JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

P27/Kip1 Is Responsible for Magnolol-Induced U373 Apoptosis *in Vitro* and *in Vivo*

Li-Ching Chen[†] and Wen-Sen Lee*[,†,‡,§]

[†]Graduate Institutes of Medical Sciences, College of Medicine, [‡]Department of Physiology, School of Medicine, College of Medicine, and [§]Cancer Research Center, Taipei Medical University, Taipei 110, Taiwan

ABSTRACT: Previously, we demonstrated that magnolol, a hydroxylated biphenyl compound isolated from the bark of *Magnolia officinalis*, at low concentrations (3−10 μM) exerted an antiproliferation effect in colon cancer, hepatoma, and glioblastoma (U373) cell lines through upregulation of the p21/Cip1 protein. Magnolol at a higher concentration of 100 μM, however, induced apoptosis and upregulated p27/Kip1 expression in U373. In the present study, we further studied whether the increased p27/Kip1 expression contributes to the magnolol-induced apoptosis in U373. Our data show that knock-down of p27/Kip1 expression significantly suppressed the magnolol-induced apoptosis, suggesting that p27/Kip1 might play an important role in the regulation of magnolol-induced apoptosis. This notion was further supported by demonstrating that magnolol induced an increase of the caspase activity in U373 *in vitro* and *in vivo*, and these effects were abolished by pretransfection of the cell with p27/Kip1 siRNA. To delineate the possible signaling pathways involved in the magnolol-induced increases of p27/Kip1 expression and apoptosis, we found that magnolol (100 μM) increased the levels of phosphorylated cSrc (p-cSrc), p-ERK, p-p38 MAP kinase (p-p38 MAPK), and p-AKT but not p-JNK in U373. Moreover, pretreatment of U373 with a cSrc inhibitor (PP2), a PI3K inhibitor (LY294002), an ERK inhibitor (PD98059), or a p38 MAPK inhibitor (SB203580) but not a JNK inhibitor (SP600125) significantly reduced the magnolol-induced increases of p27/Kip1 protein levels and apoptosis. Taken together, our data suggest that magnolol at a higher concentration of 100 μM induced apopotosis in U373 cells through cSrc-mediated upregulation of p27/Kip1.

KEYWORDS: *Magnolol, apoptosis, P27/Kip1, U373, siRNA*

INTRODUCTION

Malignant glioblastomas are the most common and aggressive primary brain tumors in humans. Despite remarkable advances in surgical techniques and treatment options, including chemotherapy, radiotherapy, radiosurgery, corticosteroids, and antiangiogenic therapy, the prognosis of malignant glioblastomas is still poor, with a median survival time of approximately 14 months, because of their diffuse invasion of the brain parenchyma.[1]

Magnolol is a widely used Chinese herbal medicine known as Hou p'u and has been reported to exert anticancer activities.[2−7] Previously, we demonstrated that treatment of the human malignant glioblastoma cell line (U373) with magnolol at a range of concentrations (10−50 μM) induces cell growth arrest at the G0/G1 phase via induction of the p21/Cip1 protein.[2] A similar molecular mechanism underlying magnolol-induced proliferation inhibition was observed in the human colon cancer cell line (COLO-205) and hepatoma cell line (Hep-G2).[6] Magnolol could also suppress tumor growth by activating apoptosis.[2,6−8] We found that magnolol at lower concentrations (10−50 μM) could inhibit proliferation, while at a higher concentration (100 μM), it could induce apoptosis in cultured U373 cells. Importantly, the pharmacokinetics of magnolol in rats showed that magnolol can cross the blood−brain barrier with a mean concentration of magnolol in the brain approximately 4-fold as high as that in the plasma after 10 min of magnolol (5 mg/kg, intravenous) administration.[9] Previous *in vitro* and *in vivo* studies have shown that magnolol exerts a neuroprotective effect.[10−12] A recent study demonstrated that magnolol has antidepressant activity in rats subjected to unpredictable, chronic, and mild stress.[8] The actions of magnolol on the central nerve system make it very attractive as a potential natural product for treating malignant glioblastomas.

Although we previously demonstrated that magnolol at a higher concentration of 100 μM induced upregulation of p27/Kip1 protein and the occurrence of apoptosis in U373 cells, the role of the increased p27/Kip1 expression in the magnolol-induced apoptosis has not been elucidated in our previous studies. In the present study, we found that knock-down of p27/Kip1 expression resulted in a reduction of the caspase activity and a decrease of the occurrence of apoptosis. Our data also suggest that magnolol induced upregulation of p27/Kip1 might be mediated via multiple signaling pathways. The findings of this study shed new light on the molecular mechanisms involved in magnolol-induced apoptosis.

MATERIALS AND METHODS

Chemicals. Magnolol was purchased from the Pharmaceutical Industry, Technology, and Development Center (Taiwan). A polyclonal antibody against p27/Kip1 was obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies against p21/Cip1, ERK, p38 MAP kinase, JNK, G3PDH, α-tubulin, phosphorylated-cSrc (p-cSrc), and AKT were purchased from Santa Cruz Biotechnology, Inc.

