

Aqueous extract of *Magnolia officinalis* mediates proliferative capacity, p21WAF1 expression and TNF- α -induced NF- κ B activity in human urinary bladder cancer 5637 cells; involvement of p38 MAP kinase

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Abstract. *Magnolia officinalis* is a commonly used herb in East Asian countries and has multiple pharmacological effects. Although *Magnolia officinalis* has a variety of pharmacological effects on certain cancer cell types, the molecular mechanisms on urinary bladder cancer are unclear. An aqueous extract of *M. officinalis* inhibited cell proliferation in cultured human urinary bladder cancer 5637 cells. Inhibition of proliferation was associated with G1 cell cycle arrest. Treatment with *M. officinalis* extract blocked the cell cycle in the G1 phase, down-regulated the expression of cyclins and CDKs and up-regulated the expressions of p21WAF1 and p27 Kip1, which are CDK inhibitors. In addition, *M. officinalis* extract induced a marked activation of p38 MAP kinase and JNK. SB203580, a p38 MAP kinase specific inhibitor, blocked the expression of *M. officinalis* extract-dependent p38 MAP kinase and p21WAF1. Blockage of the p38 MAPK kinase function reversed *M. officinalis* extract-induced cell proliferation. These data demonstrate that *M. officinalis* extract-

induced cell growth inhibition appears to be linked to the activation of p38 MAP kinase through p21WAF1 expression. Moreover, treatment of 5637 cells with *M. officinalis* extract suppressed constitutive and TNF- α -induced-nuclear factor- κ B (NF- κ B) activation. Furthermore, the transactivation of TNF- α -stimulated NF- κ B was inhibited by SB203580 treatment. Collectively, these results suggest that the p38 MAP kinase pathway contributes, at least partially, to the anti-cancer activity of *M. officinalis* extract in human urinary bladder tumor 5637 cells.

Introduction

The p38 MAP kinase is one of at least three mammalian MAPKs [the other two being extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK)] that are activated by three homologous but distinct signaling pathways (1,2). The activation is effected by dual Ser/Thr and tyrosine phosphorylation that is catalyzed by a specific upstream MAPK kinase. Proliferative potential can be regulated by various signals, blocking growth or inducing apoptosis (3). JNK and p38 MAP kinase are known examples of such stress-activated protein kinases, because they are strongly activated in response to stressful stimuli. The p38 MAP kinase has been linked to apoptosis, proliferation and differentiation (2-5).

The molecular and genetic alterations that precede morphologic changes and are responsible for tumorigenesis and progression of bladder cancer also include alterations in cell cycle regulators causing uncontrolled cancer growth. Cell proliferation depends on an ordered and tightly regulated process known as the cell cycle, involving multiple checkpoints assessing extracellular growth signals, cell size and DNA integrity (6). In general, the progression of the cell cycle in eukaryotes is a complex process including resting G0 phase and cell growth involving G1, S and G2/M phases

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Abbreviations: p38 MAP kinase, p38 mitogen activated protein kinase; PBS, phosphate-buffered saline; FBS, fetal bovine serum; TNF- α , tumor necrosis factor- α

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in a step-wise manner (7). The cell cycle is regulated by complexes composed of regulatory cyclins and catalytic cyclin-dependent kinases (CDKs), whose formation and activation promote cell cycle progression (8). The cells are stimulated to divide in response to mitogens exiting the G1 phase and entering the S phase. Cyclin D1-CDK4 and cyclin E-CDK2 predominantly act in sequence during the G1/S transition and are required for cell cycle progression through this period (9). On the other hand, cyclin-dependent kinase inhibitors act as negative regulators of the cell cycle by extinguishing the activity of CDKs (10). The kinase activity of these cyclins/CDK complexes can be negatively regulated by CDK-inhibitory protein p21WAF1, which is able to silence CDKs essential for S-phase entry, thus inhibiting cell cycle progression (11).

Nuclear factor- κ B (NF- κ B) was first described by Sen and Baltimore (12) as a ubiquitous nuclear transcription factor binding to the κ immunoglobulin-light chain enhancer and subsequently shown to be a regulator in the development of cancer. In the resting state, NF- κ B is sequestered in the cytoplasm through its tight association with a type of specific inhibitory proteins inhibitors of NF- κ B (I κ B) (13). Stimulation of cells with inducers such as TNF- α leads to rapid phosphorylation, ubiquitination, and degradation of I κ B. NF- κ B is therefore released and translocates into the nucleus to activate the expression of target genes (14).

