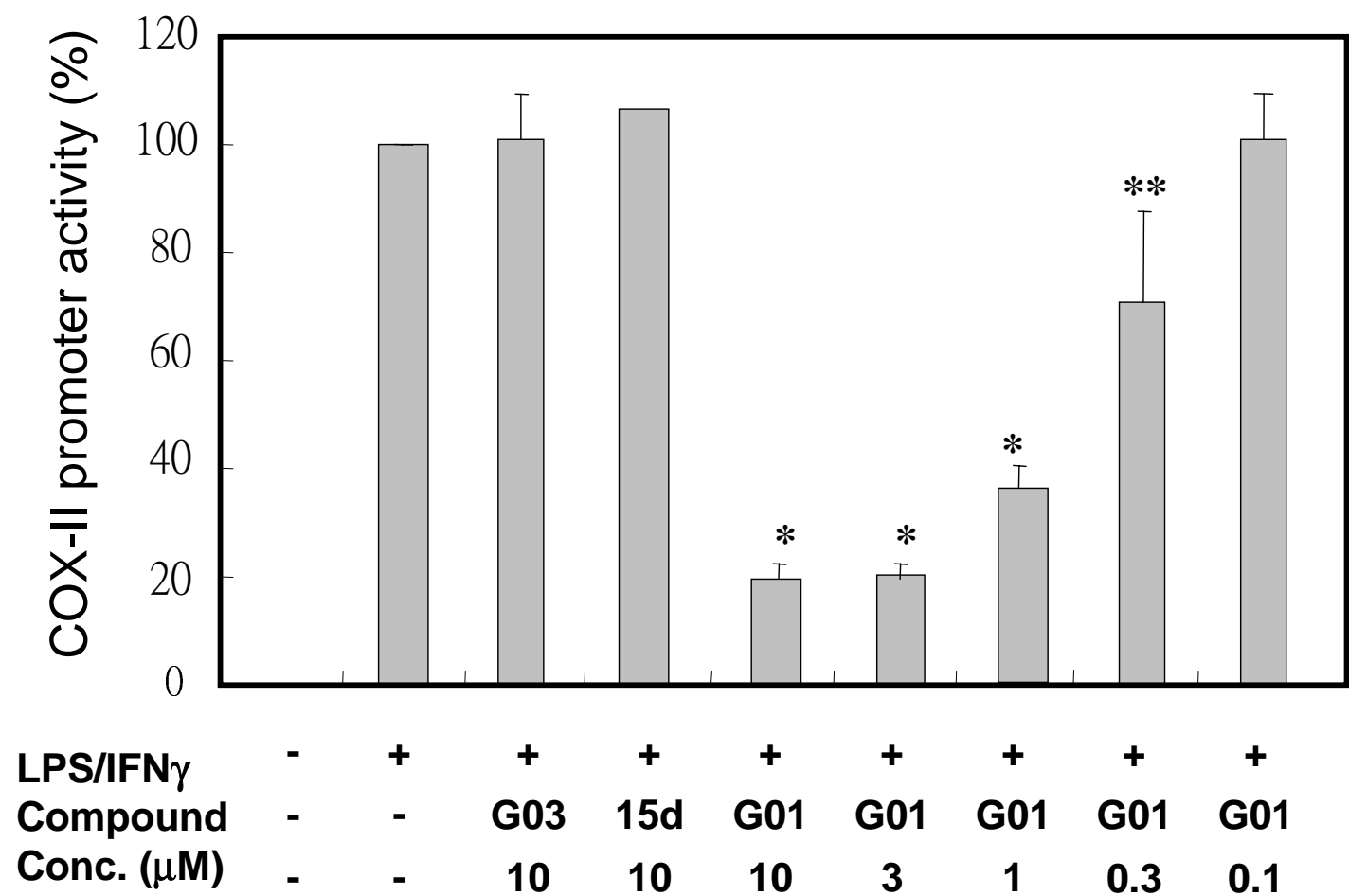


Figure 3A

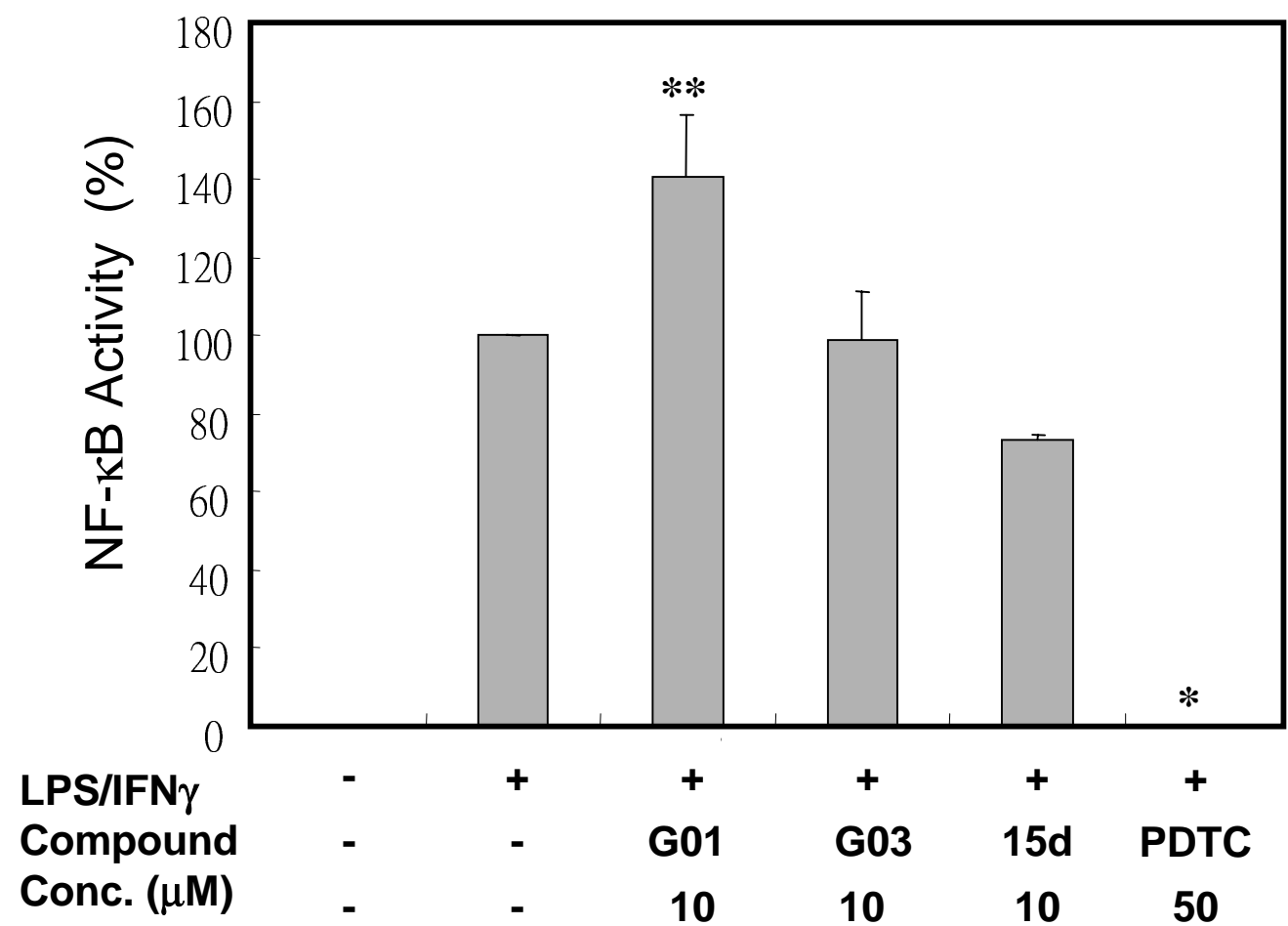