Received: February 4, 2013
Revised: February 27, 2013
Accepted: February 28, 2013
Published: February 28, 2013

dx.doi.org/10.1021/jf400542m | J. Agric. Food Chem. 2013, 61, 2811−2819

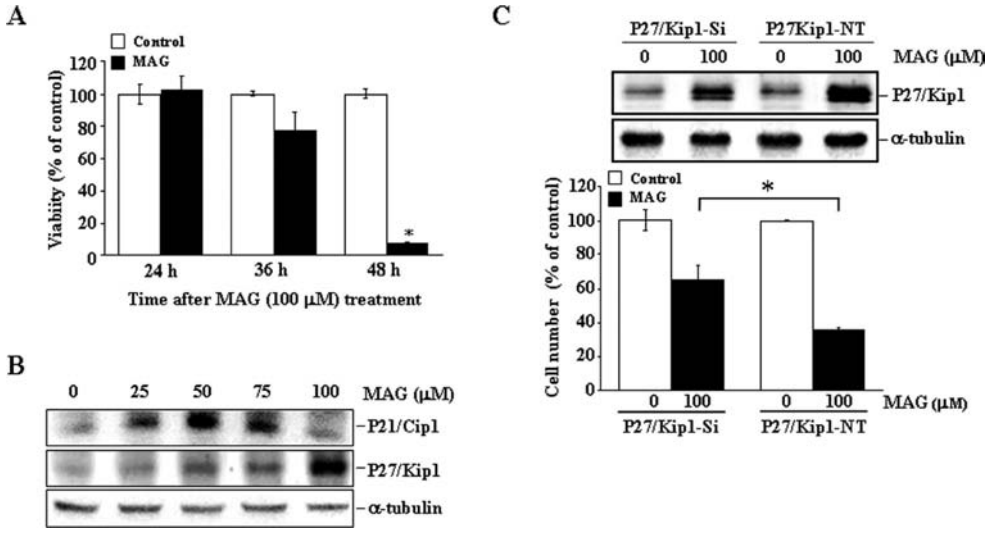

Figure 1. Involvement of p27/Kip1 in magnolol-induced decreases of U373 cell viability. (A) Magnolol at a concentration of 100 μM induced a time-dependent reduction of cell viability in U373 cells. (B) Magnolol at a higher concentration (100 μM) increased the protein levels of p27/Kip1 but not p21/Cip1 in U373. The cells were rendered quiescent by incubation in MEM containing 0.04% FBS for 24 h and then treated with MEM containing 10% FBS with or without magnolol (100 μM) for 24 h. The levels of p21/Cip1 and p27/Kip1 protein were examined using immunoblotting assays. The expression level of α-tubulin was detected as a protein-loading control. (C) Effects of magnolol-induced increases of p27/Kip1 expression and decreases of viability were partially prevented by pretransfection of the cell with siRNA p27/Kip1. U373 were transfected with p27/Kip1-Si or p27/Kip1-NT followed by treatment with 100 μM magnolol for 24 h (for protein detection) or 48 h (for cell number detection). The levels of p21/Cip1 and p27/Kip1 protein were examined using immunoblotting assays. The expression level of α-tubulin was detected as a protein-loading control. MAG, magnolol; P27/Kip1-Si, P27/Kip1 siRNA; and P27/Kip1-NT, P27/Kip1 nontargeting RNA.

(Santa Cruz, CA). Antibodies against p-ERK, p-p38 MAPK, p-JNK, and p-AKT were purchased from Cell Signaling Technology, Inc. (Danvers, MA). An antibody against cSrc was purchased from Abcam (Cambridge, MA). The JNK inhibitor (SP600125), p38 MAPK inhibitor (SB203580), PI3K inhibitor (LY294002), and ERK inhibitor (PD98059) were obtained from Tocris Bioscience (Ellisville, MO). The Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from KPL, Inc. (Gaithersburg, MD). NIR-FLIVO-747 was purchased from Immunochemistry Technologies (Bloomington, MN).

Cell Culture. The human astrocytoma cell line, U373, was obtained from the American Type Culture Collection (Rockville, MD) and cultured in minimum essential medium with Eagle's salts (MEM, Invitrogen Corp.) containing glutamine (2 mM), 1% non-essential amino acids, sodium pyruvate (1 mM), and 10% fetal bovine serum (FBS) in a humidified incubator (37 °C and 5% CO_2).

Protein Extraction and Western Blot Analysis. To prepare the protein samples, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold cell lysis buffer (50 mM Tris-HCl at pH 8.0, 120 mM $NaCl_2$, 0.5% Nonidet P-40, 100 mM sodium fluoride, and 200 μM sodium orthovanadate) containing protease inhibitors as previously described.[13] The proteins (50 μg) from each sample were resolved using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrotransferred, immunoblotted with antibodies, and then visualized by incubating with the colorigenic substrates, NBT and BCIP. The levels of G3PDH protein were detected and used as the control for equal protein loading.

Small Interfering RNA (siRNA). Expression of p27/Kip1 was knocked-down in U373 with at least three independent siRNAs. The target sequences of p27/Kip1 mRNA were selected to suppress p27/Kip1 gene expression. Nontarget (NT) sequences of each siRNA were used as controls.[14] After BLAST analysis to verify that there were no significant sequence homologies with other human genes, the selected sequences were inserted into *Bgl*II/*Hin*dIII-digested pSUPER vectors to generate the pSUPER-Si p27/Kip1 (p27/Kip1-Si) and pSUPER-nontarget (p27/Kip1-NT). Three different antisense siRNA targeted against different parts of the p27/Kip1 sequence are listed below: Si-1, 5′-GATCCCCGGAAGCGACCTGCAACCGATTCAAGAGATCGGTTGCAGGTCGCTTCCTTTTTA-3′; Si-2, 5′-GATCCCCACCCGGGAGAAAGATGTCATTCAAGAGATGACATCTTTCTCCCGGGTTTTTTA-3′; Si-3, 5′-GATCCCCGAAGCGACCTGCAACCGACTTCAAGAGAGTCGGTTGCAGGTCGCTTCTTTTTTA; and NT, 5′-GATCCCCATGGACAGACGCACGCACGTTCAAGAGACGTGCGTGCGTCTGTCCATTTTTTA-3′. All constructs were confirmed by DNA sequence analysis. The transfection protocol has been previously described.[13,14] Briefly, 2×10^5 cells were washed twice with PBS and mixed with 0.5 μg of plasmid, and then, one electric pulse was applied for 20 ms under a fixed voltage of 1.3 kV on a pipet-type MP-100 microporator (Digital Bio, Seoul, Korea).

