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Magnolol elicits activation of the extracellular signal-regulated kinase pathway by inducing p27KIP1-mediated G2/M-phase cell cycle arrest in human urinary bladder cancer 5637 cells

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ABSTRACT

Magnolol has been reported to play a role in antitumor activity. However, the relevant pathway integrating cell cycle regulation and signaling pathways involved in growth inhibition in cancer cells remains to be identified. In the present study, magnolol treatment of these cells resulted in significant dose-dependent growth inhibition together with apoptosis, G1- and G2/M-phase cell cycle arrest at a 60 μM (IC_{50}) dose in 5637 bladder cancer cells. In addition, magnolol treatment strongly induced p27KIP1 expression, and down-regulated expression of cyclin-dependent kinases (CDKs) and cyclins. Moreover, treatment with magnolol-induced phosphorylation of ERK, p38 MAP kinase, and JNK. Among the pathway inhibitors examined, only PD98059, an ERK-specific inhibitor, blocked magnolol-dependent p27KIP1 expression. Blockade of ERK function consistently reversed magnolol-mediated inhibition of cell proliferation and decreased G2/M cell cycle proteins, but not G1 cell cycle proteins. Furthermore, magnolol treatment increased both Ras and Raf activation. Transfection of cells with dominant negative Ras (RasN17) and Raf (RafS621A) mutant genes suppressed magnolol-induced ERK activity and p27KIP1 expression. Finally, the magnolol-induced reduction in cell proliferation and G2/M cell cycle proteins was also abolished in the presence of RasN17 and RafS621A mutant genes. These data demonstrate that the Ras/Raf/ERK pathway participates in p27KIP1 induction, leading to a decrease in the levels of cyclin B1/Cdc2 complexes and magnolol-dependent inhibition of cell growth. Overall, these novel findings concerning the molecular mechanisms of magnolol in 5637 bladder cancer cells provide a theoretical basis for therapeutic treatment of malignancies.

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1. Introduction

Bladder cancer is one of the most common malignancies worldwide, and more than 90% of malignant bladder cancer is transitional cell carcinoma [1]. Associated with the risk of bladder cancer are environmental factors, such as chemical carcinogens, including polycyclic aromatic hydrocarbons, aromatic amines, and *N*-nitroso compounds; some anticancer drugs; and reactive oxygen species [2–4]. At initial presentation, 70–80% of cases are superficial, while the remainder of cases present with muscle-invasive disease. Superficial bladder cancers represent a heterogeneous group of tumors, and about 60% of them will recur after transurethral resection [5]. Some of them will progress to invasive and/or metastatic tumors and are therefore potentially lethal [6]. For these people, effective preventive measures are needed.

The progression of the cell cycle in eukaryotes is controlled by the action of cyclin-dependent kinases (CDKs) and their activating subunits, cyclins [7–9]. After different growth controlling signals, cells are stimulated to divide in response to mitogens and exit the G1 phase, entering the S phase and G2/M phases. 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 [10]. A key regulator of the G2/M transition of the cell cycle is a complex of cell division cycle Cdc2 (CDK1) and a B-type cyclin [11]. Cyclin B1/Cdc2 complexes are regulated by phosphorylation and protein interaction events that tightly control the timing and extent of Cdc2 activation. On the other hand, dephosphorylation of Tyr-15 by the phosphatase Cdc25C [12] increases the kinase activity of the cyclin B1/Cdc2 complex in the M phase. The kinase activity of these cyclins/CDK complexes can be negatively regulated by CDK-inhibitory proteins, including p21WAF1 (p21) and p27KIP (p27) [10–13].

The extracellular signal-regulated kinase (ERK) pathway is known to be activated by various stimuli including mitogens and cell survival factors [14–16]. ERK activation may exert either an antiapoptotic [17,18] or proapoptotic [19,20] influence depending upon the cellular context. The activity of the MAP kinase pathway seems to be regulated by Ras/Raf expression. Activation of the Ras/Raf signal transduction pathway has been shown to contribute to both cell growth [21,22] and cell death [23,24] by influencing the cell cycle machinery in numerous types of cancer.

Magnolol, a hydroxylated biphenyl compound isolated from extracts of the commonly used Chinese medicinal herb *Magnolia officinalis*, is reported to have a variety of pharmacological activities, including antitumor, antioxidant, antimicrobial, antiallergic, antifungal, anti-inflammatory, and antiatherogenic effects [25–30]. A recent study reported that magnolol can suppress proliferation of cultured cancer cells by inhibiting DNA synthesis and inducing apoptosis [28]. It has recently been shown that magnolol activated G1 cell cycle arrest in smooth muscle cells [30]. Although studies have analyzed the effects of magnolol on tumor growth inhibition involving G1 cell cycle arrest in several cancer cell lines [28,29], available information on the effect of magnolol on cellular responses via the p27-mediated G2/M arrest and MAP kinase phosphorylation is lacking. In the present study, we report on the effect of magnolol on a relevant pathway between the cell cycle regulation and signaling pathways involved in growth inhibition in human bladder cancer 5637 cells.

2. Materials and methods

2.1. Materials

Magnolol was purchased from Wako Pure Chemical Industries, Ltd. (Tyoko, Japan). Polyclonal antibodies to cyclin E, CDK2, CDK4, cyclin B1, pCdc2, Cdc2, pCdc25c, and Cdc25c were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies to cyclin D1, p21WAF1, p53, p27, ERK, anti-Raf, phospho-Raf, phosphor-ERK, p38 MAP kinase, phosphor-p38 MAP kinase, JNK and phosphor-JNK were obtained from New England Biolabs. PD98059, SP600125 and SB203580 were obtained from Calbiochem (San Diego, CA). Anti-Ras antibody was obtained from Transduction Laboratories. The pCMV vector encoding dominant negative Ras (RasN17) and dominant negative Raf (RafS621A) were from Clontech.

2.2. Cell cultures

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% CO_2 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).

2.3. Cell viability assay

Subconfluent, exponentially growing 5637 cells in 24-well plates were incubated with *magnolol* for the indicated times. Cell viability was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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 [31], measured by light absorbance at 490 nm. Cell growth and viability were checked before and after treatment with *magnolol* using phase contrast microscopy.

2.4. [^{3}H]thymidine incorporation

For [^{3}H]thymidine-uptake experiments, cells were grown to near confluence in 24-well tissue culture plates and then made quiescent and treated with thrombin, as indicated. Cells were incubated for an additional 24 h, during which time they were labeled with [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, MA) at 1 μCi/ml. After labeling, the cells were washed with phosphate-buffered saline, fixed in cold 10% trichloroacetic acid, then washed with 95% ethanol. Incorporated [^{3}H]thymidine was extracted in 0.2 M NaOH and measured by liquid scintillation counter as previously described [31,32].

2.5. Cell cycle analysis (FACS)

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

2.6. Apoptosis detection by ELISA

This method is based on a quantification of the enrichment of mono- and oligo-nucleosomes in the cytoplasm by Cell Death Detection ELISA kit (Roche; Mannheim, Germany) [31].