Figure 3B

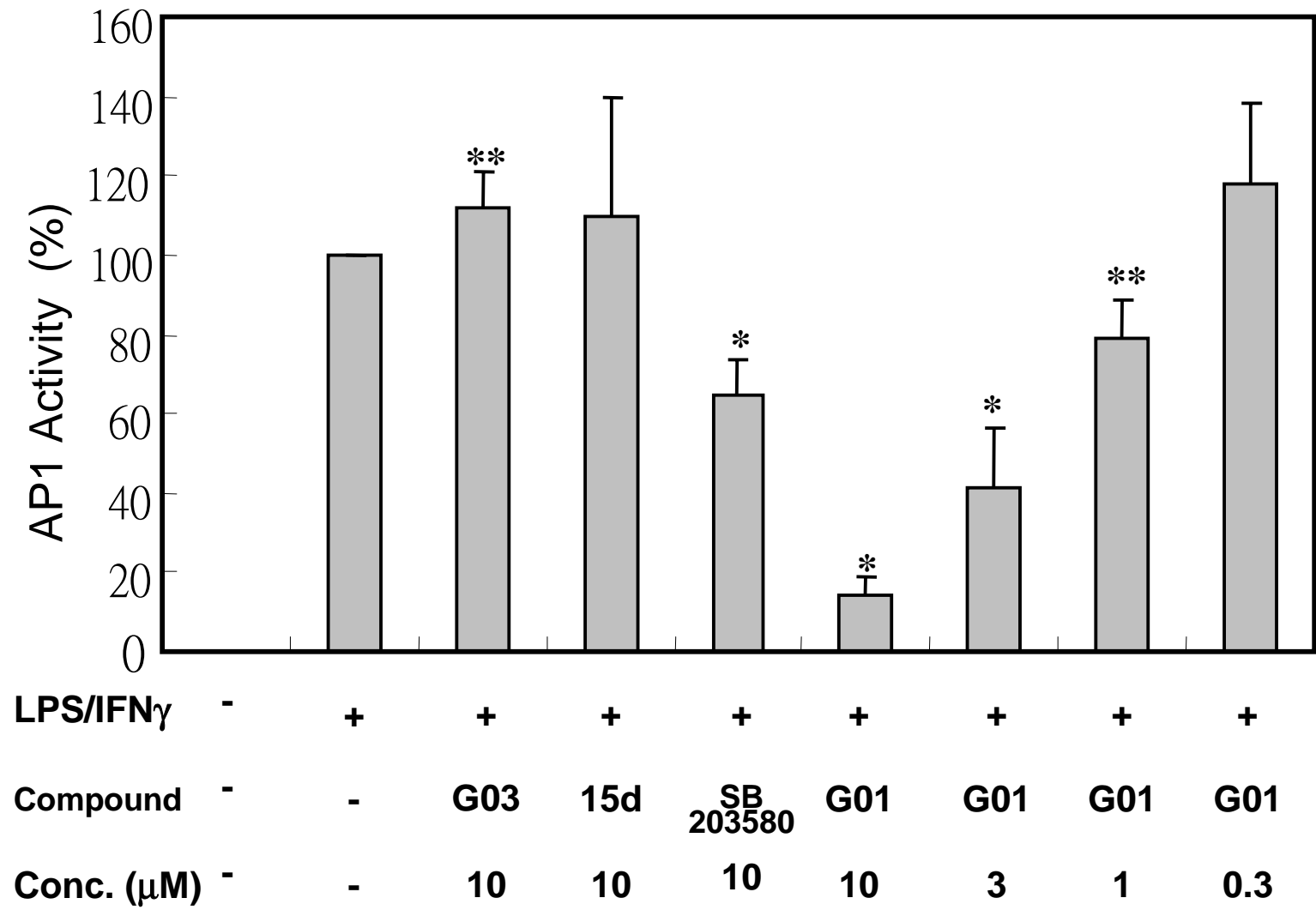


Figure 3C