The majority of bladder cancers (90%) are transitional cell carcinomas (TCC), which have been determined to result from a neoplastic lesion with environmental and occupational factors identified as causatives (15). Bladder cancer patients are at risk of recurrence and/or progression to invasive diseases. In addition, smokers and workers exposed to industrial carcinogens are at risk of developing primary bladder tumors (16). For these people, effective preventive measures are needed. One approach to the control of cancer is growth inhibition, wherein the disease is prevented, slowed or reversed substantially by the administration of complementary and alternative medicines (17). In this regard, naturally occurring medicinal plants are receiving increased attention in recent years because of their promising efficacy in several pre-clinical cancer models (18-20). *Magnolia officinalis* is used in traditional Chinese medicine for the treatment of various pharmacological activities (21-25). A number of chemical constituents such as honokiol and magnolol have been isolated from *M. officinalis*. However, the anti-tumor mechanism of *M. officinalis* in the treatment of urinary bladder cancer has thus far remained uncharacterized.

For the present study, we explored the mechanism by which cell growth is inhibited by *M. officinalis* in urinary bladder cancer 5637 cells and examined the inhibitory effects of *M. officinalis* on proliferative capacity, cell cycle regulation and TNF- α induced NF- κ B activation in 5637 cells. Its inhibitory effects are possibly dependent of the p38 MAP kinase activity, indicating its potential use in cancer prevention and therapy.

Materials and methods

M. officinalis crude extract. Air-dried and crushed *M. officinalis* (100 g) was added to distilled water, and heated at

100°C to accomplish extraction. The extract was then concentrated with a rotary evaporator and lyophilized. The final aqueous extracts, weighing 6 g (a collection rate of 6%), were diluted with saline solution.

Materials. TNF- α was obtained from R&D systems. Polyclonal antibodies to cyclin E, CDK2, CDK4 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to cyclin D1, p21WAF1, p53, p27, p38 MAP kinase, phospho p38 MAP kinase, JNK and phospho JNK were obtained from New England Biolabs. SB203580 was obtained from Calbiochem (San Diego, CA).

Cell culture. The human bladder carcinoma cell line, 5637, was obtained from the American Type Culture Collection. The cells were maintained in DMEM (4.5 g glucose/l) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C in a 5% CO₂ humidified incubator. Cells were subcultured twice a week with trypsin/EDTA solution [saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4) and 0.02% EDTA].

Cell viability assay. Subconfluent, exponentially growing 5637 cells in 24-well plates were incubated with *M. officinalis* extract for the indicated times. Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, based on the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-tetrazolium by mitochondrial dehydrogenase to a formazan product (26), measured by light absorbance at 490 nm. Cell growth and viability were checked before and after treatment with *M. officinalis* using phase contrast microscopy.

Cell counts and [³H]thymidine incorporation. For cell counts, 5637 cells were plated overnight at equivalent densities (5x10⁵ cells/plate) in 100 mm plates. At intervals after plating, the cells were trypsinized, and cell numbers were determined using a Coulter Counter. In [³H]thymidine uptake experiments, cells were grown to near confluence in 24-well tissue culture plates and treated with *M. officinalis* extract as indicated. The cells were incubated for an additional 24 h and labeled with [methyl-³H]thymidine (New England Nuclear, Boston, MA, USA) at a concentration of 1 μ Ci/ml for the final 12 h of this period. After labeling, the cells were washed with phosphate-buffered saline (PBS), fixed in cold 10% trichloroacetic acid, and then washed with 95% ethanol. The incorporated [³H]thymidine was extracted with 0.2 M NaOH and measured in a liquid scintillation counter as previously described (26). Values are expressed as the means of 6 wells from three separate experiments.

Cell cycle analysis. Cells were harvested and fixed in 70% ethanol and stored at -20°C. The cells were then washed twice with ice-cold PBS and incubated with RNase and DNA intercalating dye propidium iodide, and a cell cycle phase analysis was performed by flow cytometry using a Becton-Dickinson Facstar flow cytometer and Becton-Dickinson cell fit software.

SPANDIDOSlot analysis. 5637 cells were treated with *M.* extract for the specified periods of time at 37°C. Cell

lysates were prepared, and immunoblotting was performed as described previously (26). Briefly, to prepare the whole-cell extract, cells (1x10⁷ cells per 100 mm dish) were detached and washed once in cold PBS and suspended in 100 µl lysis buffer (10 mM Tris-HCl, pH 8, 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT and 1 mM phenylmethyl-sulfonyl fluoride). The suspension was put on ice for 20 min and then centrifuged at 5000 rpm for 20 min at 4°C. Total protein content was determined by a Bio-Rad protein assay reagent using bovine serum albumin as the standard. Protein extracts were reconstituted in the sample buffer [0.062 M Tris-HCl, 2% SDS, 10% glycerol and 5% (vol/vol) β-mercaptoethanol], and the mixture was boiled for 5 min. Equal amounts (50 µg) of the denatured proteins were loaded into individual lanes and separated on 10% SDS polyacrylamide gels, followed by transfer of the proteins to membranes overnight.

Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were allowed to react with primary antibodies for 2 h. The membranes were then incubated with a horseradish peroxidase-conjugated, goat anti-rabbit or the anti-mouse antibody for 2 h before being developed using enhanced chemiluminescence.