2.7. Immunoblotting

Growth-arrested cells were treated with magnolol for specified time periods at 37 °C. Cell lysates were prepared, and immunoblotting was performed as described previously [31,32].

2.8. Immunoprecipitation and immune complex kinase assays

Cell lysates were prepared with ice-cold lysis buffer (containing, in mM/l, HEPES [pH 6.0] 50, NaCl 150, EDTA 1, EGTA 2.5, DTT 1, β-glycerophosphate 10, NaF 1, Na_3VO_4 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 [from KONTES], 30% power, two times for 10 s each time). Lysates were clarified by centrifugation at 10 000 × *g* for 5 min, and the supernatants were precipitated by treatment with protein A-Sepharose beads precoated with saturating amounts of the indicated antibodies at 4 °C for 2 h. When monoclonal antibodies were used, protein A-Sepharose was pretreated with rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories). Proteins immunoprecipitated on the beads were washed four times with 1 ml of lysis buffer and twice with kinase buffer (containing, in mM/l, HEPES 50, $MgCl_2$ 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 H_1 (Life Technologies, Inc.), 20 μM/l ATP, and 5 μCi of [γ^{32}P]ATP (4500 μCi/mmol; ICN) and incubated for 20 min at 30 °C with occasional mixing. The reaction was terminated by the addition of 25 μl of 2× concentrated Laemmli sample buffer and separated on 10% or 12.5% SDS-polyacrylamide gels. Migration of histone H_1 or GST-pRb was determined by Coomassie blue staining; phosphorylated pRb and histone H_1 were visualized.

2.9. Affinity precipitation of Ras active form (Ras-GTP)

Cells seeded on 100 mm dishes were serum starved at subconfluency (5×10^4 cells/cm^2) and treated with 100 ng/ml of TNF-α for the indicated times. After washing with ice-cold PBS once, the cells were lysed by adding 500 ∝l of lysis buffer (25 mM HEPES, 10 mM EDTA, 1% Igepal CA630, complete protease inhibitor cocktail (Roche Diagnostics), 1 mM sodium orthovanadate, 10% glycerol). The lysate was clarified by centrifugation for 15 min at 14,000 × *g* and the protein concentration of the lysate was determined by a BCA assay (Pierce, Rockford, IL). Equal amounts of cell lysates (500 μg) were subjected to affinity precipitation for Ras-GTP with 10 μl of an agarose suspension conjugated with GST fusion protein, which corresponds to the human Ras-binding domain of c-Raf (GST-RBD) (Upstate Biotechnology, Lake Placid, NY). After 1 h incubation at 4 °C, the agarose was washed three times with lysis buffer and boiled with 30 μl SDS sample buffer. The product was resolved by 15% SDS-PAGE, followed by immunoblotting with the anti-Ras antibody.

2.10. Statistical analysis

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

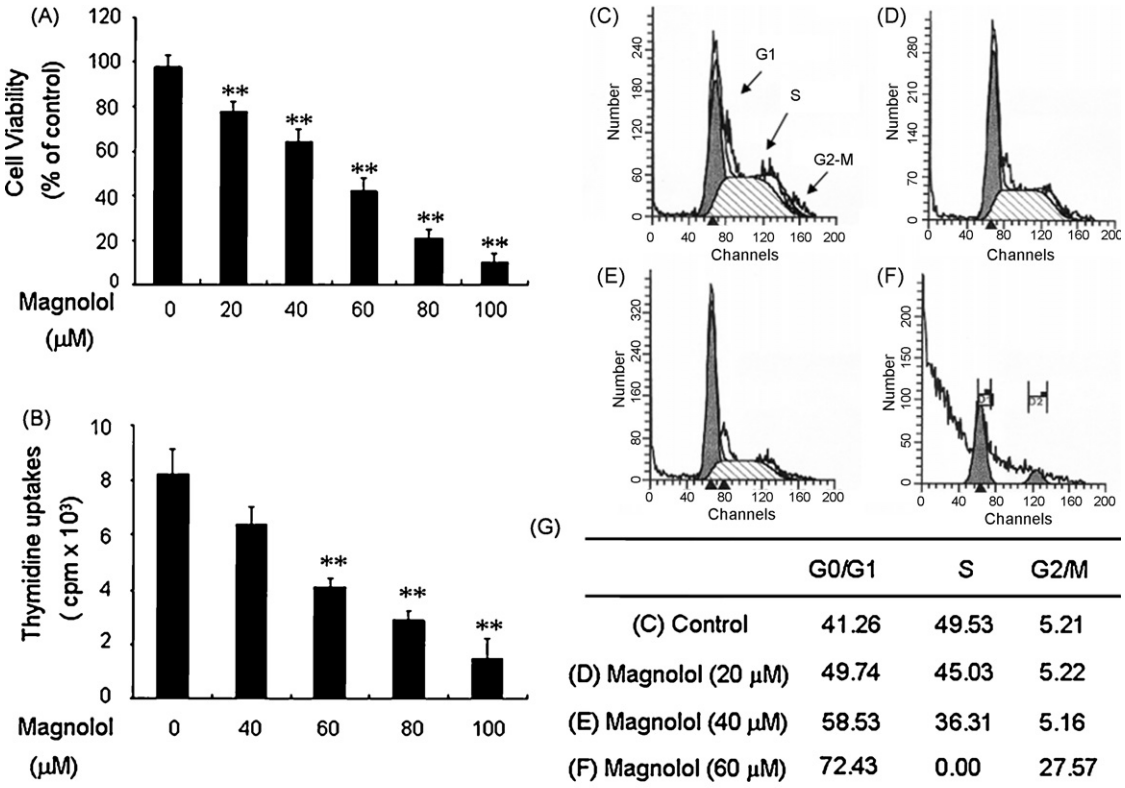
3. Results

3.1. Magnolol inhibits the proliferation of human urinary bladder cancer cells

In order to investigate the proliferation inhibitory effect of magnolol on bladder cancer cells, 5637 cells were grown in 10% FBS containing medium in the absence or presence of various concentrations of magnolol (0–100 μM) for 24 h. As shown in Fig. 1A, magnolol significantly inhibited cell viability, as evidenced by a MTT assay, in a concentration-dependent manner. In addition, the effect of magnolol on DNA synthesis was determined by [^{3}H]thymidine incorporation after treatment with various concentrations of magnolol for 24 h. Magnolol had a concentration-dependent inhibitory effect on 5637 cell growth (Fig. 1B). The vehicle (DMSO) alone had no effect on the basal levels of cell viability and thymidine incorporation (data not shown).

3.2. Magnolol induces G1- and G2/M-phase cell cycle arrest

Flow cytometric analysis was performed to determine whether the magnolol-induced cell growth inhibition was due to cell cycle arrest at a specific point in the cell cycle. 5637 cells were cultured in the presence of 0, 20, 40, or 60 μM magnolol for 24 h and subsequently cell cycle distribution was analyzed by flow cytometry. Upon treatment with 60 μM magnolol (at its IC_{50} concentration, as assessed by MTT assay and [^{3}H]thymidine incorporation), a flow cytometric analysis demonstrated that magnolol (60 μM) induced the accumulation of significant numbers of cells in the G1 and G2/M phase of the cell cycle, and the G1- and G2/M-phase accumulation was accompanied by a corresponding reduction in the percentages of cells in the S phase, suggesting that the observed growth inhibitory effects of magnolol in 5637 cells were due to cell cycle arrest (Fig. 1C–G).