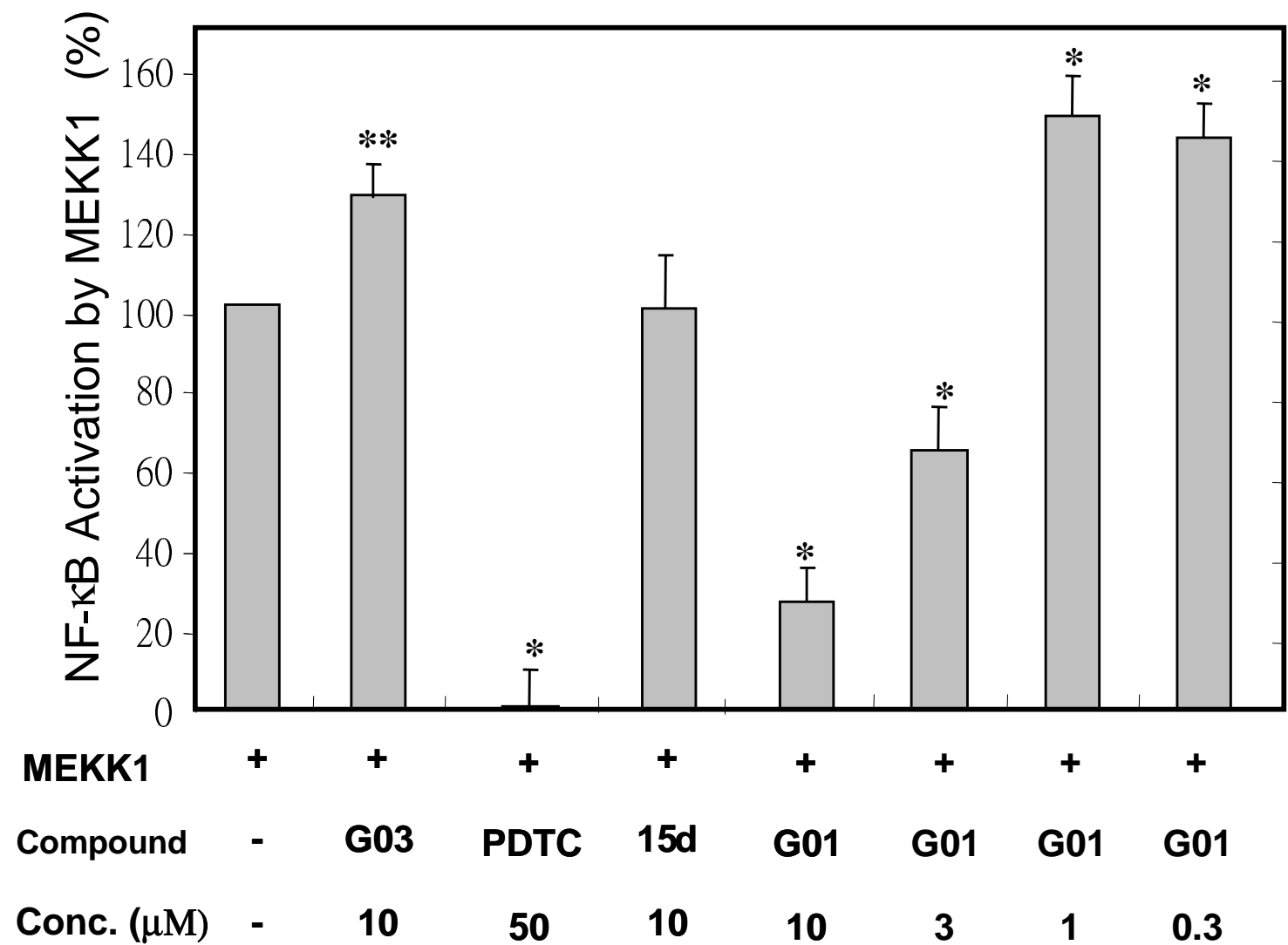


Figure 3D

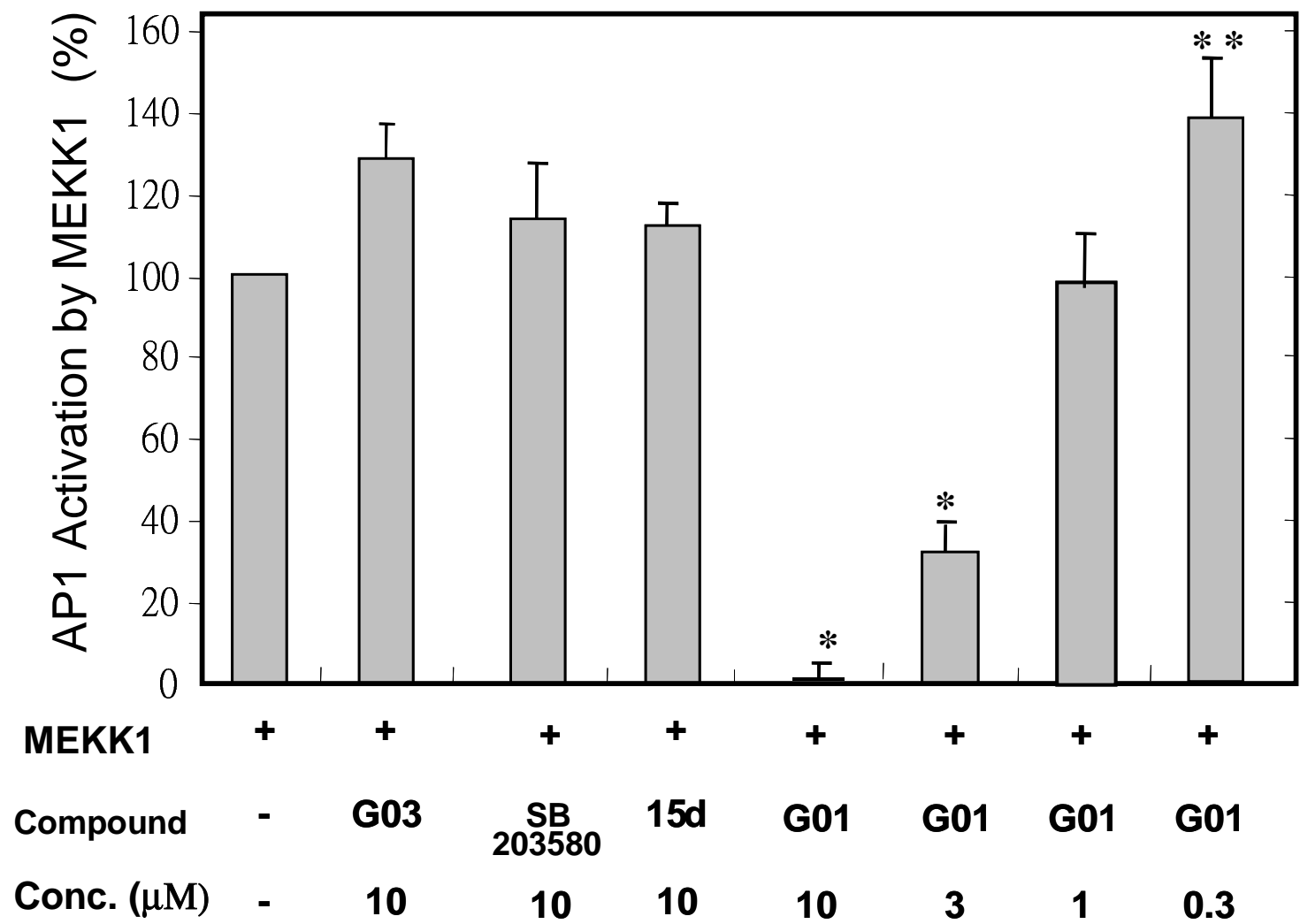


Figure 4A