Generation of Stable p27/Kip1-Knocked-down U373. At least three clones of the U373 cell line stably expressing the siRNA were generated. All experiments were performed using multiple subclones of each cell line with consistent results. The p27/Kip1-Si and p27/Kip1-NT plasmids were transfected into U373, and stable integrants were selected at 72 h later with Geneticin (G418, 4 mg/mL). After 30 days in selective medium, three Geneticin (G418)-resistant clones, referred to as p27/Kip1-Si and p27/Kip1-NT, were isolated. The p27/Kip-Si-transfected clone showed more than 80% reduction in mRNA and protein levels when compared to the control clones (p27/Kip1-NT).

U373-Derived Xenografts. U373 (1.5×10^7) was resuspended in 0.1 mL of MEM and subcutaneously injected between the scapulae of each male nude mouse (4-week-old females, n = 5 per group) purchased from the National Science Council Animal Center, Taipei, Taiwan. After transplantation, the tumor size was measured using calipers and the tumor volume was estimated as follows: tumor volume (mm^3) = $1/2LLW^2$, where L is the length and W is the width of the tumor.[15,16] Once the tumor reached a volume of 100 mm^3, the animals received intraperitoneal injections of normal saline (50 μL) or magnolol (5 mg/50 g body weight) every other day 5 times. Tumor-bearing mice were then adapted for the *in vivo* fluorescently labeled polycaspase inhibitor (FLIVO) apoptosis assay. At the end of the experiment, the animals were sacrificed using an overdose of ether and the subcutaneous tumor masses were dissected. The xenografts were weighed, snap-frozen in dry ice, and stored at −80 °C until western blot analysis. The surgery was performed under isoflurane anesthesia

