



Inhibition of NF- κ B/Rel by Paclitaxel in Mouse Macrophages

Jin Soo Lim¹, Seog Ki Lee¹ and Young Jin Jeon²

¹Department of Thoracic and Cardiovascular Surgery, Chosun University College of Medicine, Kwangju 501-709

²Department of Pharmacology, Chosun University College of Medicine, Kwangju 501-709, Korea

Received December 9, 2006; Accepted February 9, 2007

We demonstrate that paclitaxel, an antitumor agent derived from yew tree, inhibits LPS- and IFN- γ -induced NF- κ B/Rel activation in RAW 264.7 cells. Previously, paclitaxel (> 10 μ M) has been known to induce iNOS gene expression in macrophages. However, in the previous report we described that the pretreatment of macrophages with low concentration of paclitaxel (0.1 μ M) for 8 h inhibited LPS-induced iNOS gene expression. Pretreatment of RAW 264.7 cells with paclitaxel significantly inhibited NF- κ B/Rel transcriptional activation. Electrophoretic mobility shift assay further confirmed that pretreatment of macrophages with paclitaxel inhibited NF- κ B/Rel DNA binding. Taxotere, a semisynthetic analog of paclitaxel, also inhibited LPS- and IFN- γ -induced iNOS gene expression. Collectively, these series of experiments indicate that paclitaxel inhibits iNOS gene expression by blocking NF- κ B/Rel activation.

Key words: Paclitaxel, Macrophages, iNOS, NF- κ B/Rel

INTRODUCTION

Lipopolysaccharide (LPS) is a potent immune system activator which induces local inflammation, antibody production in severe infectious diseases such as septic shock (Rietschel and Brade, 1992). Macrophages play a central role in a host's defense against bacterial infection and are major cellular targets for LPS action. Stimulation of murine macrophages by LPS results in the expression of an iNOS, which catalyzes the production of large amounts of nitric oxide (NO) from L-arginine and molecular oxygen (Palmer *et al.*, 1988). NO, in turn, participates in the inflammatory response of macrophages (Hibbs *et al.*, 1987). The promoter of the murine gene encoding iNOS contains two κ B binding sites, located at 55 and 971 bp upstream of the TATA box, respectively (Lowenstein *et al.*, 1993). It has been reported that protein binding to the κ B site is necessary to confer inducibility by LPS (Xie *et al.*, 1994).

Paclitaxel, isolated from the bark of the yew tree, is one of the more promising agents for treatment of breast cancer (Rowinsky, 1994) and is shown to block cells at the G₂/M junction of the cell cycle (Blagosk-

lonny *et al.*, 1996). The primary mechanism of action of paclitaxel is attributed to its ability to bind to microtubules and to prevent their assembly. In addition to the blockage of mitosis, paclitaxel also triggers cellular responses that mimic those induced by a potent activator of the innate immune system, LPS, such as tyrosine phosphorylation of mitogen-activated protein kinases, translocation of NF- κ B, and induction of gene expression (Perera *et al.*, 1996; Das and White, 1997). In murine macrophages, paclitaxel can induce the expression of a series of cytokines, such as IL-1 α , IL-1 β , TNF- α , and iNOS (Manthey *et al.*, 1992; Kim and Paik, 2005). However, in the previous report we described that the pretreatment of macrophages with paclitaxel (0.1 μ M) for 8 h inhibited LPS-induced iNOS gene expression (Li *et al.*, 2006).

The objective of this study was to investigate the mechanism by which paclitaxel inhibits iNOS gene expression in mouse macrophages. Since iNOS gene expression requires NF- κ B/Rel activation, we analyzed the effect of paclitaxel on the transcriptional activation and DNA binding activity of NF- κ B/Rel.