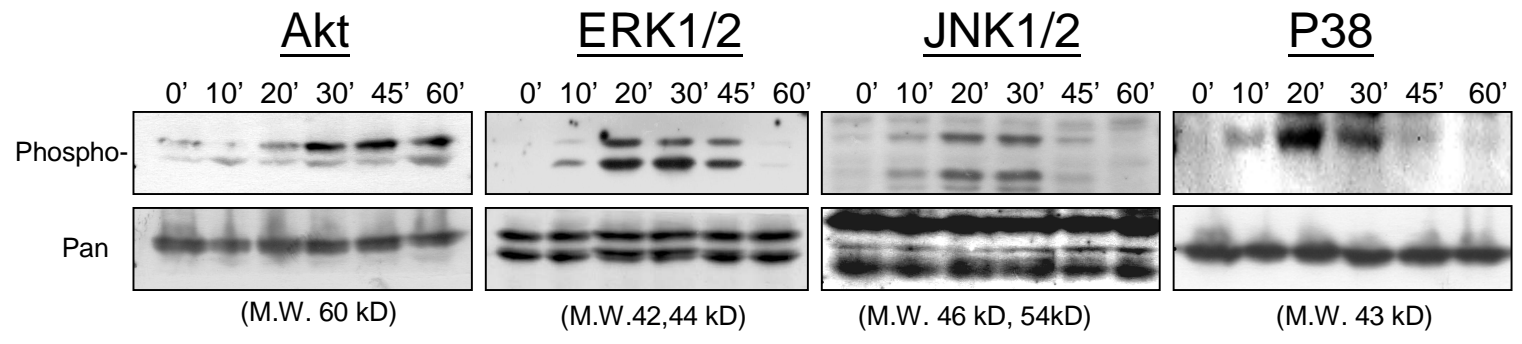


Figure 4B-1

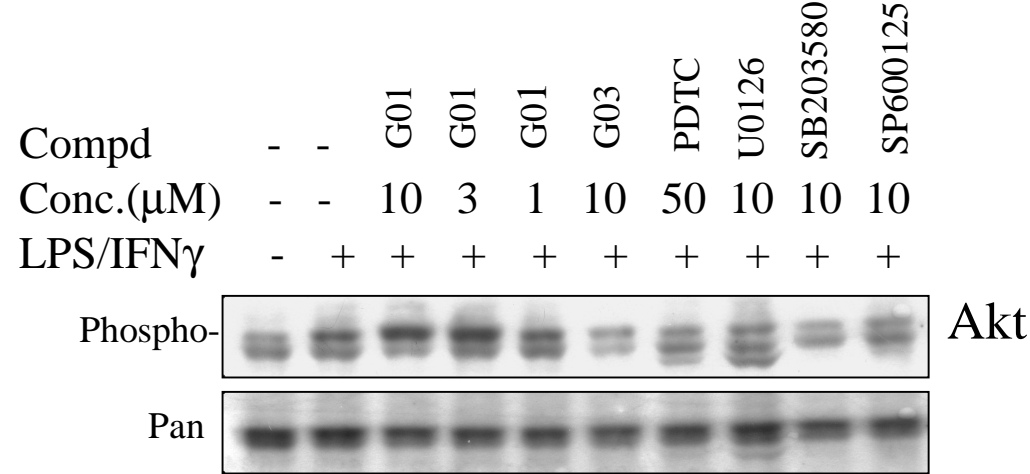