Immune complex kinase assays. Cell lysates were prepared with ice-cold lysis buffer [containing in mM/l: HEPES (pH 7.5) 50, NaCl 150, EDTA 1, EGTA 2.5, DTT 1, β-glycerophosphate 10, NaF 1, Na₃VO₄ 0.1, and phenylmethylsulfonyl fluoride 0.1 and 10% glycerol, 0.1% Tween-20, 10 µg/ml of leupeptin and 2 µg/ml of aprotinin] and sonicated at 4°C [Micro ultrasonic cell disrupter (Kontes), 30% power, two times for 10 sec each time]. Lysates were clarified by centrifugation at 10000 g for 5 min, and the supernatants were precipitated by treatment with protein A-Sepharose beads precoated with saturating amounts of the indicated antibodies for 2 h at 4°C. When monoclonal antibodies were used, the protein A-Sepharose was pretreated with rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories). The immunoprecipitated proteins on the beads were washed 4 times with 1 ml of lysis buffer and twice with kinase buffer (containing in mM/l: HEPES 50, MgCl₂ 10, DTT 1, β-glycerophosphate 10, NaF 1 and sodium orthovanadate 0.1). The final pellet was resuspended in 25 µl of kinase buffer containing either 1 µg of glutathione S-transferase (GST)-pRb C-terminal (pRb amino acids 769-921) fusion protein (Santa Cruz Biotechnology) or 5 µg of histone H₁ (Life Technologies, Inc.), 20 µM/l ATP, and 5 µCi of [γ-³²P]ATP (4500 Ci/mmol; ICN), followed by incubation for 20 min at 30°C with occasional mixing. The reaction was stopped by the addition of 25 µl of 2X concentrated Laemmli sample buffer and separated on 10 or 12.5% SDS-polyacrylamide gels. The migration of histone H₁ or GST-pRb was determined by Coomassie blue staining. Phosphorylated pRb and histone H₁ were visualized and quantified with a BAS 2000 bioimaging analyzer.

Nuclear extracts and electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared essentially as described previously (43). Cultured cells were collected by

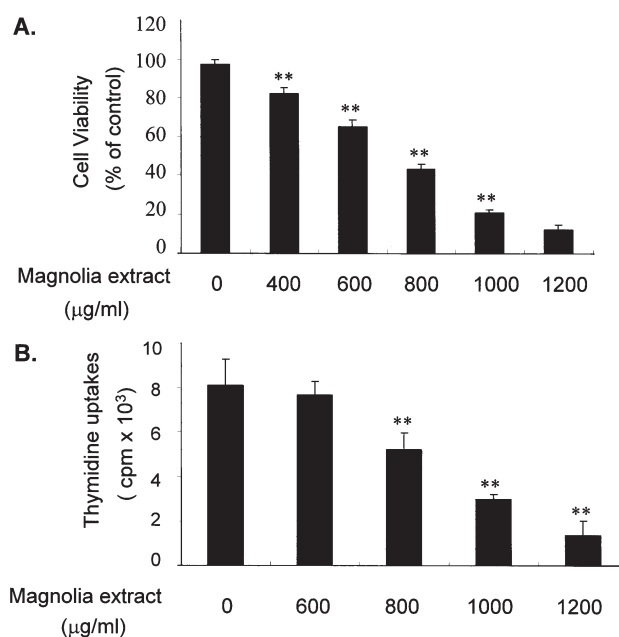


Figure 1. Effects of *Magnolia officinalis* extract on cell viability and DNA synthesis in 5637 cells. (A) Cell viability was evaluated by a modified MTT assay. (B) DNA replication was measured by a thymidine uptake experiment. Indicated values are means of triplicate wells. **P<0.01 compared with no *Magnolia officinalis* extract treatment.

centrifugation, washed and suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet NP-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF for 15 min at 4°C. The nuclear extract (2 µg) was preincubated at 4°C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the *cis* element of interest. The sequences were as follows: NF-κB, CAGTGGAAATCCCCAGCC. After this time, the reaction mixture was incubated at 4°C for 20 min in a buffer (25 mM HEPES buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl and 2.5% glycerol) with 2 µg of poly dI/dC and 5 fmol (2x10⁴ cpm) of a Klenow end-labeled (³²P-ATP) 30-mer oligonucleotide, which spans the DNA binding site. The reaction mixture was electrophoresed at 4°C in a 6% polyacrylamide gel using a TBE (89 mM Tris, 89 mM boric acid and 1 mM EDTA) running buffer. The gel was rinsed with water, dried and exposed to X-ray film overnight.

Statistical analysis. When appropriate, data were expressed as means ± SE. Data were analyzed by factorial ANOVA and Fisher's least significant difference test where appropriate. Statistical significance was accepted at P<0.05.