MATERIALS AND METHODS

Materials. Paclitaxel and LPS from *Salmonella thyposa* were purchased from Sigma (St. Louis, MO,

Correspondence to: Young Jin Jeon, Department of Pharmacology, Chosun University College of Medicine, 375 Susukdong, Kwangju 501-759, Korea
E-mail: yjeon@chosun.ac.kr

USA). Mouse recombinant IFN- γ was purchased from R&D systems (Minneapolis, MN, USA). Reagents used for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Rabbit anti-mouse iNOS antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Paclitaxel was dissolved in Dimethylsulfoxide (DMSO), and the final concentration of DMSO was 0.1%.

Cell culture. RAW 264.7 cells (murine macrophage line) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were then cultured in the presence of 5% CO₂ at 37°C.

Nitrite quantitation. NO₂⁻ accumulation was used as an indicator of NO production in the medium as previously described (Green *et al.*, 1982). Cells were plated at 5 \times 10⁵ cells/ml in 96-well culture plates and treated with LPS and/or paclitaxel. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Using NaNO₂ to generate a standard curve, nitrite production was measured by an O.D. reading at 550 nm.

Transient transfection of RAW 264.7 cells. Vector constructions were performed as previously described (Jeon *et al.*, 1998). RAW 264.7 cells were transfected using the DEAE-dextran method (Xie *et al.*, 1993b), diluted to 5 \times 10⁵ cells per 1 ml of complete media, plated on 24 well plates, and then incubated in the presence of 5% CO₂ at 37°C for 24 hr. The transfectants were treated with LPS and radicicol. Eighteen hours later the cells were lysed with lysis buffer (250 μ l). The lysates were centrifuged (12,000 \times g for 10 min at 4°C), and the supernatant was assayed for the expression of CAT enzyme using CAT ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assay (EMSA) was performed as previously described (Jeon *et al.*, 1996). Nuclear extracts were prepared as previously described (Xie *et al.*, 1993a). Treated and untreated RAW 264.7 cell line was lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, pH 7.5) and nuclei were pelleted by centrifugation at 3,000 \times g for 5 min. Nuclear lysis was per-

formed using a hypertonic buffer (30 mM HEPES, 1.5 mM MgCl₂, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μ g/ml of aprotinin, and 1 μ g/ml of leupeptin). Following lysis, the samples were centrifuged at 14,500 \times g for 15 min, and supernatant was retained for use in the DNA binding assay. The double-stranded oligonucleotides were end-labeled with [γ -³²P]-ATP. Nuclear extracts (5 μ g) were incubated with poly (dI-dC) and the [³²P]-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM MgCl₂, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μ g/ml of aprotinin, and 1 μ g/ml of leupeptin) for 10 min. DNA binding activity was separated from free probe using a 4% polyacrylamide gel in 0.5 \times TBE buffer. Following electrophoresis, the gel was dried and subjected to autoradiography.

Western immunoblot analysis. Whole cell lysates (20 μ g) were separated by 10% SDS-PAGE and then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline (TBS), pH 7.6 containing 0.05% Tween-20 and 3% bovine serum albumin. The nitrocellulose membranes were incubated with iNOS-specific antibody. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

Statistical analysis. The mean \pm SD was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared to the vehicle control using a Dunnett's two-tailed *t* test (Dunnett, 1955).

RESULTS

Inhibition of iNOS gene expression by pre-treatment with paclitaxel in macrophages. In the previous study, we demonstrate that iNOS protein production was inhibited by paclitaxel treatment in a pre-incubation time-dependent manner (Li *et al.*, 2006). Since 8 h pre-incubation strongly inhibited LPS-induced iNOS expression, we chose 8 h pre-treatment and assessed the effect of paclitaxel pretreatment on LPS and/or IFN- γ -induced iNOS expression. We treated RAW 264.7 cells with low dose of paclitaxel (0.01, 0.1, and 1 μ M) for 8 h before the treatment with LPS (200 ng/ml) for 24 h. When culture supernatants were isolated and analyzed for nitrite production, we found that pretreatment with paclitaxel significantly inhibited LPS-induced nitrite gen-