Figure 4B-2

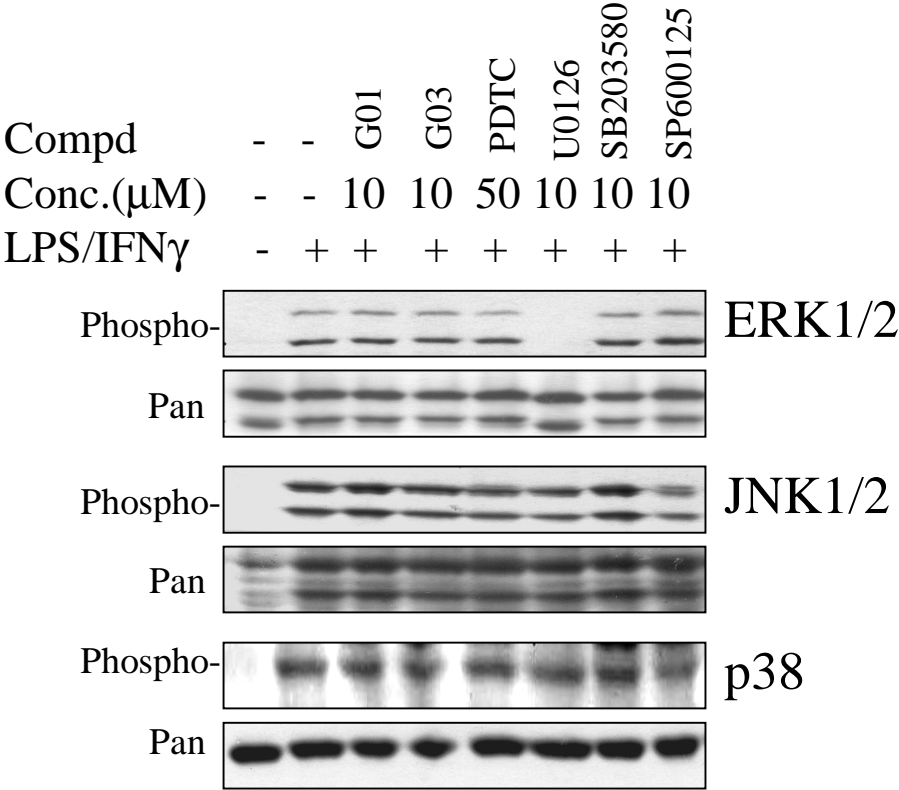


Figure 5A