Results

***M. officinalis* extract induces cell growth inhibition in 5637 cells.** To determine the growth inhibitory activity of aqueous extract of *M. officinalis*, human urinary bladder cancer 5637 cells were treated with *M. officinalis* extract (400-1200 µg/ml) for 24 h and viable cells were measured by MTT. The

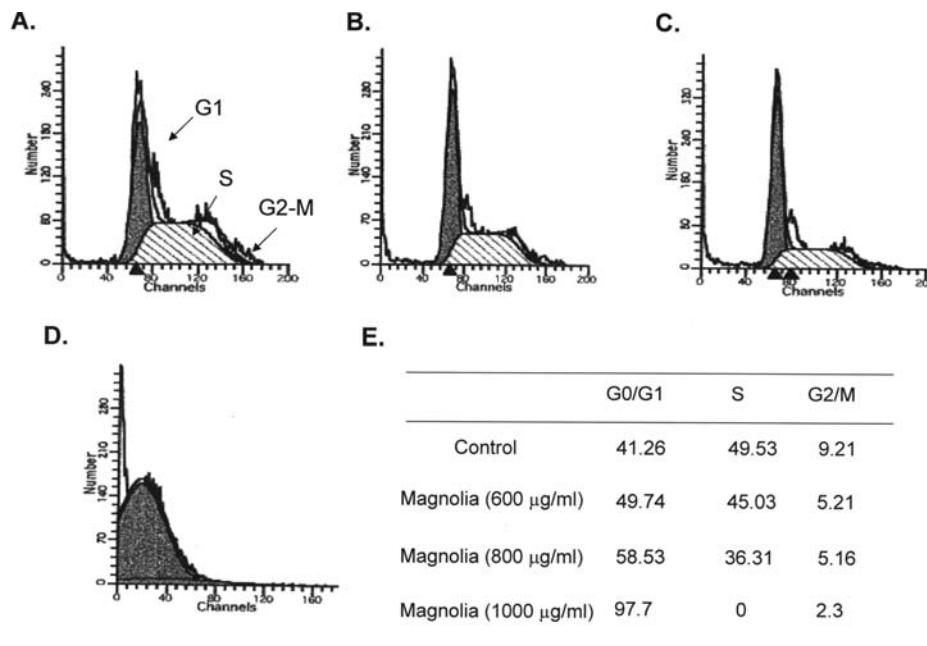


Figure 2. *M. officinalis* extract induces G1 cell cycle arrest in 5637 cells. Cells were treated with 0 (A), 600 (B), 800 (C) and 1000 µg/ml of *M. officinalis* extract for 24 h (D). Cells were subjected to flow cytometric analysis to determine the effect of *M. officinalis* extract on cell cycle distribution. (E) The percentage of cells in each population is shown as the means \pm SE from three triplicate experiments.

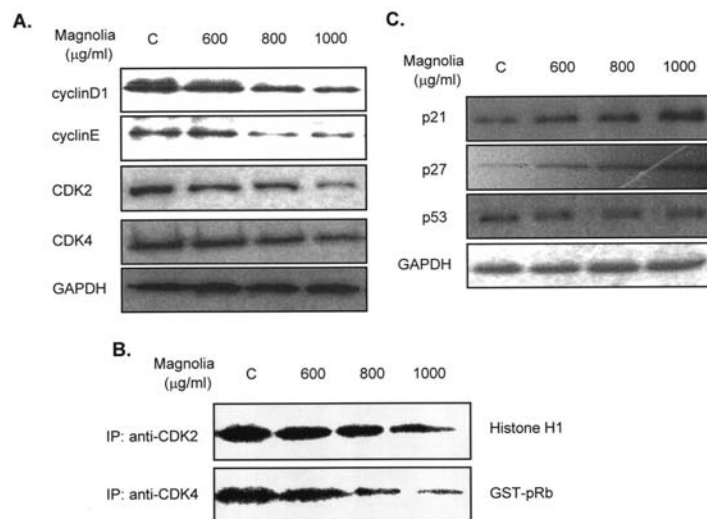


Figure 3. Effects of *Magnolia officinalis* on cell cycle regulators. (A) 5637 cells were treated with *M. officinalis* extract as indicated, and immunoblot analysis was performed with antibodies specific for cyclin D1, cyclin E, cdk2 and cdk4. (B) The kinase assay was performed using histone H1 (for CDK2) or GST-Rb (for CDK4) as the substrate. (C) Effects of *M. officinalis* extract on p21/WAF1, p27 and p53 expression. The results from representative experiments were normalized to GAPDH expression. The indicated values are the means of three triplicate experiments.

effects of *M. officinalis* extract on 5637 cell growth and DNA synthesis were investigated. Treatment of *M. officinalis* extract to 5637 cells resulted in a significant decrease in viable cells in a concentration-dependent manner (Fig. 1A) as compared to untreated control cells. In addition, the effect of *M. officinalis* extract on cell proliferation was determined by [³H]thymidine incorporation after treatment with various concentrations of *M. officinalis* extract for 24 h. As shown in Fig. 1B, *M. officinalis* extract had a concentration-dependent inhibitory effect on 5637 cell growth.

M. officinalis extract induces G1 cell cycle arrest. A flow cytometric analysis (Fig. 2) demonstrated that *M. officinalis* extract (800 and 1000 µg/ml) induced the accumulation of

significant numbers of cells in the G1 phase of the cell cycle, suggesting that the observed growth inhibitory effects of *M. officinalis* extract in 5637 cells were due to cell cycle arrest (Fig. 2).