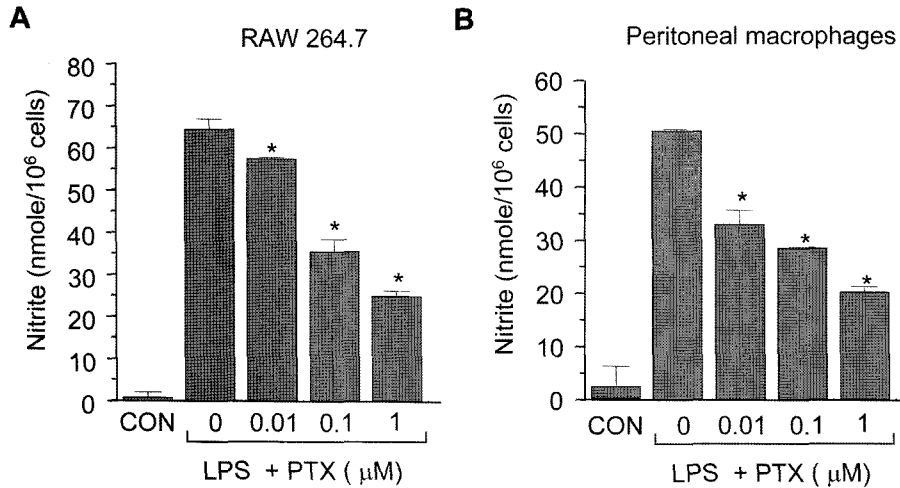


Fig. 1. Inhibition of nitrite production by paclitaxel in macrophages. RAW 264.7 cells (A) and mouse peritoneal macrophages (B) were pretreated with paclitaxel (0.01, 0.1, and 1 μ M) for 8 h before the treatment with LPS (200 ng/ml) for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each value shows the mean \pm S.D. of triplicate determinations. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$. CON; control, PTX; paclitaxel.

eration (Fig. 1A). To confirm the effect of paclitaxel on NO production, we isolated peritoneal macrophages and treated with paclitaxel for 8 h before the treatment with LPS for 24 h. The nitrite generation in peritoneal macrophages was also significantly inhibited by the pretreatment with paclitaxel (Fig. 1B). These results indicate that paclitaxel decreases the gene expression of iNOS, which is involved in inflammation (Hibbs *et al.*, 1987).

Inhibition of NF- κ B/Rel by pretreatment with paclitaxel in LPS-stimulated macrophages. To further investigate the molecular mechanism of paclitaxel-mediated inhibition of macrophage, we focused on the transcription factors whose binding sites are in the promoter of iNOS gene. Since it has been reported that protein binding at the κ B binding site is necessary to confer inducibility by LPS of iNOS (Xie *et al.*, 1994), we assessed the effect of paclitaxel on NF- κ B/Rel using a transient transfection assay. When RAW 264.7 cells were transiently transfected with p(NF- κ B/Rel)₃-CAT, the CAT gene expressions were found to be induced by LPS and IFN- γ , (Fig. 2). Basal levels of CAT expression in unstimulated RAW 264.7 cells were $< 4 \pm 1.1$ pg/ml (mean \pm standard deviation, two experiment). On LPS- and IFN- γ -stimulation, CAT expression by RAW 264.7 cells increased by 10.1- and 4.9-fold, respectively. Pretreatment of paclitaxel significantly inhibited both LPS- and IFN- γ -induced CAT expression. The synergism between LPS and IFN- γ on CAT expression was inhibited partially by paclitaxel.