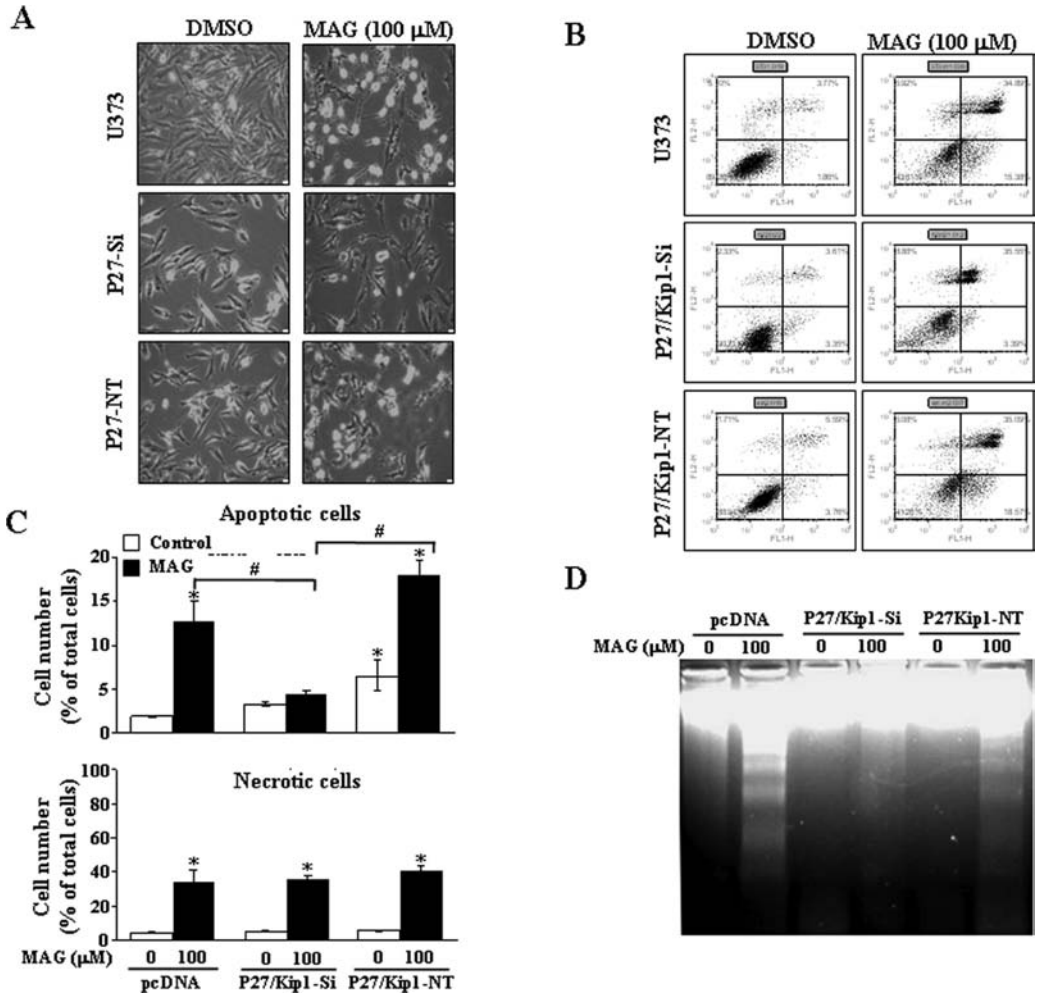

Figure 2. Involvement of p27/Kip1 on the magnolol-induced cell death in U373. (A) U373 were treated without (left) or with (right) 100 μM magnolol. After 24 h of treatment, cytoplasmic condensation and cell shrinkage are visible in many U373. The magnolol-induced apoptosis was greatly reduced in the cell pretransfected with p27/Kip1-Si but not p27/Kip1-NT RNA. (B) To confirm the role of p27/Kip1 in the magnolol-induced apoptotic cell death, U373 was treated with the same regimens as described in part A and the apoptotic cells were determined using an Annexin V staining method and then detected by flow cytometry analysis. (C) Magnolol significantly increased the percentage of the apoptotic (upper panel) and necrotic (lower panel) cell populations. The magnolol-induced increases of the apoptotic cell populations were abolished by pretransfection of the cell with p27/Kip1-Si but not with p27/Kip1-NT. The magnolol-induced increases of the necrotic cell populations were not affected by pretransfection of the cell with p27/Kip1-Si or p27/Kip1-NT. Values represent the mean value ± standard error of the mean (n = 3). (*) $p < 0.05$, different from the corresponding control. (#) $p < 0.05$, different from the p27/Kip1-Si group treated with magnolol (100 μM). (D) Typical DNA ladder pattern associated with apoptosis was seen in U373 treated with 100 μM magnolol for 48 h. Magnolol-induced DNA fragmentation was reduced by pretransfecting the cell with p27/Kip1-Si but not with p27/Kip1-NT. MAG, magnolol; P27/Kip1-Si, P27/Kip1 siRNA; and P27/Kip1-NT, P27/Kip1 nontargeting RNA.

to minimize suffering. All mouse protocols were performed according to an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-approved protocol.

Apoptosis Assay. *In Vitro Assay.* The occurrence of apoptosis was examined using a DNA fragmentation assay and flow cytometric detection of phosphatidylserine externalization using Annexin V staining. The U373 cell transfected with pcDNA, p27/Kip1-Si, or p27/Kip1-NT was rendered quiescent by incubation in MEM containing 0.04% FBS for 24 h and then treated with or without magnolol (100 μM) in MEM supplemented with 10% FBS for an additional 24 h. For some experiments, the U373 cells were pretreated with inhibitors specific against candidate molecules for 1 h, followed by 100 μM magnolol for an additional 24 h. Cells treated with a vehicle (DMSO) were used as a control. The cells were harvested and processed for Annexin V staining according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA). Briefly, the cells were washed twice with 10 μM binding buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 140 mmol/L NaCl, and 5 mmol/L $CaCl_2$ at pH 7.4], stained with anti-Annexin V antibody (Alexa Fluor 350), counterstained with 50 mg/mL propidium iodide (PI) for 15 min at room temperature, and then processed for flow cytometric analysis using a Becton Dickinson FACSCalibur (Franklin Lakes, NJ). The extent of apoptosis was quantified as the percentage of Annexin V-positive cells.

In Vivo Assay. The apoptotic cells in the U373 tumor were detected using the commercial NIR-FLIVO-747 *in vivo* apoptosis detection kit.[17] The *in vivo* binding of a fluorescently labeled polycaspase inhibitor (FLIVO, FAM-VAD-FMK) in the apoptosis cells was detected at 24 h after intravenous injection with NIR-FLIVO-747 reagent or NIR-FLIVO-747 free dye (control) dissolved in 50 μL of dimethyl sulfoxide (DMSO) and then diluted with 50 μL of sterilized PBS (pH 7.4) prior to intravenous injection via the lateral tail vein. After injection, the FLIVO reagent was allowed to circulate for 24 h before analysis. Fluorescent images were detected using an IVIS Lumina XR (Xenogen Corporation−Caliper, Alameda, CA). Because it can be imaged non-invasively, NIR-FLIVO-747 is a powerful

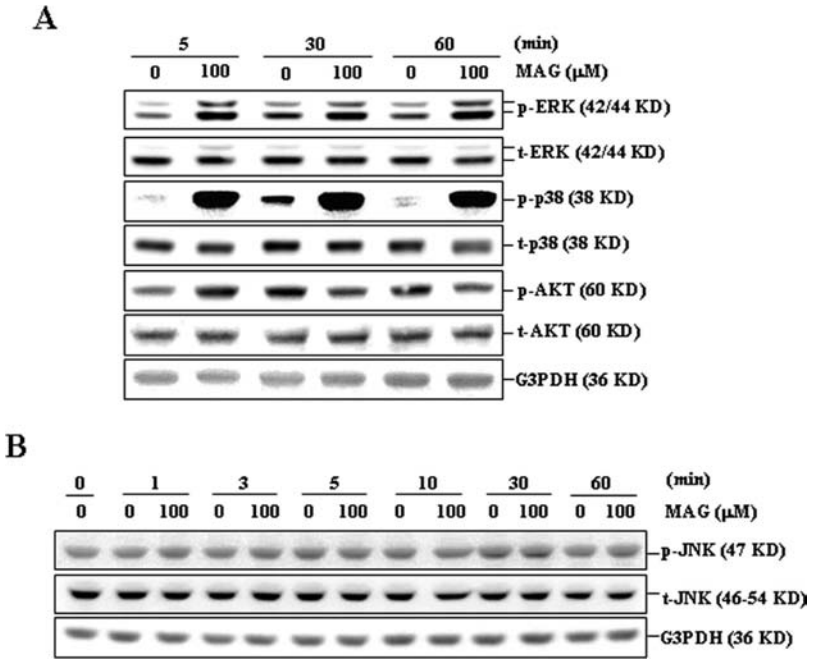

Figure 3. Effect of magnolol on the activity of signaling molecules in U373. U373 cells were switched to media with 0.04% FBS for 24 h to render them quiescent and to synchronize their cell-cycle activities. Then, they were returned to media with 10% FBS supplemented with vehicle (control) or magnolol (100 μM), and at various times thereafter, they were processed for protein extraction. The protein levels were determined by immunoblotting analysis. The expression level of G3PDH was detected as a protein-loading control. Magnolol at a concentration of 100 μM increased the level of (A) phosphorylated ERK, p38 MAPK, and AKT but not (B) JNK. p-ERK, phosphorylated ERK; t-ERK, total ERK; p-p38, phosphorylated p38; t-p38, total p38; p-AKT, phosphorylated AKT; t-AKT, total AKT; p-JNK, phosphorylated JNK; and t-JNK, total JNK.

imaging tool, allowing for longitudinal studies and eliminating false positives that may arise from the *ex vivo* manipulation of tissue.