Next, the effects of *M. officinalis* extract on cell cycle regulatory molecules that are operative in the G1 phase of the cell cycle were examined. Treatment of 5637 cells with *M. officinalis* extract at 24 h resulted in a dose-dependent decrease in the expression of cyclin D1 and cyclin E as well as CDK2 and CDK4 (Fig. 3A).

M. officinalis extract-induced cell cycle arrest is associated with a decrease of kinase activities associated with cyclins. Due to their ability to activate cyclins, the kinase activities of

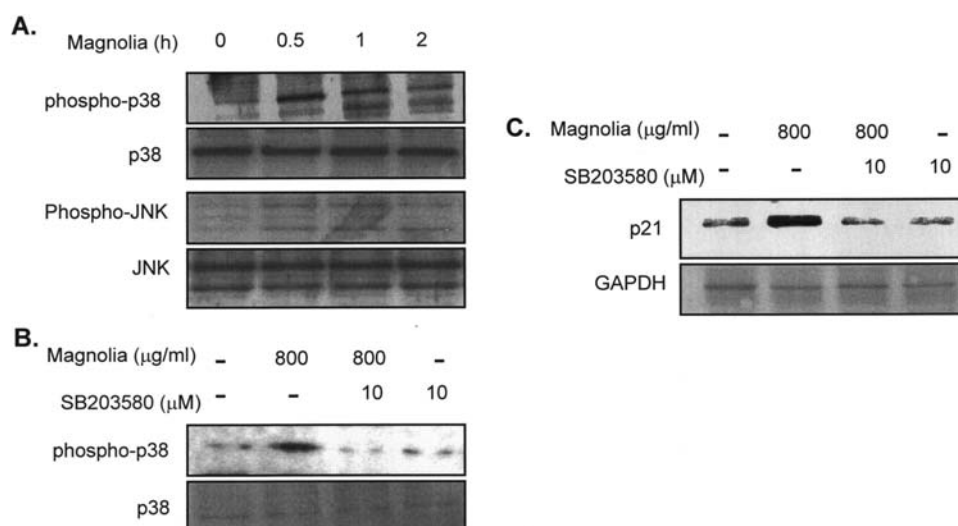


Figure 4. *Magnolia officinalis* extract-induced p38 MAP kinase phosphorylation is associated with p21WAF1 induction. 5637 cells were treated for the indicated times with 800 μg/ml *Magnolia officinalis* extract. (A) Cells were harvested, lysed, and the phosphorylation levels of JNK and p38 were detected by immunoblot analysis using antibodies phosphospecific for JNK and p38. (B) Cells were pretreated for 40 min with 10 μM SB203580 before cells were treated with 800 μg/ml *Magnolia officinalis* extract at 30 min. (C) Cells were pretreated for 40 min with the indicated concentrations of SB203580 before stimulating the cells with 800 μg/ml *Magnolia officinalis* extract at 24 h. Proteins were resolved by SDS-PAGE and immunoblotted with antibodies to p21WAF1. The results are expressed as the mean from three triplicate experiments.

CDKs are the driving force for the progression of the cell cycle through the transition checkpoints, which are essential components of cyclin-CDK complexes (6-9). Therefore, the effects of *M. officinalis* extract treatment on the kinase activities associated with CDK2 and CDK4 (Fig. 3B) were assessed. *M. officinalis* extract inhibited the kinase activities of both the CDK2- and CDK4-immunoprecipitates in a dose dependent manner at 24 h (Fig. 3B).

M. officinalis extract-induced cell cycle arrest is associated with the up-regulation of the CKIs, p21WAF1 and p27Kip1. Next, the effect of *M. officinalis* extract on the induction of p21WAF1, which is known to regulate the entry of cells at the G1-S phase transition checkpoint (10,11), was assessed. An immunoblot analysis revealed that treatment of 5637 cells with *M. officinalis* extract resulted in a significant dose-dependent induction in p21WAF1 compared with the basal levels (Fig. 3C). Treatment of cells with *M. officinalis* extract also showed up-regulation of p27Kip1 protein levels. However, under similar experimental conditions, the levels of expression of p53 tumor suppressor protein were decreased slightly, suggesting that it is unlikely that p53 is involved in the cell cycle arrest induced by *M. officinalis* extract (Fig. 3C).