(G)

	G0/G1	S	G2/M
(C) Control	41.26	49.53	5.21
(D) Magnolol (20 μM)	49.74	45.03	5.22
(E) Magnolol (40 μM)	58.53	36.31	5.16
(F) Magnolol (60 μM)	72.43	0.00	27.57

Fig. 1 – Magnolol suppressed cell proliferation via G1 and G2/M cell cycle arrest in 5637 cells. (A) Subconfluent, exponentially growing cells were incubated with magnolol for 24 h at indicated concentrations in 10% DMEM. Cell viability was determined by using a modification of the MTT assay. (B) Measurement of DNA replication by thymidine uptake as a marker of cell proliferation. Cells were grown to near confluence in 24-well tissue culture plates and treated with magnolol as indicated. Cells were incubated for an additional 24 h and labeled with [methyl-^{3}H]thymidine at 1 μCi/ml during the last 12 h of this time period. Results are presented as mean ± S.E. from three triplicate experiments. $^{**}P < 0.01$ compared with no magnolol treatment. Magnolol induces G1 and G2/M cell cycle arrest in 5637 cells. Cells were treated with 0 μM (C), 20 μM (D), 40 μM (E), and 60 μM magnolol (F). Cells were subjected to flow cytometric analysis to determine the effect of magnolol on cell cycle distribution. (G) The percentage of cells in each population is shown as the mean ± S.E. from three triplicate experiments.

We next examined the effect of magnolol on cell cycle regulatory molecules that are operative in the G1 phase of the cell cycle. To determine whether the observed growth inhibitory effects of magnolol in 5637 cells were due to decreased activation of cell cycle machinery, the expression of cell cycle regulatory molecules was examined using immunoblot and kinase assays. Treatment of cells with magnolol 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. 2A). The kinase activities associated with the CDKs drive cell cycle progression through the transition checkpoints because they activate cyclin-essential components of cyclin-CDK complexes. Therefore, kinase activities associated with CDK2 and CDK4 were assessed in magnolol-treated cells. CDK complexes were immunoprecipitated using specific anti-CDKs antibodies, and the levels of CDK-associated kinase activities were measured using Rb protein or histone H1 as the substrate. The kinase activities of both CDK2- and CDK4-immunoprecipitates were markedly inhibited after treatment with magnolol in the 5637 cells (Fig. 2B).

To elucidate mechanisms underlying the G2/M arrest observed after addition of magnolol, we next conducted a detailed analysis of the molecules involved in the G2/M phase of the cell cycle. As shown in Fig. 2C, magnolol treatment of cells also resulted in a decrease in pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15) and Cdc2 protein levels. Similar magnolol treatment also resulted in a strong decrease in cyclin B1 protein levels in 5637 cells (Fig. 2C). Taken together, these results suggest that changes in the expression of G2/M regulatory proteins in 5637 cells by magnolol are related to its overall efficacy in inducing G2/M arrest in these cells.

3.3. *Magnolol-induced cell cycle arrest is associated with the up-regulation of the CKI, p27KIP1*

We next assessed the effect of magnolol on the induction of p27, which is known to regulate the entry of cells at the G1-S phase transition checkpoint [7,10,13]. An immunoblot analysis revealed that treatment of 5637 cells with magnolol resulted in significant dose-dependent induction of p27 compared with

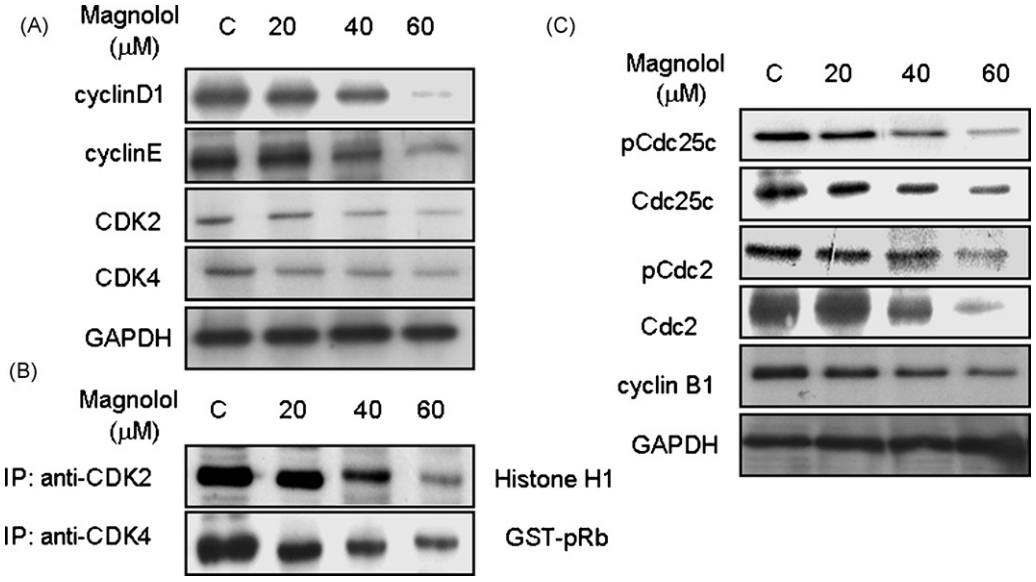


Fig. 2 – Effect of magnolol on G1 and G2/M cell cycle regulator cyclins and CDKs. (A and C) 5637 cells were treated with magnolol at indicated concentration and immunoblot analysis was performed with antibodies specific for cyclin D1, cyclin E, cyclin B1, CDK2 CDK4, pCdc2, Cdc2, pCdc25c, and Cdc25c. The results from representative experiments were normalized to GAPDH expression. (B) Cells were treated with magnolol at the indicated concentrations at 24 h, and the cells were then harvested. Total cell lysates were then immunoprecipitated with anti-CDK2 and anti-CDK4 antibodies. The kinase reaction was performed using histone H1 (for CDK2) or GST-Rb (for CDK4) as substrate.

the non-treated cells (Fig. 3A). However, magnolol had no effect on induction of p21. Moreover, under similar experimental conditions, the levels of expression of p53 tumor suppressor protein were unaffected, suggesting that it is unlikely that p21 and p53 are involved in the cell cycle arrest induced by magnolol (Fig. 3A).

Based on our findings showing that magnolol strongly induces CDKI, p27, expression in 5637 cells, and since such an induction in CDKI has been shown to result in an increased interaction with CDKs leading to a decrease in their kinase activity [7,10,13], we next assessed whether magnolol causes an increased interaction between induced CDKI and CDKs. To assess the effect of magnolol on this binding, cell extracts were subjected to immunoprecipitation using CDK2, CDK4 or Cdc2 antibody, and after SDS-PAGE and blotting, membranes were probed with anti-p27. In magnolol treated cells, the association of CDK2 with p27 was maintained at high levels. Levels of the p27/CDK4 and p27/Cdc2 complexes were also increased in 5637 cells at 24 h after magnolol treatment (Fig. 3B). These results suggest that an increased interaction between induced levels of CDKI with CDKs may play an important regulatory role in inhibiting CDK kinase activity leading to G1 and G2/M arrest by magnolol in the cell cycle progression of human bladder 5637 cells. Collectively, these results demonstrate that magnolol induces the expression of p27 protein.