Statistics. All data were expressed as the mean value ± standard error of the mean. Three to four samples were analyzed in each experiment. Comparisons were subjected to one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $p < 0.05$.

3. RESULTS

Effect of p27/Kip1 Upregulation on the Magnolol-Induced Cell Death in U373. Previously, we demonstrated that magnolol concentration-dependently (0−40 μM) decreased the cell number in a cultured U373 and arrested the cells at the G0/G1 phase of the cell cycle. Here, we further investigated whether magnolol at a higher concentration could cause U373 cell death. As illustrated in Figure 1A, treatment of U373 with magnolol at a concentration of 100 μM for 24−48 h time-dependently decreased the cell number. To study whether an increase of the p27/Kip1 protein level could contribute to the magnolol-induced decreases of the cell number, the level of p27/Kip1 protein in U373 was examined. As shown in Figure 1B, magnolol (25−100 μM) concentration-dependently increased the level of p27/Kip1 protein in U373. However, transfection of U373 with p27/Kip1 siRNA (p27/Kip1-Si) reduced the magnolol-induced increases of p27/Kip1 protein (top panel of Figure 1C) and decreased the cell number (bottom panel of Figure 1C) compared to transfection with nontargeting siRNA (p27/Kip1-NT). Taken together, these data suggest that upregulation of p27/Kip1 might contribute to magnolol-induced decreases of the cell number in U373.

Involvement of p27/Kip1 on the Magnolol-Induced Apoptosis in U373. To examine whether cell death contributes to the magnolol-induced decreases of the cell number, the experiments shown in Figure 2 were conducted. After 24 h of magnolol (100 μM) treatment, cytoplasmic condensation and cell shrinkage were observed in many U373 (Figure 2A), suggesting that treatment with magnolol at a concentration of 100 μM for 24 h induced apoptosis in U373. The magnolol-induced apoptosis was greatly reduced in U373 pretransfected with p27/Kip1 siRNA but not with p27/Kip1-NT (part B and top panel of part C of Figure 2). However, the magnolol-induced increases of the necrotic cell populations were not significantly affected by pretransfection of the cell with p27/Kip1 siRNA or p27/Kip1-NT (part B and bottom panel of part C of Figure 2). The effect of p27/Kip1 induction on the magnolol-induced apoptosis was confirmed using the fragmentation analysis. As shown in Figure 2D, treatment of U373 with 100 μM magnolol for 36 h induced DNA laddering formation and this effect was abolished by pretransfection of the cell with p27/Kip1 siRNA but not with p27/Kip1-NT.

Signaling Pathways Involved in the Magnolol-Induced p27/Kip1 Upregulation and Apoptosis in U373. To study the possible signaling pathways involved in the magnolol-induced p27/Kip1 upregulation and apoptosis in U373, we examined the effect of magnolol on the activation of MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways. As shown in Figure 3A, the levels of p-ERK, p-p38 MAPK, and p-AKT in U373 were increased at 5 min after treatment with 100 μM magnolol. In contrast, magnolol (100 μM) treatment did not significantly affect the levels of p-JNK (Figure 3B). We further examined whether the magnolol-induced activation of MAPK and PI3K signaling pathways in U373 contributed to magnolol-induced upregulation of p27/Kip1 and apoptosis. As illustrated in Figure 4A, pretreatment of U373 with a PI3K inhibitor, LY294002, or an ERK inhibitor, PD98059, reduced the magnolol-induced upregulation of p27/Kip1 in U373 cells. In contrast, pretreatment of the cell with a