Effects of M. officinalis extract on JNK and p38 MAP kinase activation in 5637 cells. To clarify whether *M. officinalis* extract affects MAP kinase activation, we examined the effect of various time courses for *M. officinalis* extract on JNK and p38 MAP kinase activation. Cells were treated with *M. officinalis* extract for the indicated times. Fig. 4A shows a Western blot analysis of 5637 cells treated with *M. officinalis* extract, using antibodies specific for phosphorylated JNK and p38 MAP kinase. These experiments indicate that JNK and p38 MAP kinase are significantly activated by *M. officinalis* extract. *M. officinalis* extract increased the amount of phospho-

rylated JNK and p38 MAP kinase at 3 h, suggesting that it induces cell growth inhibition via the activation of JNK and p38 MAP kinase. This *M. officinalis* extract-induced p38 MAP kinase activity was blocked by SB203580, a p38 MAP kinase specific inhibitor (Fig. 4B). This result indicates that *M. officinalis* extract-induced cell growth inhibition signaling likely requires a p38 MAP kinase mediated pathway.

M. officinalis extract-induced p21WAF1 is blocked by SB203580, a specific inhibitor of p38 MAP kinase. p38 MAP kinase has recently been shown to be involved in the regulation of the cell cycle (26,27) and in the spindle assembly checkpoint and mitotic arrest (28). The observations that p38 MAP kinase activity is induced by *M. officinalis* extract prompted consideration of the possibility that p38 MAP kinase activity is related to the induction of p21WAF1. To elucidate the signal cascade triggered by *M. officinalis* extract, the possible requirement of p38 MAP kinase activity for the induction of p21WAF1 was investigated. Thus, 5637 cells were pretreated for 40 min with or without 10 μM SB203580, followed by exposure to 800 μg/ml *M. officinalis* extract. As shown in Fig. 4C, the *M. officinalis* extract-induced increase in p21WAF1 expression was reduced to the control level by SB203580. These results strongly suggest that p38 MAP kinase is required in the regulation of p21WAF1 induction in response to *M. officinalis* extract.

M. officinalis extract-induced cell growth inhibition is reversed by p38 MAP kinase inhibition. In order to investigate the effect of p38 MAP kinase activation on *M. officinalis* extract-induced cell growth inhibition, the cells were treated with SB203580, and the MTT assay, [³H]thymidine incorporation assay and immunoblot experiments were performed (Fig. 5). The 5637 cells were pretreated for 40 min with or without 10 μM SB203580, followed by treatment with 800 μg/ml

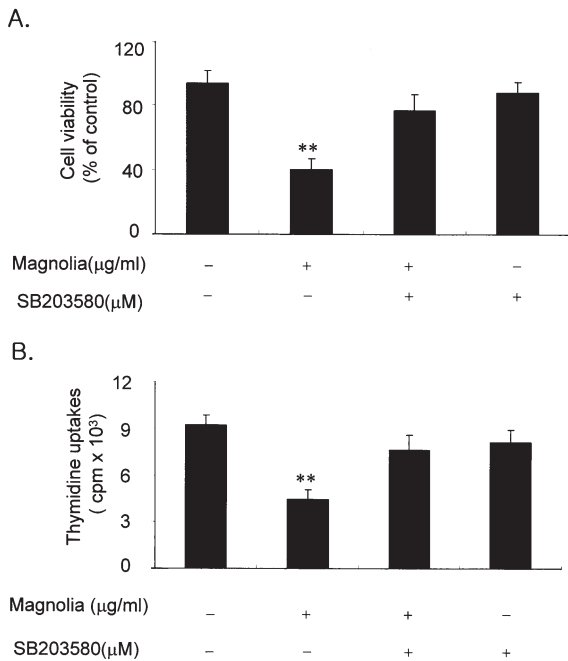


Figure 5. Inhibition of p38 MAP kinase activity reverses *Magnolia officinalis* extract-induced cell growth inhibition. Cells were plated in plates and were pre-incubated for 30 min in the absence or presence of 10 μ M SB203580. Cells were then treated with 800 μ g/ml *Magnolia officinalis* extract. MTT assay and thymidine uptake experiments were determined as described in Materials and methods. Indicated values are means of triplicate wells. ** $P < 0.01$ compared with no *Magnolia officinalis* extract treatment.

M. officinalis extract in the presence of 10% serum. As shown in Fig. 5, the inhibition of cell viability and [³H]thymidine incorporation by *M. officinalis* extract were reversed by the pretreatment with SB203580, suggesting that the p38 MAP

kinase signaling pathway in 5637 cells was involved in the cell growth inhibition by *M. officinalis* extract.

M. officinalis extract decreases constitutive and TNF- α -induced NF- κ B activation. The transcription factor NF- κ B mediates tumor promotion, angiogenesis and metastasis through the expression of genes participating in malignant conversion and tumor promotion particularly in inflammation-associated cancer models (29-31). That phenomenon is observed in various cell types upon stimulation with cytokines such as TNF- α (14,33). To determine the effect of *M. officinalis* extract on constitutive and TNF- α -induced NF- κ B activity, an electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts of 5637 cells treated with TNF- α (100 ng/ml) in the presence or absence of *M. officinalis* extract. In the EMSA, the nuclear extracts were incubated with a radiolabeled double-stranded oligonucleotide probe containing the consensus sequence for the NF- κ B binding site and electrophoresed in a 5% non-denaturing polyacrylamide gel. A nuclear extract from 5637 cells treated with TNF- α showed an increased binding to the NF- κ B binding activity (Fig. 6B). Moreover, as shown in Fig. 6A and B, constitutive and TNF- α induced NF- κ B binding activity was almost abolished in 5637 cells by treatment with *M. officinalis* extract.