3.4. *Magnolol induces apoptosis in 5637 cells*

Next, we used an ELISA-based assay (see Section 2) to measure *magnolol*-induced apoptosis. As shown in Fig. 3C, using a quantitative assay, 60 μM *magnolol* significantly increased the cytoplasmic DNA–histone complex in 5637 cells.

3.5. *Effects of magnolol on ERK, JNK and p38 MAP kinase activation in 5637 cells*

Several studies have reported that MAPK signaling pathways may play an important role in a host of cellular functions, including cell growth, differentiation, development, apoptosis, and cell growth arrest [14,16,18]. To clarify whether magnolol affects MAP kinase activation, time course experiments, measuring ERK1/2, JNK and p38 MAP kinase activation in response to magnolol, were performed in 5637 cells. The results of these experiments indicated that ERK1/2, JNK and p38 MAP kinases were significantly activated by magnolol (Fig. 4A). Magnolol increased the amount of phosphorylated ERK1/2, JNK and p38 MAP kinase at 12 h, suggesting that magnolol induces cell growth inhibition via activation of the ERK1/2, JNK and p38 MAP kinase pathways. The effects of specific kinase inhibitors on the activation of MAP kinase then were analyzed. PD98059 is known to selectively block the activity of MAP kinase kinase (MEK), which activates ERK1/2 kinases. SB203580 is a specific inhibitor of p38 MAP kinase, and SP600125 inhibits JNK activity. Magnolol-induced phosphorylation of the MAP kinases (ERK1/2, p38 MAPK and JNK) was inhibited by PD98059, SB203580 and SP600125 (Fig. 4B). These results suggest that magnolol induced the activation of ERK1/2, p38 MAP kinase and JNK in 5637 cells.

3.6. *Magnolol-induced p27KIP1 is blocked by PD98059, a specific inhibitor of ERK*

Our observations that MAPK activity is induced by magnolol led us to consider whether the MAPK activity is related to the induction of p27. To elucidate the signal cascade triggered by MAPK, we investigated whether the MAPK signaling pathway

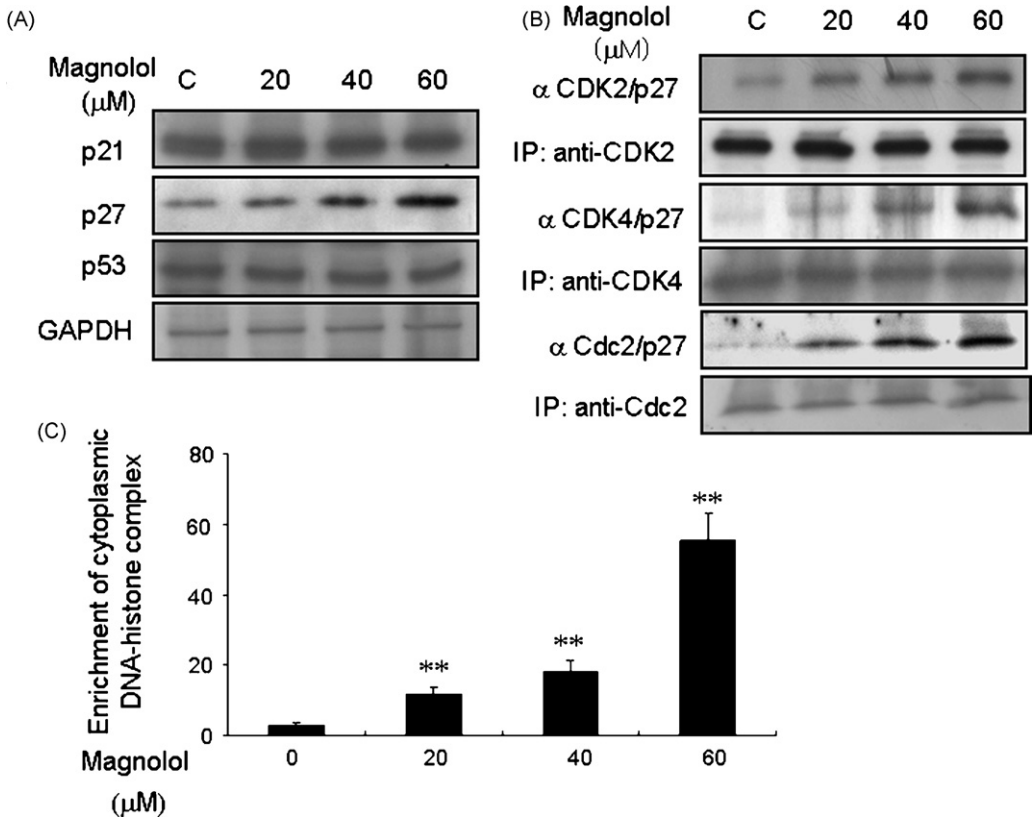


Fig. 3 – Induction of p27 expression and apoptosis in 5637 cells by treatment with magnolol. (A) Effect of magnolol on p21, p27 and p53 expression. (B) Equal amounts of cell lysates were subjected to immunoprecipitation with anti-CDK2, anti-CDK4 and anti-Cdc2 antibodies. The immunoprecipitates were examined by SDS-PAGE. After electrophoresis, the samples were transferred to a nitrocellulose membrane, followed by immunoblot analysis with an anti-p27 antibody. The results from representative experiments were normalized to immunoprecipitated CDK2, CDK4 and Cdc2 expression. (C) Detection of apoptosis in cells treated with magnolol. 5637 cells were incubated in 10% serum medium, followed by addition of magnolol, and cultured for 24 h, and cytoplasmic DNA–histone complex was measured by ELISA. Results are presented as mean ± S.E. from three triplicate experiments. $^{**}P < 0.01$ compared with no magnolol treatment.

was required for the induction of p27. Thus, 5637 cells were pretreated for 40 min with or without several kinase inhibitors, such as PD98059, SB203580 and SP600125, followed by exposure to 60 μM magnolol. As shown in Fig. 5A, the magnolol-induced increase in p27 expression was reduced to the control level by PD98059. SP600125 had a slight reverse effect on magnolol-mediated p27 expression. However, SB203580 had no apparent reverse effect on magnolol-induced p27 levels. In addition, protein levels of Cdc2 and Cdc25 were also reversed after PD98059 treatment for 24 h (Fig. 5C). Moreover, under similar experimental conditions, the levels of expression of CDK2 and CDK4 protein were unaffected. These results strongly suggest that the ERK signaling pathway is required in the regulation of p27-mediated G2/M-phase cell cycle arrest in response to magnolol.