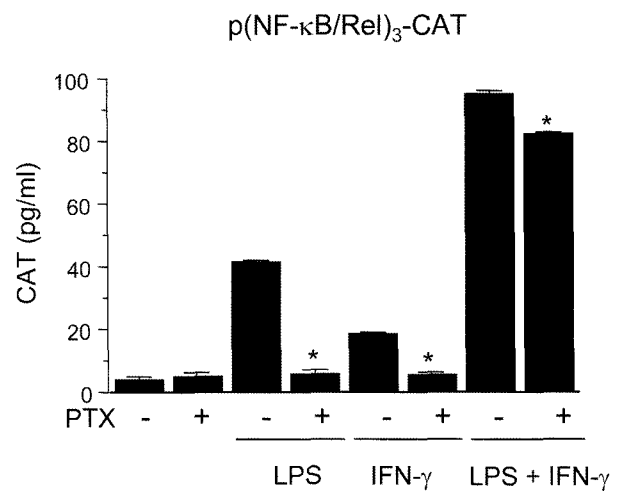


Fig. 2. Inhibition of NF- κ B/Rel transcriptional activation by paclitaxel in RAW 264.7 cells. RAW 264.7 cells were transfected with p(NF- κ B/Rel)₃-CAT by DEAE dextran method. Twenty-four hours after transfection, cells were pretreated with paclitaxel for 8 h before the treatment LPS (200 ng/ml), IFN- γ (100 ng/ml), and LPS plus IFN- γ for 18 h. Cell extracts were then prepared and analyzed for the expression of CAT using CAT ELISA kit. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$. PTX; paclitaxel

We further assessed the effect of paclitaxel on the NF- κ B/Rel whose binding motif is in the promoter of iNOS gene using EMSA. LPS treatment of RAW 264.7 cells induced a marked increase in NF- κ B/Rel binding to its cognate site. And the induction of NF- κ B/Rel binding was inhibited by paclitaxel pretreatment in a dose-

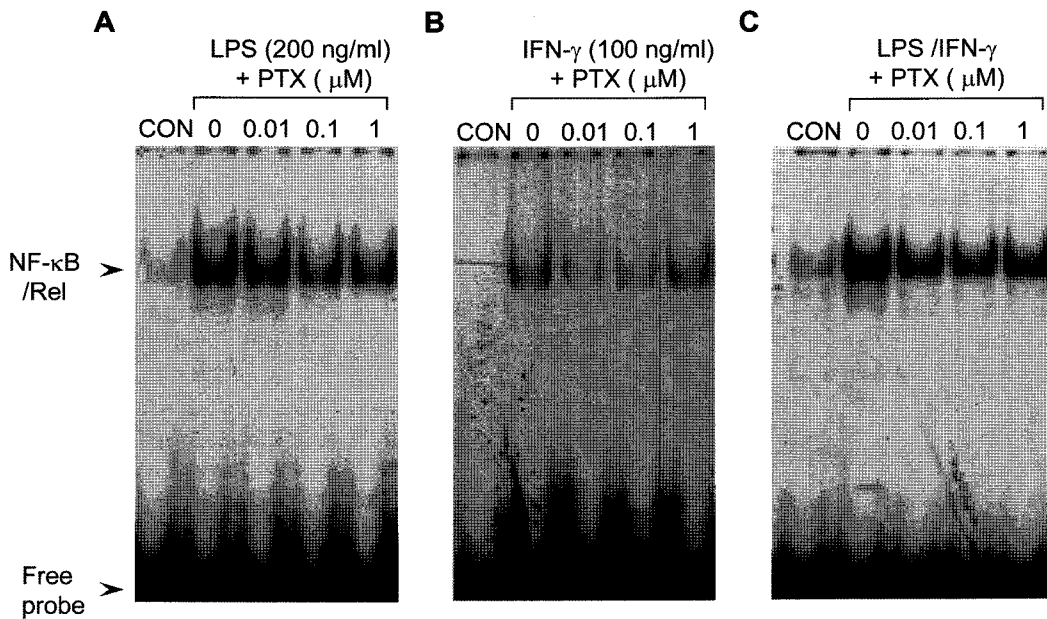


Fig. 3. Inhibition of NF- κ B/Rel DNA binding by paclitaxel in RAW 264.7 cells. Cells were pre-incubated with indicated concentrations of paclitaxel for 8 h before the treatment with LPS (200 ng/ml) (A), IFN- γ (100 ng/ml) (B), and LPS plus IFN- γ (C) for 2 h. Nuclear extracts were then isolated and analyzed for the activity of NF- κ B/Rel. CON; control, PTX; paclitaxel.