TNF- α induces NF- κ B activation through p38 MAP kinase activation. p38 MAP kinase has recently been shown to be involved in the regulation of the transcription factors (32,33). The observations that p38 MAP kinase activity is induced by *M. officinalis* extract led to the consideration of whether the p38 MAP kinase activity is related to the induction of TNF- α mediated NF- κ B. To address this hypothesis, we performed

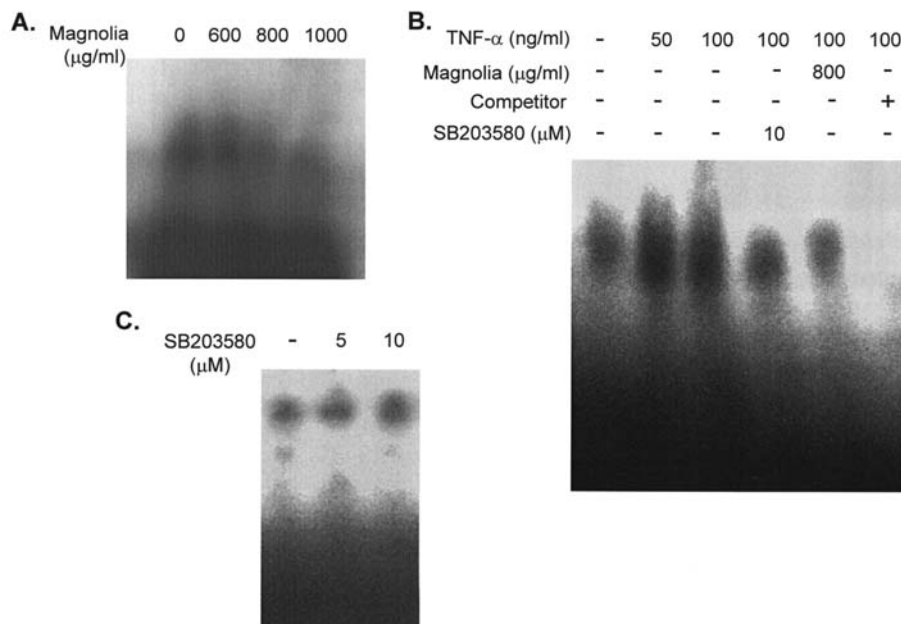



Figure 6. Inhibitory effects of *Magnolia officinalis* extract on constitutive and TNF- α -induced NF- κ B activation in 5637 cells. (A) Nuclear extracts from cells incubated with indicated concentration of *Magnolia officinalis* extract for 24 h were subjected to EMSA to assess NF- κ B DNA binding activity. (B) Cells were pretreated with the indicated concentrations of *Magnolia officinalis* extract or SB203580 for 40 min in serum-free medium and then incubated with TNF- α (50, 100 ng/ml) for 24 h. After incubation, nuclear extracts from the cells were analyzed by EMSA for activated NF- κ B using radiolabeled oligonucleotide probes. (C) Nuclear extracts from cells incubated with indicated concentration of SB203580 for 24 h were subjected to EMSA to assess NF- κ B DNA binding activity.

 SPANDIDOS PUBLICATIONS assays as described in Materials and methods.

effectively inhibited the increased NF- κ B binding activity in response to TNF- α (Fig. 6B). However, under treatment of SB203580 the control level of NF- κ B binding activity remained essentially unchanged (Fig. 6C). These results suggest that TNF- α -induced NF- κ B is at least partially involved in the p38 MAP kinase-mediated control in 5637 cells.

Discussion

The data show that *M. officinalis* extract treatment inhibited the growth of 5637 bladder cancer cells. This is consistent with other studies showing that *M. officinalis* extract treatment induces cell growth inhibition in several cell lines (24,25,35). The inhibition was due to cell cycle arrest in G1 phase. Because this study demonstrated that treatment of 5637 cells with *M. officinalis* extract resulted in the G1 phase arrest of the cell cycle (Fig. 2), the effect of *M. officinalis* extract on cell cycle regulatory molecules that are operative in the G1 phase of the cell cycle was examined. In this experiment, the treatment of 5637 cells with *M. officinalis* extract resulted in a significant down-modulation of cyclin D1/CDK4 and cyclin E/CDK2, although to different extents. *M. officinalis* extract treatment was found to result in the dose-dependent inhibition of kinase activities associated with all of the CDKs examined.