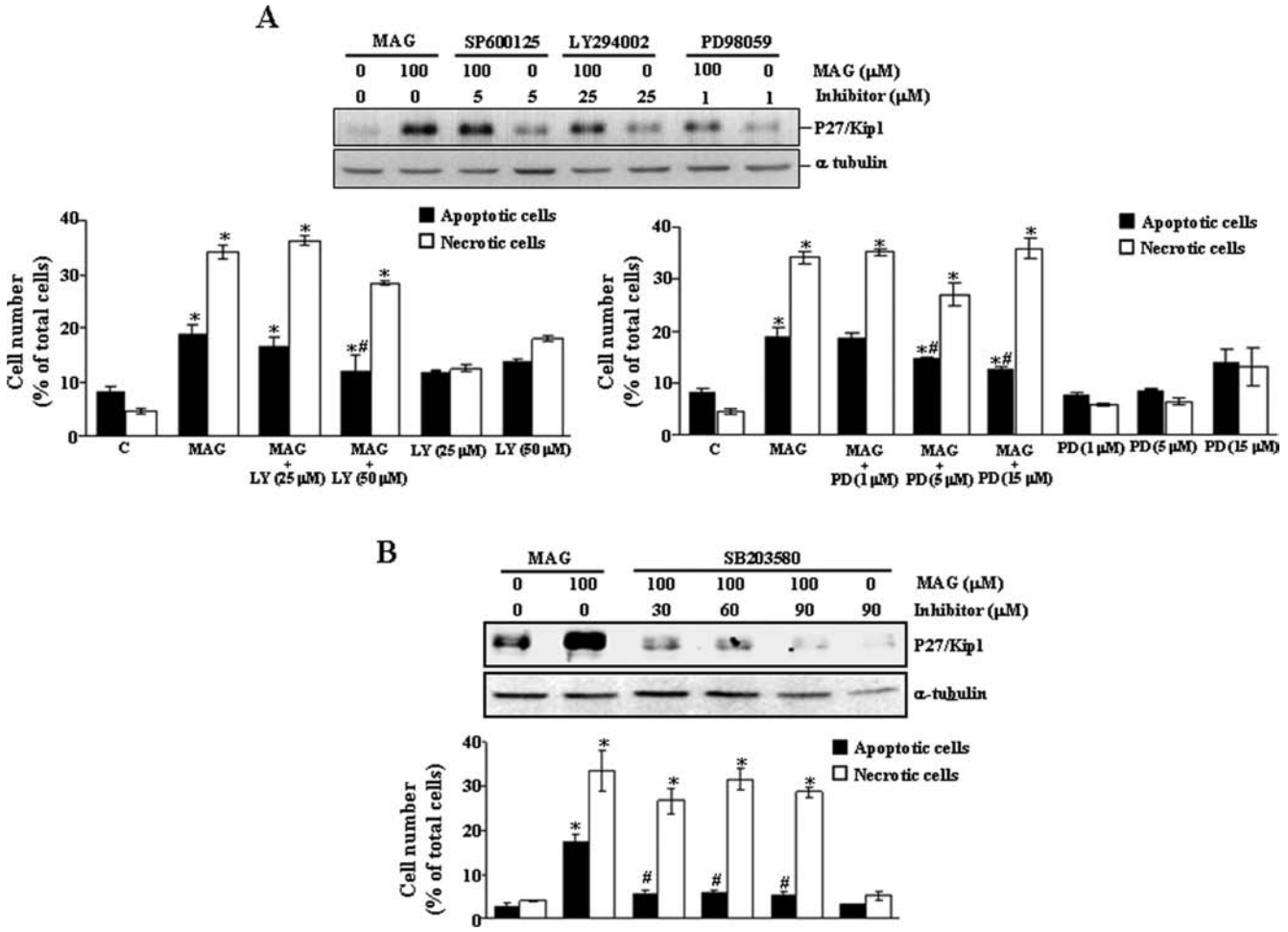

Figure 4. Magnolol-induced apoptotic cell death resulting from a JNK-independent pathway. The magnolol-induced increases of the level of p27/Kip1 protein (top panel) and apoptotic cell death (black columns in the bottom panel) in U373 were reduced by pretreatment of the cell with (A) a PI3K inhibitor (LY294002) and an ERK inhibitor (PD98059) but not (B) a JNK inhibitor (SP600125). The magnolol-induced increases of necrotic cell death in U373 (white columns in the bottom panels of parts A and B) were not significantly affected by these inhibitors. The U373 cells were pretreated with a p38 MAPK-specific inhibitor (30−90 μM) for 60 min, followed by 100 μM magnolol for an additional 24 h. The cell-treated vehicle was used as a control. The p27/Kip1 protein expression levels were determined by immunoblotting analysis. The expression level of α-tubulin was detected as a protein-loading control. Cells treated by the same regimens were adapted for the Annexin V staining assay, and the populations of necrotic and apoptotic cells were determined by flow cytometric analysis. The significance of the differences in the apoptotic and necrotic cell numbers among the groups was analyzed using the *t* test, and each pairwise comparison was performed using the two-way ANOVA. Values represent the mean value ± standard error of the mean ($n = 3$). (*) $p < 0.05$, different from the corresponding control. (#) $p < 0.05$, different from the cell treated with magnolol (100 μM). LY, LY294002; and PD, PD98059.

JNK inhibitor, SP600125, did not affect the magnolol-induced increases of upregulation of p27/Kip1 in U373. These findings suggest that ERK and PI3K signaling pathways might contribute to the magnolol-induced upregulation of p27/Kip1 and apoptosis in U373. We further applied a p38 MAPK inhibitor, SB203580, to confirm this notion. As shown in Figure 4B, the magnolol-induced increases of p27/Kip1 protein levels and apoptotic cell populations but not necrotic cell populations were significantly reduced by SB203580.

Involvement of cSrc Activation in the Magnolol-Induced Activation of ERK, AKT, and p38 MAPK. Because cSrc has been suggested to be the upstream molecule regulating the activity of ERK, AKT, and p38 MAPK, we further examined the involvement of cSrc activation in the magnolol-induced activation of ERK, AKT, and p38 MAPK. As shown in Figure 5A, treatment with magnolol (100 μM) for 2 min induced cSrc activation in U373. Pretreatment of U373 with a cSrc inhibitor, PP2 (30 μM), reduced the magnolol-induced activation of ERK and upregulation of p27/Kip1 (Figure 5B). As illustrated in Figure 5C, pretreatment of U373 with PP2 reduced the magnolol-induced activations of p38 MAPK and AKT. However, pretreatment with an ERK inhibitor, PD98059 (5 μM), reduced the magnolol-induced activation of p38 but not AKT. To confirm the involvement of cSrc activation on magnolol-induced apoptosis, the experiment illustrated in Figure 5D was conducted. Figure 5D shows that pretreatment of U373 with PP2 (30 μM) reduced the magnolol-induced increases of apoptosis but not necrosis.

Effect of Inhibition of the p27/Kip1 Protein Expression on the Magnolol-Induced Apoptosis *in Vivo*. As described above, our *in vitro* studies demonstrated that inhibition of the p27/Kip1 protein expression in U373 by either p27/Kip1-Si (Figure 1C) or specific inhibitors for ERK, AKT, or p38 MAPK (parts A and B of Figure 4) significantly attenuated the magnolol-induced apoptosis. To examine whether these effects observed in the *in vitro* studies also