3.7. *Magnolol-induced cell growth inhibition is reversed by ERK inhibition*

In order to investigate the effect of ERK activation on magnolol-induced cell growth inhibition, we pretreated the cells with PD98059 and performed a [^{3}H]-thymidine incorporation assay (Fig. 5B). 5637 cells were pretreated for 40 min with or without 40 μM PD98059, followed by treatment with 60 μM magnolol in the presence of 10% serum. As shown in Fig. 5B, the inhibition of [^{3}H]-thymidine incorporation by magnolol was reversed by pretreatment with PD98059. However, SB203580 and SP600125 had no effect on magnolol-induced cell growth inhibition (data not shown), suggesting that the ERK signaling pathway in 5637 cells was involved in the cell growth inhibition induced by magnolol.

3.8. *Magnolol-induced G2/M-phase cell cycle arrest is reversed by ERK inhibition*

To confirm the effect of ERK activation on magnolol-induced cell cycle distribution, we pretreated the cells with PD98059 and examined a flow cytometry analysis (Fig. 6A–E). The induction of G2/M-phase cell cycle arrest by magnolol was reversed by pretreatment with PD98059. Moreover, ERK inhibition had a slight reversible effect on S-phase cell cycle distribution in response to magnolol. However, PD98059 had no effect on magnolol-induced G1-phase cell cycle arrest. These data suggested that the ERK signaling pathway was involved in the magnolol-induced G2/M-phase cell cycle arrest in 5637 cells.

3.9. *Effects of a dominant negative RasN17 mutant gene on magnolol-mediated ERK, p27KIP1, growth inhibition and decreased Cdc2 levels*

The results described above led to an examination of whether or not Ras, an activator of the ERK1/2 signaling pathway, is involved in magnolol-induced growth inhibition. We first examined whether magnolol was able to induce the activation of Ras in 5637 cells. For this purpose, cellular levels of the Ras-active form (Ras-GTP) were analyzed via its ability to specifically bind to an immobilized c-Raf-GST-conjugated agarose. The bound Ras-GTP was then assayed by immuno-

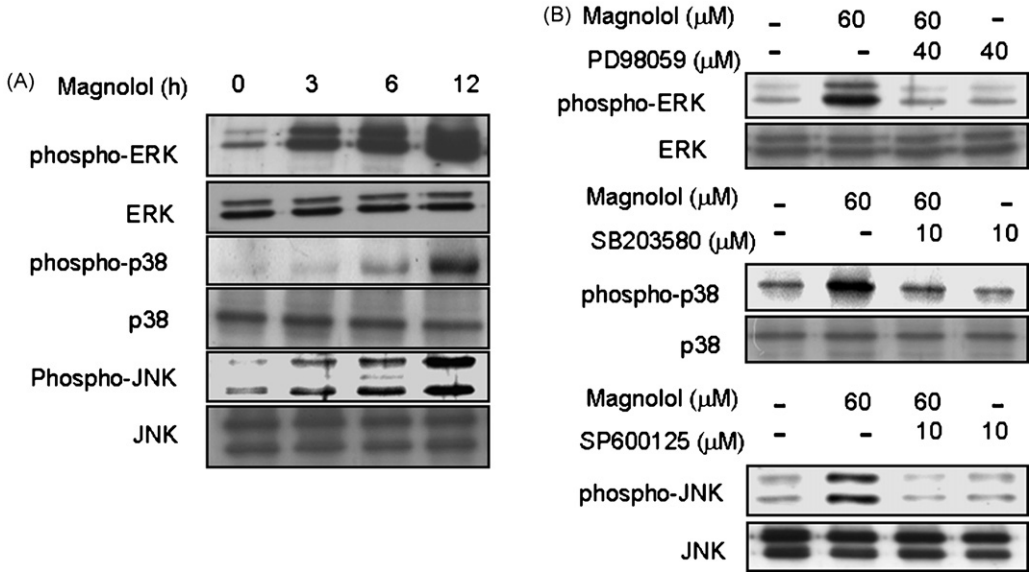


Fig. 4 – Magnolol-induced ERK1/2, JNK and p38 MAP kinase phosphorylation. (A) Cells were harvested, lysed and the phosphorylation levels of ERK1/2, JNK and p38 MAP kinase were detected by immunoblot analysis using antibodies phospho-specific for ERK1/2, JNK and p38 MAP kinases. (B) 5637 cells were pretreated for 40 min with PD98059 (40 μM), SB203580 (10 μM), and SP600125 (10 μM) before cells were treated with magnolol (60 μM) at 12 h.

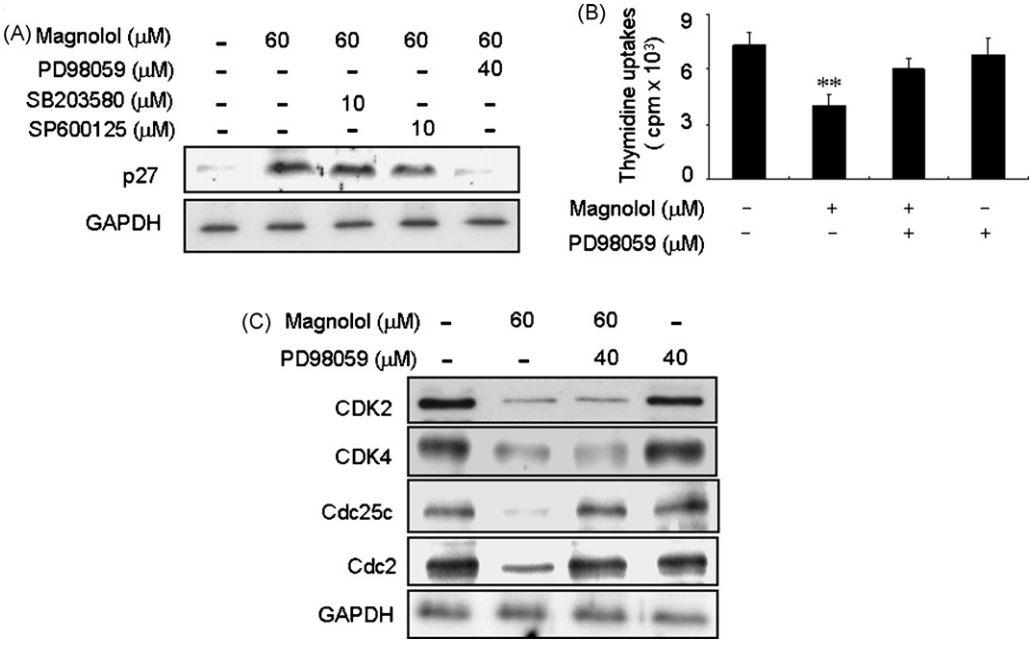
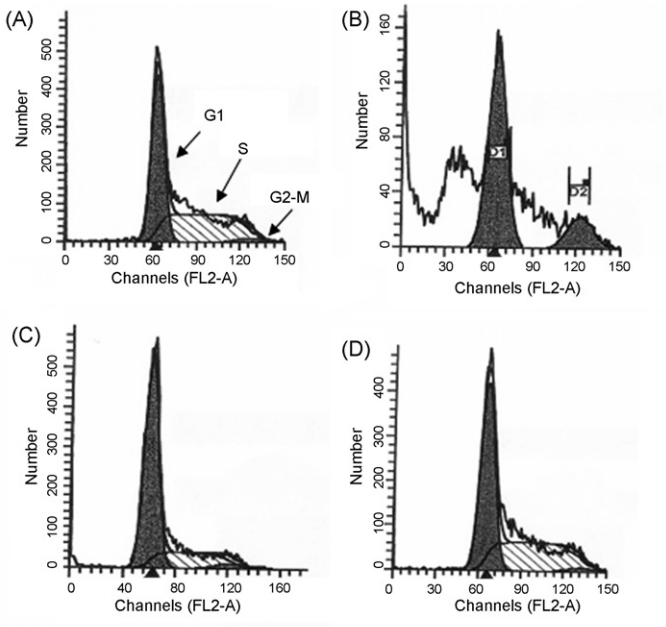