The data demonstrated a significant up-regulation in the p21 WAF1 and p27 Kip1, CKIs, during G1-phase arrest of 5637 cells by *M. officinalis* extract. Many studies have shown that the regulation of G1 cell cycle arrest can be attributed to a number of cellular proteins, including p53 (34). However, *M. officinalis* extract had no effect on p53 protein levels in 5637 cells, as determined by immunoblot analysis, suggesting that the *M. officinalis* extract-induced accumulation of p21 WAF1 and p27 Kip1 could also be responsible for G1 phase arrest. Although reports have appeared showing that *M. officinalis* extract induces G1 cell cycle arrest and apoptosis through a p53-independent pathway in several cell lines (25,35), to our knowledge, this is the first systematic study showing the involvement of each component of the CKI-cyclin-CDK machinery during the cell cycle arrest of 5637 cells induced by *M. officinalis* extract.

The MAP kinase superfamily plays a crucial role in regulation of cell growth, differentiation or even programmed cell death in response to diverse extracellular stress in eukaryotic cells (36,37). The exposure of 5637 cells to *M. officinalis* extract resulted in the up-regulation of the JNK and p38 MAP kinase phosphorylation. SB203580, a specific inhibitor of p38 MAP kinase, markedly inhibited *M. officinalis* extract-induced p38 MAP kinase phosphorylation, suggesting that *M. officinalis* extract induces cell growth inhibition via the activation of p38 MAP kinase. However, the issue of whether JNK is also associated with *M. officinalis* extract-induced cell growth inhibition remains to be determined.

Abundant evidence has shown that the MAP kinase pathway was involved in cell growth inhibition (2) and/or the regulation of the cell cycle (26,27). It is noteworthy that the role of p38 MAP kinase in cell growth inhibition is dependent on the cell type and the stimuli used (38), but its role in 5637

cells in response to *M. officinalis* extract is unclear. The findings herein show that *M. officinalis* extract induces the phosphorylation of p38 MAP kinase in 5637 cells. The observation that p21WAF1 induction was observed as the result of *M. officinalis* extract treatment prompted an examination into the role of p38 MAP kinase in the regulation of p21WAF1 expression. The specific inhibitor of p38 MAP kinase, SB203580, prevented the *M. officinalis* extract-induced p21WAF1 of 5637 cells, indicating that *M. officinalis* extract induces p21WAF1 in 5637 cells through the activation of p38 MAP kinase. These results are consistent with recent reports that have demonstrated a requirement of the p38 MAP kinase pathway in the agent-induced G1 phase cell cycle arrest followed by cell growth inhibition (26,39). Collectively, the present results suggest that p38MAP kinase is involved in *M. officinalis* extract-induced cell growth inhibition through the induction of p21WAF1 in 5637 cells. However, a limitation of our study is that a clear understanding of relationships between the activation of the JNK pathway and the regulation of p21WAF1 has not been developed. The exact mechanism needs to be studied further.

The effects of *M. officinalis* extract on 5637 cell proliferation were confirmed by inhibiting p38 MAP kinase. Consistent with the observations that *M. officinalis* extract treatment inhibited cell proliferation, blockade of p38 MAP kinase with SB203580 rescued 5637 cell proliferation. These data provide evidence that p38 MAP kinase is an important mediator for *M. officinalis* extract-induced 5637 cell proliferation.

Numerous lines of evidence indicate that NF- κ B promotes tumorigenesis (29-31). The activation of NF- κ B is thought to be part of a stress response as it is activated by a variety of stimuli that includes cytokine TNF- α (14,33). A number of reports have demonstrated that NF- κ B activation can maintain tumor cell viability, and that inhibiting NF- κ B activation alone can be sufficient to induce cell death (40-42). In agreement with these reports, the results of this study indicate that *M. officinalis* extract significantly inhibits constitutive and inducible NF- κ B activation.

In an attempting to understand how TNF- α mediates NF- κ B activation, this study focused on p38 MAP kinase because p38 MAP kinase has recently been shown to be involved in the regulation of the transcription factors (32,33). The study herein clearly shows that the ability of the p38 MAP kinase inhibitor, SB203580, to reduce NF- κ B activation in 5637 cells is achieved via a reduced NF- κ B binding (Fig. 6), thus suggesting that p38 MAP kinase activity may play a role in the TNF- α -mediated regulation of NF- κ B on 5637 cells.

In conclusion, this study has demonstrated that *M. officinalis* extract inhibited the growth of 5637 bladder cancer cells. The inhibition was due to cell cycle arrest in the G1 phase and the findings show that the inhibition of cyclin D1/CDK4 and cyclin E/CDK2 activities as the result of the increased expression of p21WAF1, p27Kip1 and the activation of p38 MAP kinase may be the primary mechanisms by which *M. officinalis* inhibits 5637 cell proliferation. These data demonstrate a link between p38 MAP kinase activation and p21WAF1 induction, and suggest that p38 MAP kinase is required for the inhibition of cell growth by *M. officinalis*. In addition, these results showed that the inhibitory effects

of *M. officinalis* on constitutive and TNF- α induced NF- κ B activation. Finally, this study examined how TNF- α modulates NF- κ B activation through the p38 MAP kinase signaling pathway in 5637 cells. These findings may, in part, explain the therapeutic benefits of *M. officinalis* extract for urinary bladder cancer.

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