dx.doi.org/10.1021/jf400542m | J. Agric. Food Chem. 2013, 61, 2811−2819

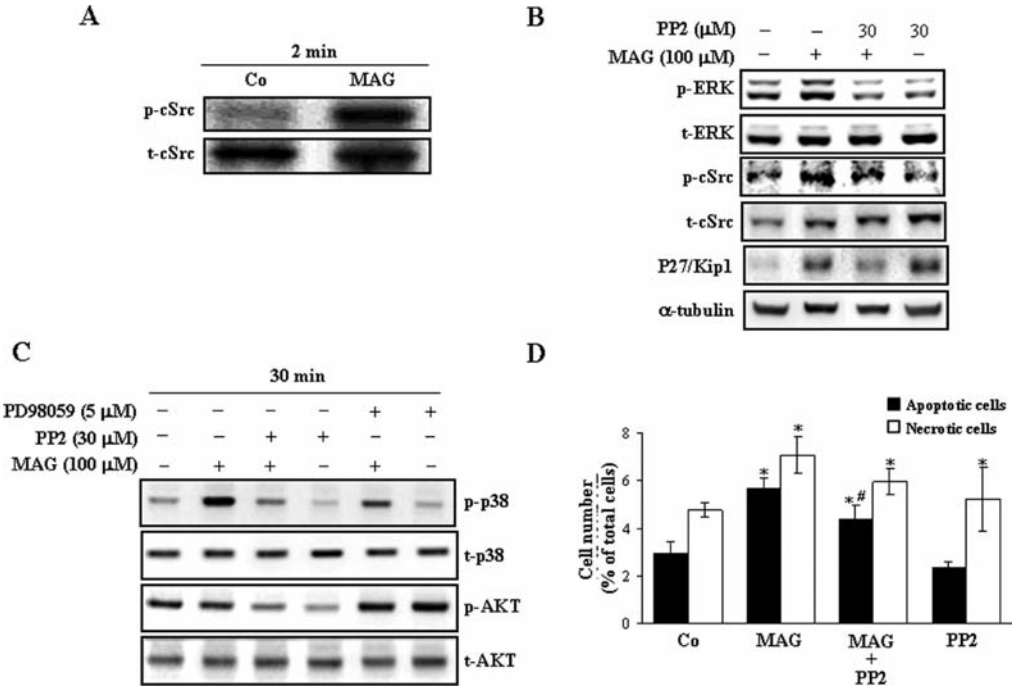

Figure 5. Involvement of cSrc activation in the magnolol-induced activation of ERK, AKT, and p38 MAPK. (A) Treatment of U373 with magnolol (100 μM) for 2 min induced cSrc activation. (B) Pretreatment of U373 with a cSrc inhibitor, PP2 (30 μM), reduced the magnolol-induced activations of cSrc and ERK and p27/Kip1 upregulation. (C) Pretreatment of U373 with PP2 reduced the magnolol-induced activations of p38 MAPK and AKT. However, pretreatment with an ERK inhibitor, PD98059 (5 μM), reduced the magnolol-induced activation of p38 but not AKT. (D) Pretreatment of U373 with PP2 (30 μM) for 1 h reduced the magnolol-induced increases of apoptosis but not necrosis. The apoptotic and necrotic cells were determined by flow cytometry analysis. The significant difference in the apoptotic and necrotic cell numbers among the groups was analyzed using the *t* test, and each pairwise comparison was performed using the two-way ANOVA. Values represent the mean value ± standard error of the mean (n = 3). (*) $p < 0.05$, different from the corresponding control. (#) $p < 0.05$, different from the cell treated with magnolol (100 μM). Co, control; MAG, magnolol; p-cSrc, phosphorylated cSrc; and t-cSrc, total cSrc.

occurred in an *in vivo* setting, nude mice bearing the U373 tumor were pretransfected with p27/Kip1-Si or p27/Kip1-NT, followed by magnolol treatment (5 mg/50 g intraperitoneal injection, every other day 5 times). As shown in parts B and D of Figure 6 (solid bar 2 versus solid bars 1 and 3), apparently apoptotic (FLIVO positive) cells were detected in mice bearing the p27/Kip1-NT-transfected U373 tumor-treated magnolol. However, the magnolol-induced apoptosis in the p27/Kip1-Si-transfected U373 tumor was significantly reduced. At the end of experiments, the animal was sacrificed and the tumors were excised for the NIR-FLIVO-747 caspase activity examination. The tumor tissues were also processed for protein extraction and western blot analyses. The results revealed that magnolol treatment significantly decreased the levels of p27/Kip1 protein in the p27/Kip1-Si-transfected U373 tumor than in the p27/Kip1-NT-transfected U373 tumor (lane 3 versus lanes 1 and 5 in Figure 6C). Moreover, magnolol caused a smaller region of the apoptotic (FLIVO positive) cells in the p27/Kip1-Si-transfected U373 tumor than in the p27/Kip1-NT-transfected U373 tumor (lane 3 versus lanes 1 and 5 in Figure 6C).

DISCUSSION

Malignant glioblastomas represent the most common type of primary brain tumor. Despite remarkable advances in surgical techniques and treatment options, including chemotherapy and radiotherapy, the prognosis of malignant glioblastomas is still poor because of their diffuse invasion of the brain parenchyma, which makes total surgical resection difficult.[18] Accordingly, there is an urgent need for new therapeutic strategies for malignant glioblastomas. Magnolol has been reported to exert antitumor activities in various types of cancer cells.[3−5] Recently, we have demonstrated that magnolol at lower concentrations (0−40 μM) arrests the growth of human glioblastoma (U373) cancer cells through induction of the p21/Cip1 expression.[2] In the present study, we further showed that magnolol induced apoptosis in U373 when the concentration of magnolol was increased to 100 μM and this effect was due to an increase of p27/Kip1 expression (Figure 2C). These results indicate that p27/Kip1, which is known to function as a cyclin-dependent kinase (CDK) inhibitor,[19] might also play an important role in magnolol-induced apoptosis in U373.

P27/Kip1, a cyclin-dependent kinase inhibitor, is known as a negative regulator of the cell cycle. Previously, it has been shown that quercetin, a flavonoid widely distributed in nature, could arrest primary human foreskin keratinocytes in the G1 phase through upregulation of the p27/Kip1 protein.[20] Using p27/Kip1 knockout mice, it has been demonstrated that p27/Kip1-deficient mice show a more rapid clonal expansion.[21] In addition to the cell cycle regulation activity, p27/Kip1 has also been demonstrated to be involved in the regulation of cancer cell differentiation[22] and apoptosis.[23] In the present study, we showed that upregulation of p27/Kip1 might contribute to the magnolol-induced apoptosis of U373 *in vitro* and *in vivo*.