Fig. 5 – MEK1/2 inhibitor reversed p27 expression, decreased growth inhibition and G2/M-phase cell cycle-associated proteins by magnolol. (A and C) Cells were plated in plates and were pre-incubated for 40 min in the absence or presence of PD98059 (40 μM), SB203580 (10 μM), and SP600125 (10 μM). Cells were then treated with 60 μM magnolol, followed by immunoblot analysis performed with antibodies specific for p27, CDK2, CDK4, Cdc2 and Cdc25c. The results from representative experiments were normalized to GAPDH expression. (B) Cells were pretreated for 40 min with 40 μM PD98059 before cells were treated with 60 μM magnolol at 24 h. Thymidine uptake experiments were determined as described in Section 2. Indicated values are means of triplicate wells. $^{**}P < 0.01$ compared with no magnolol treatment.



(E)

	G0/G1	S	G2/M
(C) Control	47.43	43.72	8.85
(D) Magnolol (60 μM)	72.69.	0.00	27.31
(E) Magnolol (60 μM) + PD98059 (40 μM)	71.45	21.10	7.45
(F) PD98059 (40 μM)	48.62	42.23	9.15

Fig. 6 – MEK1/2 inhibitor reversed G2/M-phase cell cycle arrest by magnolol. Cells were treated with 0 μM (A), 60 μM magnolol (B), 60 μM magnolol + 40 μM PD98059 (C), and 40 μM PD98059 (D). Cells were subjected to flow cytometric analysis to determine the effect of magnolol on cell cycle distribution. (E) The percentage of cells in each population is shown as the mean ± S.E. from three triplicate experiments.

blotting with an anti-Ras antibody, which was proportional to the amount of the active form of Ras. As shown in Fig. 7A, magnolol is capable of inducing the activation of Ras in 5637 cells, while the expression level of Ras remained unchanged after treatment with magnolol.

To elucidate whether the activation of Ras is required for MAPK phosphorylation induced by magnolol, 5637 cells were transfected with a dominant negative Ras (RasN17) or an empty vector (EV) and then treated with magnolol. As shown in Fig. 7B, magnolol treatment of 5637 cells or of cells transfected with the empty vector (EV) induced ERK1/2 activity. These stimulatory effects were nearly completely suppressed by transfection with RasN17, suggesting that magnolol might activate MAPK through a Ras-dependent pathway in 5637 cells.

We further investigated the involvement of the Ras molecule in p27 expression and cell growth inhibition induced by magnolol, a dominant-negative RasN17 mutant gene was transfected into 5637 cells. As shown in Fig. 7C and D, magnolol treatment of 5637 cells or of cells transfected with EV-induced p27 expression and decreased [^{3}H]thymidine incorporation, Cdc2 and Cdc25c. RasN17 gene transfectants blocked the magnolol-induced p27 expression and prevented the magnolol-induced decrease of [^{3}H]thymidine incorporation, Cdc2 and Cdc25c. The results showed that magnolol regulates p27 expression, growth inhibition and decreased Cdc2 in 5637 cells by activating a Ras-dependent pathway. Collectively, these results suggest that the Ras/ERK signaling pathway must be involved in p27-mediated G2/M-phase cell cycle arrest and growth inhibition in 5637 cells in response to magnolol.

3.10. *RafS621A mutant gene reversed the effects of growth inhibition, decreased Cdc2 levels, ERK activation and p27KIP1 expression by magnolol*

The requirement for the activation of the Ras, Raf and ERK pathway, associated with cell proliferation, for cell function has been demonstrated by various stimuli [17–23]. The activation of the Raf molecule, which is a downstream molecule of Ras was also observed in the magnolol-treated 5637 cells. Fig. 8A shows an immunoblot analysis of 5637 cells

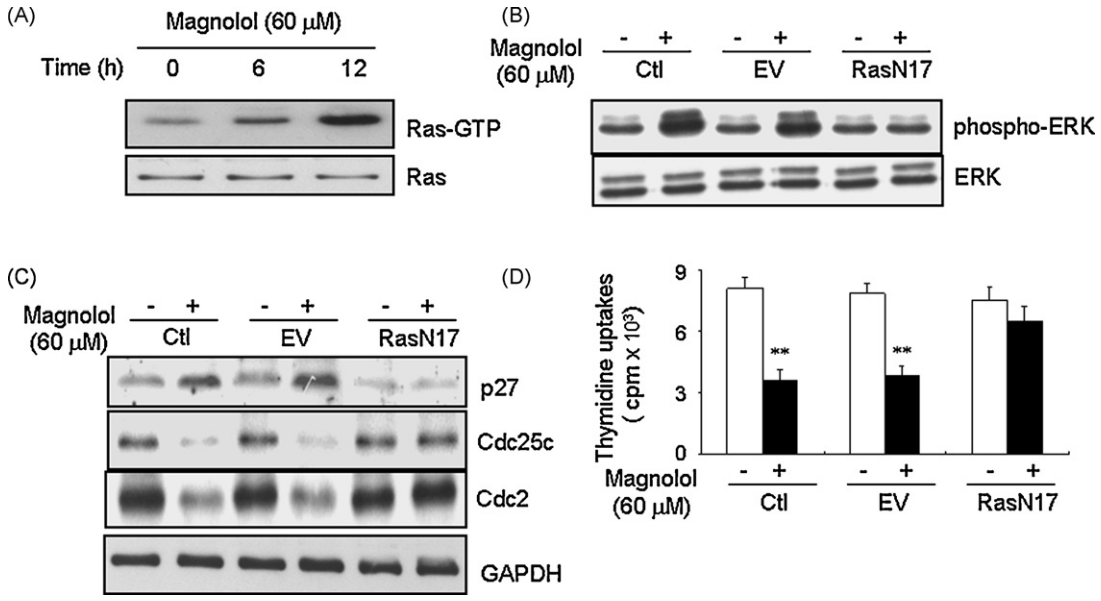


Fig. 7 – Effects of a dominant negative RasN17 mutant gene on magnolol-mediated cell growth inhibition. (A) Time course for magnolol-induced Ras activation. 5637 cells were stimulated by magnolol for 6 or 12 h and lysed. Cellular content of Ras active form (Ras-GTP) was analyzed by immunoblotting as described in Section 2. (B and C) Immunoblot of ERK1/2, p27, Cdc2 and Cdc25c in cells after 12 h treatment with magnolol. Cells were transfected with an empty vector (EV), or with RasN17. Cellular content of phosphorylated ERK1/2, p27, Cdc2 and Cdc25c was analyzed by immunoblotting as described in Section 2. (D) Thymidine uptake experiments were determined as described in Section 2. Results are presented as mean ± S.E. from three triplicate experiments. $^{**}P < 0.05$ compared with no magnolol treatment in RasN17 mutant gene transfected 5637 cells.