related manner (Fig. 3A). IFN- γ also induced NF- κ B/Rel binding, which was inhibited by paclitaxel pretreatment in a dose-related manner (Fig. 3B). The synergism between LPS and IFN- γ on NF- κ B/Rel DNA binding was inhibited partially by paclitaxel (Fig. 3C). The specificity of the retarded bands was confirmed by the addition of an excess of 32 P-unlabeled double-stranded κ B that competed for protein binding (data not shown). These results indicate that paclitaxel decreases DNA binding of NF- κ B/Rel, which is important in the regulation of iNOS gene expression.

Inhibition of iNOS gene expression by pre-treatment with taxotere in macrophages. To further investigate the mechanism by which pretreatment with paclitaxel inhibits iNOS gene expression, we used paclitaxel analogue, taxotere. Taxotere, a semisynthetic taxoid, is more potent than taxol as an inducer of microtubule bundling, but does not activate mouse macrophages (Manthey *et al.*, 1993; Cassidy *et al.*, 2002). RAW 264.7 cells were pretreated with taxotere (0.1 μ M) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN- γ (100 ng/ml) for 24 h. When culture supernatants were isolated and analyzed for nitrite production, we found that pretreatment with taxotere (0.1 μ M) significantly inhibited both LPS- and IFN- γ -induced nitrite generation (Fig. 4A). The synergism between LPS and IFN- γ on nitrite generation was inhibited slightly by the pretreatment with taxotere. To analyze the effect of pre-

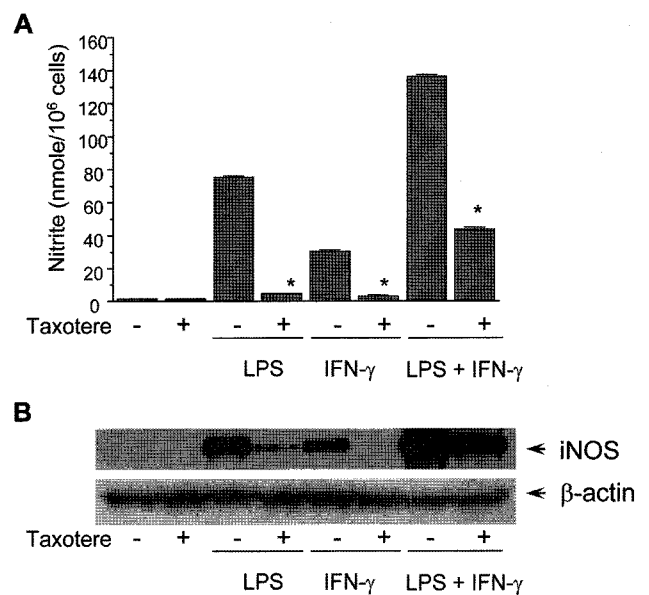


Fig. 4. Inhibition of nitrite production by taxotere in RAW 264.7 cells. (A) Cells were pretreated with taxotere (0.1 μ M) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN- γ (100 ng/ml) for 24 h. Culture supernatants were subsequently isolated and analyzed for nitrite production. Each value shows the mean \pm S.D. of triplicate determinations. (B), Cells were pretreated with taxotere (0.1 μ M) for 8 h before the treatment with LPS (200 ng/ml) and/or IFN- γ (100 ng/ml) for 16 h. Cell extracts were isolated and subjected to Western immunoblot assay using iNOS- and β -actin-specific antibody. *, response that is significantly different from the control group as determined by Dunnett's two-tailed *t* test at $P < 0.05$.

treatment with taxotere on iNOS gene expression, we treated RAW 264.7 cells with low dose of taxotere (0.1 μ M) for 8 h before the treatment with LPS (200 ng/ml) for 16 h. The expression level of iNOS gene was monitored by Western immunoblot analysis. As shown in Fig. 4B, taxotere significantly inhibited iNOS protein production by both LPS and IFN- γ (100 ng/ml). Co-treatment of LPS and IFN- γ synergistically increased the amount of iNOS, which was reduced partially by taxotere pretreatment (Fig. 4B). These results demonstrate that paclitaxel and its analogue, taxotere, inhibits iNOS gene expression in mouse macrophages.