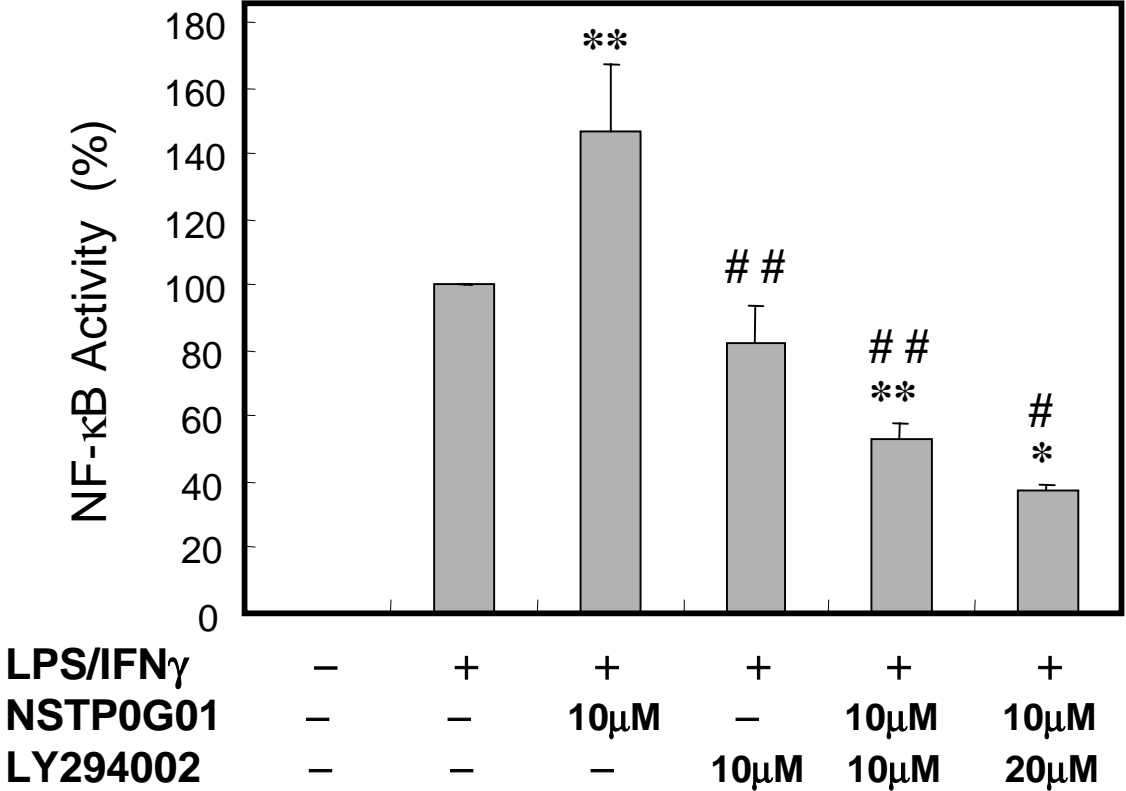


Figure 5B

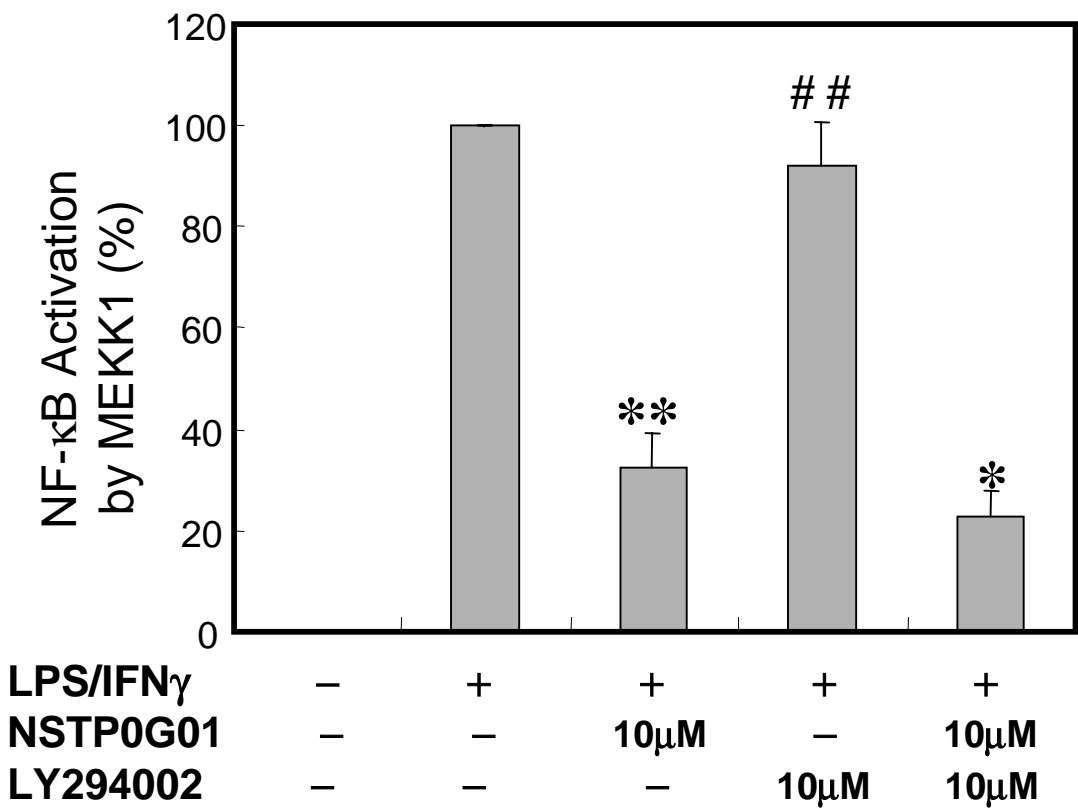