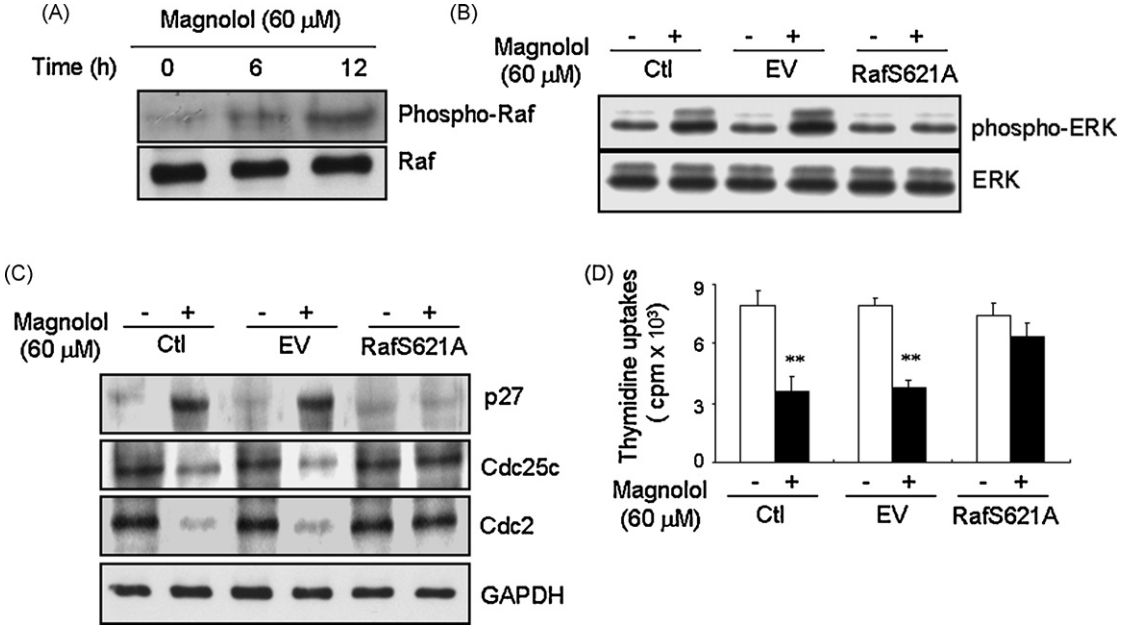


Fig. 8 – Effects of a dominant negative RafS621A mutant gene on magnolol-mediated cell growth inhibition. (A) The levels of phosphorylated Raf protein were increased in 5637 cells after 12 h treatment with magnolol (60 μM). (B and C) Immunoblot of ERK1/2, p27, Cdc2 and Cdc25c in cells after 12 h treatment with magnolol. Cells were transfected with an empty vector (EV), or with RafS621A mutant gene. Cellular content of phosphorylated ERK1/2, p27, Cdc2 and Cdc25c was analyzed by immunoblotting as described in Section 2. (D) Thymidine uptake experiments were determined as described in Section 2. Results are presented as mean ± S.E. from three triplicate experiments. $^{**}P < 0.05$ compared with no magnolol treatment in RafS621A mutant gene transfected 5637 cells.

treated with magnolol, using antibodies specific for phosphorylated Raf. This experiment indicates that Raf is significantly activated by magnolol. Experiments were subsequently carried out to demonstrate direct involvement of Raf activation in the magnolol-induced ERK and p27 expression in 5637 cells. 5637 cells were transiently transfected with either EV or the RafS621A mutant gene and stimulated with magnolol. As shown in Fig. 8B and C, magnolol treatment of either 5637 cells or of cells transfected with EV induces ERK activity and p27 expression. This activation of ERK and p27 was decreased in cells transfected with the RafS621A mutant gene. In addition, the RafS621A mutant gene transfectants prevented the magnolol-induced decrease of [^{3}H]thymidine incorporation into Cdc2 and Cdc25c levels (Fig. 8C and D). The results showed that magnolol can regulate ERK activation, p27 expression, growth inhibition and decreased Cdc2 levels in 5637 cells by activating a Raf-dependent pathway. Collectively, these results suggest that the Ras/Raf/ERK signaling pathway must be involved in magnolol-induced growth inhibition via activation of the p27-mediated G2/M cell cycle arrest in 5637 cells.

4. Discussion

Understanding how naturally occurring compounds regulate proliferation and cell survival is important to the development of new agents to prevent and treat cancer with low toxicity. Despite many studies to determine the variety of biological functions associated with natural compounds, the precise molecular mechanisms underlying their cell cycle regulation and signaling pathway remain largely known. In the present study, we demonstrated that magnolol suppressed cell growth by inducing expression of p27-mediated G2/M cell cycle arrest through the Ras/Raf/ERK signaling pathway in bladder cancer 5637 cells.

Treatment of 5637 cells with magnolol resulted in decreased cell viability and increased apoptosis. The result of thymidine uptakes as an index of DNA synthesis in 5637 cells after magnolol treatment indicated that there is a cessation of DNA synthesis. The present study showed that magnolol inhibits both cell proliferation and thymidine uptakes in 5637 cells. Several studies have investigated the effects of magnolol on cancer cells. Consistent with the present results, other studies reported that magnolol inhibited cell growth in different cancer cell lines [28,29]. Previously, magnolol was reported to induce G1 cell cycle arrest in several cell lines, such as vascular smooth muscle cells, colon and liver cancer cells [28,30]. However, our study showed that magnolol promoted both G1 and G2/M arrest in 5637 bladder cancer cells. These findings may explain the cell type species-differences in cell cycle regulation by magnolol. The results of the present study indicate that magnolol caused G1 and G2/M-phase cell cycle arrest and a decrease in CDK2, CDK4 and Cdc2, which are involved in cell-cycle progression from the G1- to the S- and G2/M phases. We showed that, in addition to inhibiting a variety of CDKs, magnolol treatment led to the downregulation of protein levels including cyclin D1, cyclin E and cyclin B_1 that are required for CDK4, CDK2 and Cdc2 activation and progression through the G1 and G2/M checkpoints. The negative regulation of these positive regulators of cell cycle progression would impair CDK activities and contribute to the increase in G1 and G2/M arrest following addition of magnolol. The data demonstrate that significant up-regulation of p27 occurred during the G1- and G2/M-phase arrest in 5637 cells treated with magnolol. However, magnolol had no effect on the expression of p21 and p53 as determined by immunoblot analysis, suggesting that magnolol-induced accumulation of p27 may also be responsible for the G1- and G2/M-phase arrest in 5637 cells. Recent work showed that magnolol caused G1 cell cycle arrest through induced expression of p21 in colon cancer cells [39]. Although other findings indicated that magnolol or honokiol treatment enhanced HL-60 human leukemia cell differentiation via enhancing G1 cell cycle arrest and increasing the expression of p27 [29], this is the first known systematic study examining the involvement of p27 in the cyclin-CDK machinery during magnolol-induced G1- and G2/M-phase cell cycle arrest, which is related to cell growth inhibition.