DISCUSSION

We demonstrate that paclitaxel treatment significantly attenuates LPS-induced NO production and iNOS transcription through the blocking of NF- κ B/Rel pathway in the macrophage cell line RAW 264.7. The effect of paclitaxel on iNOS immune cells is controversial. Ding *et al.* (1990) described that paclitaxel induces the secretion of tumor necrosis factor and down-regulation of tumor necrosis factor receptors in murine macrophages. Although the structure of paclitaxel is quite different from that of LPS, paclitaxel has been shown to possess many LPS-like activities, such as tyrosine phosphorylation of microtubule-associate protein kinases (Manthey *et al.*, 1992) and activation of NF- κ B/Rel (Perera *et al.*, 1996). Paclitaxel also activates iNOS gene expression in astrocytes (Cvetkovic *et al.*, 2004) and macrophages (Kim and Paik, 2005). However, our data showed that pretreatment with paclitaxel for 8 h inhibited LPS-induced iNOS gene expression and NF- κ B/Rel activation. Our data also demonstrated that pretreatment with paclitaxel inhibited IFN- γ -induced NF- κ B/Rel activation. This is the first report showing that paclitaxel inhibits iNOS gene expression through the blocking of NF- κ B/Rel in macrophage line RAW 264.7 cells. To further investigate the mechanism by which pretreatment with paclitaxel inhibits iNOS gene expression, we used paclitaxel analogue, taxotere. Since taxotere, which has no LPS-mimic activity against mouse macrophages, also inhibits iNOS gene expression in macrophages, the inhibition of macrophage by the pretreatment of paclitaxel may not be related to the activation mechanism. The mechanism how paclitaxel causes macrophages to hypo-responsive state to LPS, should be further studied.

Our study showed that NF- κ B/Rel is positively regulated by LPS for iNOS gene expression, and paclitaxel pretreatment of RAW 264.7 cell significantly inhibited both LPS- and IFN- γ -induced NF- κ B/Rel activity. The NF- κ B/Rel is pleiotropic regulator of many genes involved

in immune and inflammatory responses, including iNOS (Xie *et al.*, 1994). NF- κ B/Rel exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, I κ B. Macrophage activation by certain external stimuli results in the phosphorylation of I κ B, thus releasing the active DNA-binding form of NF- κ B/Rel to translocate to the nucleus to bind κ B motifs in the regulatory region of a variety of genes. EMSA studies showed strong induction by LPS of κ B binding complexes at 2 h. Paclitaxel inhibited activation of the κ B binding complex (Fig. 3). Transient transfection assay also showed LPS and IFN- γ -induced transcriptional activation of NF- κ B/Rel was inhibited by paclitaxel pretreatment (Fig. 2). However, the synergism between LPS and IFN- γ on CAT expression was inhibited partially by paclitaxel. One possible mechanism for the synergism between LPS and IFN- γ is the production of cytokines including TNF- α , which is a potent activator of NF- κ B/Rel. No effect on TNF- α production by paclitaxel may be the reason why paclitaxel only partially inhibited the synergistic induction of NF- κ B/Rel activation by LPS and IFN- γ . This possibility remains to be tested.

In summary, these experiments demonstrate that paclitaxel inhibits LPS-induced expression of iNOS gene in RAW 264.7 cells. Based on our findings, the most likely mechanism that can account for this biological effect involves the inhibition of NF- κ B/Rel.

ACKNOWLEDGEMENTS

This research was supported by research funds from Chosun University, 2006.