Figure 6A

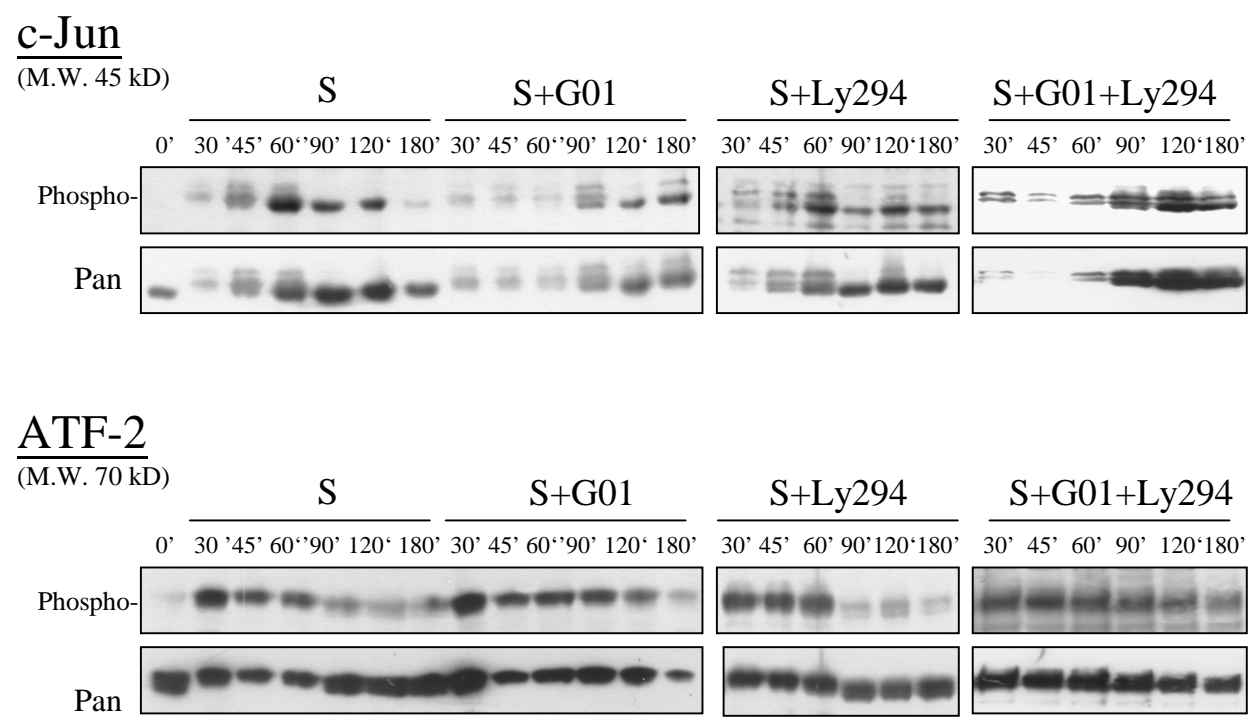
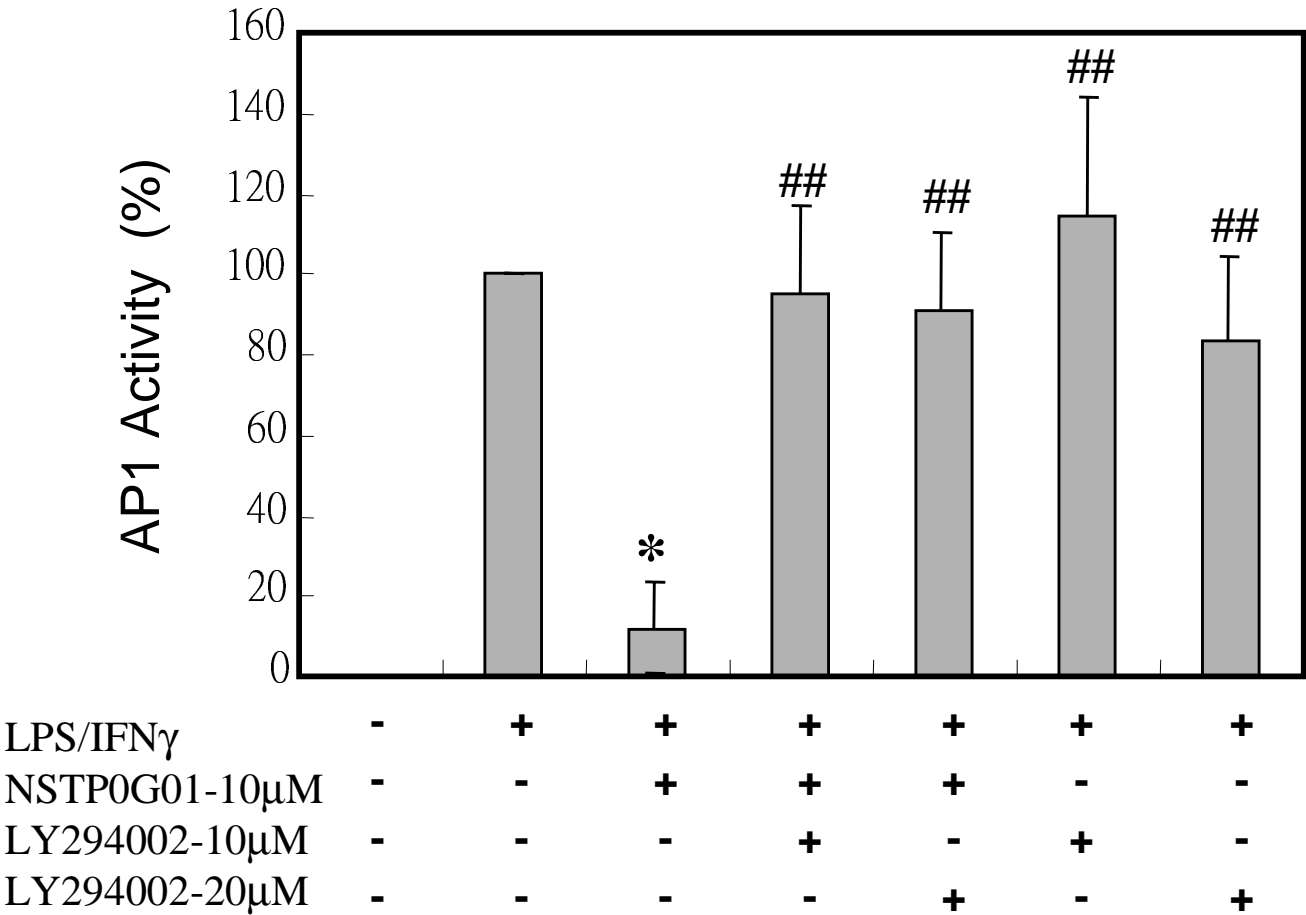