The importance of MAPK signaling pathways in regulating inhibition of cell growth during conditions of stress has been widely investigated [14,19,20]. The effect of magnolol on early signal transduction pathways was examined using ERK, p38 MAP kinase and JNK. Magnolol treatment resulted in up-regulation of ERK, JNK and p38 MAP kinase phosphorylation. Previous studies have demonstrated that the MAPK pathway is involved in cell growth inhibition [14,19,20] and/or the regulation of the cell cycle [21–24]. Because magnolol treatment induced p27, the role of MAPK in the regulation of p27 expression was investigated using pharmacological inhibitors (PD98059, SB203580 and SP600125). Of considerable interest was the marked decrease in magnolol-induced expression of p27 only by PD98059 treatment. Under the same experimental conditions, SB203580 and SP600125 treatment had no effect on magnolol-induced p27 expression (Fig. 5A). These results suggest that activation of these two kinases is not required for magnolol-induced p27 expression, although magnolol activates both p38 MAP kinase and JNK. The ERK signaling pathway appears to involve multiple signal transduction pathways, used to accomplish a variety of functions [17–23]. Many studies have supported the general view that activation of the ERK pathway delivers a survival signal that counteracts the pro-apoptotic effects of JNK and p38MAP kinase activation [17,18]. However, recent reports have maintained that sustained activation of ERK also is involved in apoptosis [19,20]. Robust ERK stimulation has been reported to suppress cell-cycle progression, via induction of cell-cycle inhibitor proteins, including p21 and p27 [35]. The results of the present study suggest that ERK signaling is involved in magnolol-induced inhibition of cell growth due to induction of p27 in 5637 cells.

The effects of magnolol on cell proliferation and on the cell-cycle-associated proteins, CDK2, CDK4 and Cdc2, were confirmed by inhibition of ERK. Consistent with the observations that magnolol treatment inhibited cell growth and modulated cell-cycle-associated proteins, blockade of ERK with PD98059 rescued cell proliferation and G2/M cell-cycle-associated proteins, Cdc2 and Cdc25c. However, ERK inhibition did not reverse G1 cell-cycle-associated proteins CDK2 and CDK4. These data provide the first evidence that ERK is a

key mediator of magnolol-induced inhibition of cell growth via G2/M cell cycle arrest.

The results showed that the ERK inhibitor, PD98059, could not antagonize the inhibitory effects of magnolol on the G1-phase CDKs. However, the growth inhibitory effect was antagonized. The results from flow cytometry analysis (Fig. 6A–E) in the 5637 cells indicate that the S-phase cell-cycle distribution by magnolol was reversed by pretreatment with PD98059. It has been reported that ERK inhibition suppressed apoptosis in cisplatin-induced growth inhibition in HeLa cells [19]. Recent study suggested that sustained activity of ERK is required for the down-regulation of many antiproliferative genes throughout the whole G1 phase of the cell cycle [21]. Considering these things, our results suggest that ERK activation is not sufficient by itself to promote G1 cell cycle entry in magnolol-induced cell growth inhibition. Because the growth inhibitory effects need not be limited to G1 cell cycle proteins, further studies are awaited to resolve this question.

The ERK cascade has been implicated in a wide variety of processes in cells including the regulation of both cell proliferation and survival [14–18]. Ras, a GTP-binding protein, is a common upstream activator of several signaling pathways, including Raf/MEK/ERK [33–38]. The Raf-1 serine/threonine kinase is the best-characterized effector of Ras [22,23]. Activated Ras binds to and promotes the activation of Raf, which activates the MEK1/2 dual specificity kinases, which in turn phosphorylate and activate the p42/p44 ERK mitogen-activated protein kinases (MAPKs). Ultimately, ERK activation is typically associated with cell proliferation [33–38]. In contrast, activated Ras or Raf-induced cell growth arrest has been shown to occur in the presence of induced levels of CDKI, such as p16INK4a, p21WAF1, p27KIP1, tumor suppressor p53, and of reduced phosphorylation of Rb or of the E2F family [19–24]. There are a number of chemotherapeutic agents that induce Ras/Raf/ERK signaling [19,20]. Recently, the involvement of Ras/Raf/ERK signaling has been demonstrated for the regulation of magnolol-induced G1 cell cycle arrest via p21 expression in colon cancer cells [39]. However, the mechanism by which Ras or Raf activation, in response to magnolol induction, promotes p27-mediated G2/M cell growth arrest remains to be elucidated. The involvement of a Ras/Raf-mediated pathway in magnolol-induced inhibition of 5637 cell growth was examined because both Ras and Raf were activated by magnolol. In this study, to determine whether Ras/Raf is required for magnolol-induced activation of ERK, 5637 cells were transfected with dominant negative Ras (RasN17) and dominant negative Raf (RafS621A). Magnolol-induced ERK activation was suppressed by transfection with the dominant negative mutants of RasN17 and RafS621A, suggesting that Ras/Raf plays a key role in magnolol-induced activation of the ERK signaling cascade in 5637 cells.

To better understand the mechanism of magnolol-mediated cell growth inhibition in 5637 cells, the influence of Ras/Raf activation on the ERK1/2 kinase cascades that control cell growth and G2/M cell cycle machinery proteins was examined. Transfection of cells with RasN17 and RafS621A mutant genes abolished the magnolol-induced inhibition of cell growth, together with up-regulation of p27 levels and inhibition of Cdc2 expression. The results obtained for the RasN17 and RafS621 overexpression experiments provide evidence that the Ras/Raf signaling pathway is involved in magnolol-induced inhibition of cell growth via p27-mediated G2/M cell-cycle regulation in 5637 cells. Thus, a Ras/Raf-dependent ERK signaling pathway may be responsible for magnolol-induced inhibition of cell proliferation in bladder cancer 5637 cells.

The present study provides important new insight into the molecular mechanisms of the effects of magnolol in 5637 cancer cells. First, magnolol reduces cell viability and cell proliferation. In addition, magnolol arrests the cell cycle at the G1 and G2/M-phase in 5637 cells and this effect can be attributed to the inhibition of cyclin D1/CDK4, cyclin E/CDK2 and cyclin B1/Cdc2 complexes by the increased expression of p27. Finally, magnolol-induced inhibition of cell growth appears to be linked to activation of Ras/Raf/ERK through p27-mediated G2/M-phase cell cycle arrest. The present findings may, in part, explain the therapeutic effects of magnolol for treatment of urinary bladder cancer. Further studies will be required to investigate the issue of whether the Ras/Raf/ERK signaling cascades are involved in the magnolol-induced growth inhibition in other bladder cancer cell lines.

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