REFERENCES

- Blagosklonny, M.V., Schulte, T., Nguyen, P., Trepel, J. and Neckers, L.M. (1996). Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involved c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. *Cancer Res.*, **56**, 1851-1854.
- Cassidy, P.B., Moos, P.J., Kelly, R.C. and Fitzpatrick, F.A. (2002). Cyclooxygenase-2 induction by paclitaxel, docetaxel, and taxane analogues in human monocytes and murine macrophages: structure-activity relationships and their implications. *Clin. Cancer Res.*, **8**, 846-855.
- Cvetkovic, I., Miljkovic, D., Vuckovic, O., Harhaji, L., Nikolic, Z., Trajkovic, V. and Mostarica Stojkovic, M. (2004). Taxol activates inducible nitric oxide synthase in rat astrocytes: the role of MAP kinases and NF- κ B. *Cell. Mol. Life Sci.*, **61**, 1167-1175.
- Das, K.C. and White, C.W. (1997). Activation of NF- κ B by antineoplastic agents. *J. Biol. Chem.*, **272**, 14914-14920.
- Ding, A.H., Porteu, F., Sanchez, E. and Nathan, C.F. (1990). Shared actions of endotoxin and taxol on TNF receptors

- and TNF release *Science*, **248**, 370-372.
- Dunnnett, M. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Statist. Assoc.*, **50**, 1096-1121.
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131-138.
- Hibbs, J.B., Jr., Taintor, R.R. and Vavrin, Z. (1987). Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science*, **235**, 473-476.
- Jeon, Y.J., Han, S.H., Lee, Y.W., Yea, S.S. and Yang, K.H. (1998). Inhibition of NF-kappa B/Rel nuclear translocation by dexamethasone: mechanism for the inhibition of iNOS gene expression. *Biochem. Mol. Biol. Int.*, **45**, 435-441.
- Jeon, Y.J., Yang, K.H., Pulaski, J.T. and Kaminski, N.E. (1996). Attenuation of inducible nitric oxide synthase gene expression by delta 9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-kappa B/Rel activation. *Mol. Pharmacol.*, **50**, 334-341.
- Kim, Y.M. and Paik, S. (2005). Induction of expression of inducible nitric oxide synthase by taxol in murine macrophage cells. *Biochem. Biophys. Res. Commun.*, **326**, 410-416.
- Li, M.H., Kang, J.S., Kim, H. and Jeon, Y.J. (2006). Pretreatment of macrophages with paclitaxel inhibits iNOS expression. *J. Toxicol. Pub. Health*, **22**, 103-107.
- Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W. and Murphy, W.J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 9730-9734.
- Manthey, C.L., Qureshi, N., Stutz, P.L. and Vogel, S.N. (1993). Lipopolysaccharide antagonists block taxol-induced signaling in murine macrophages. *J. Exp. Med.*, **178**, 695-702.
- Palmer, R.M., Ashton, D.S. and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664-666.
- Perera, P.Y., Qureshi, N. and Vogel, S.N. (1996). Paclitaxel (Taxol)-induced NF-kB translocation in murine macrophages. *Infect. Immun.*, **64**, 878-884.
- Rietschel, E.T. and Brade, H. (1992). Bacterial endotoxins. *Sci. Am.*, **267**, 54-61.
- Rowinsky, E.K. (1994). Update on the antitumor activity of paclitaxel in clinical trials. *Ann. Pharmacother.*, **28**, S18-S22.
- Xie, H., Chiles, T.C. and Rothstein, T.L. (1993a). Induction of CREB activity via the surface Ig receptor of B cells. *J. Immunol.*, **151**, 880-889.
- Xie, Q.W., Kashiwabara, Y. and Nathan, C. (1994). Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.*, **269**, 4705-4708.
- Xie, Q.W., Qhisnant, R. and Nathan, C. (1993b). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon-g and bacterial lipopolysaccharide. *J. Exp. Med.*, **177**, 1779-1784.