Fig. 6B



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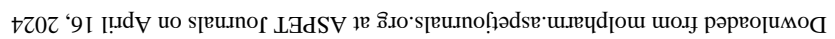


Fig. 7

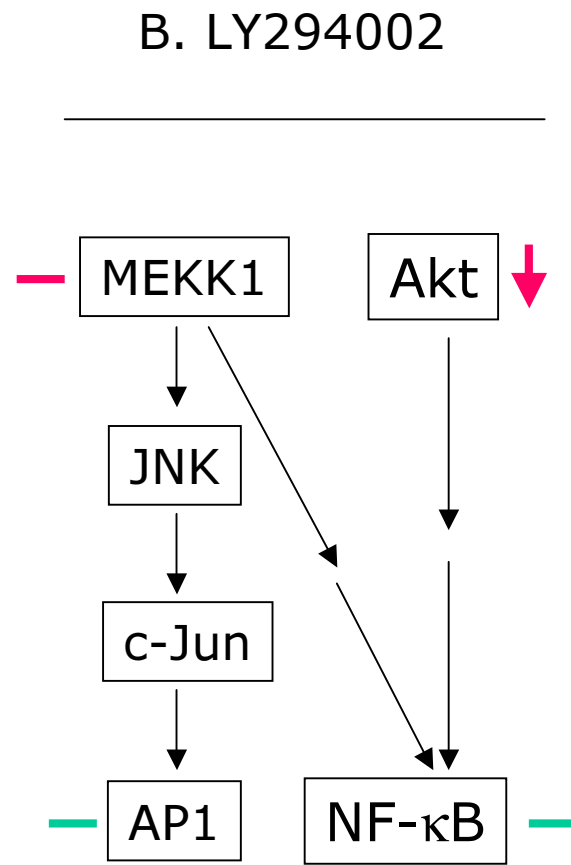


Fig. 7