The Raf-MEK-ERK-signaling pathway couples with the signals from growth factors as well as mitogenic and extracellular matrix signals to direct the cell to undergo growth, proliferation, migration, or differentiation.[24] A phase II trial was undertaken to evaluate the efficacy of the Ras-MAPK-signaling pathway inhibitor (TLN-4601) in patients with highly

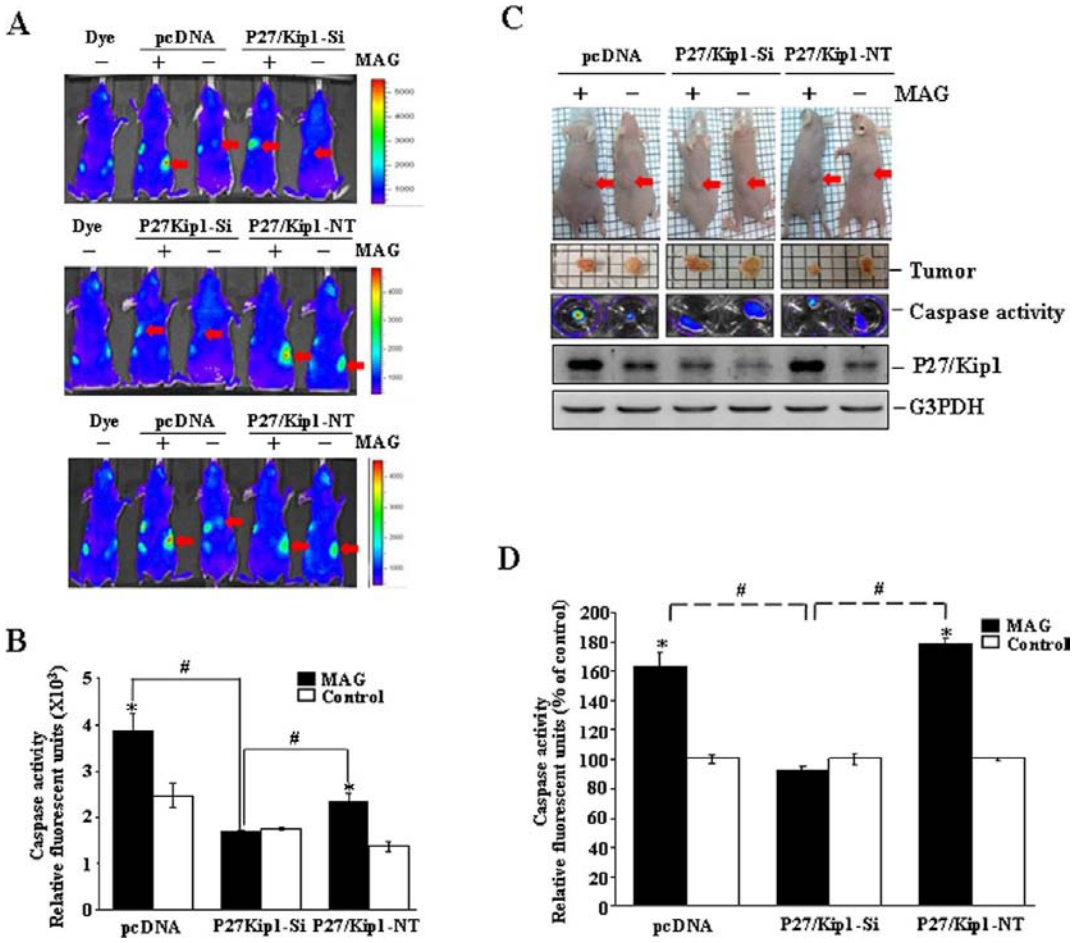

Figure 6. Role of p27/Kip1 protein in the magnolol-induced increases of caspase activity in the solid tumor derived from the implanted U373. Nude mice were subcutaneously injected with U373 cells (1.5×10^7) transfected with pcDNA (control), p27/Kip1-Si, or p27/Kip1-NT. Once the tumor reached a volume of 100 mm^3, the animal received intraperitoneal injections of normal saline (50 μL) or magnolol (5 mg/50 g body weight) every other day 5 times. U373 tumor-bearing mice were then adapted for *in vivo* fluorescently labeled polycaspase inhibitor (FLIVO) apoptosis assay. The fluorescent scale bar represents the caspase activity level. (A) Gross appearance of subcutaneous tumors with fluorescent dye and (B) quantitation of these data demonstrated that magnolol increased the caspase activity in the U373 tumor, and this effect was significantly reduced by pretransfection of U373 cells with p27/Kip1-Si but not p27/Kip1-NT. Values represent the mean value ± standard error of the mean ($n = 4$). (*) $p < 0.05$, different from the corresponding control. (#) $p < 0.05$, different from the p27/Kip1-Si group treated with magnolol (5 mg/50 mg). Red arrows indicated the tumor. (C) At the end of experiments, the U373 xenografts were dissected for detecting the caspase activity and the p27/Kip1 protein level. Red arrows indicated the tumor. The tumor size was decreased, and the levels of p27/Kip1 protein and caspase activity were increased in magnolol-treated U373 tumors. These increases were reduced by pretransfection of U373 with p27/Kip1-Si but not p27/Kip1-NT. (D) Significance of the differences in the caspase activity among the groups was analyzed using the *t* test, and each pairwise comparison was performed using the two-way ANOVA. Values represent the mean value ± standard error of the mean ($n = 4$). (*) $p < 0.05$, different from the corresponding control. (#) $p < 0.05$, different from the cell pretransfected with P27/Kip1 siRNA, followed by treatment with magnolol (100 μM). MAG, magnolol; P27/Kip1-Si, P27/Kip1 siRNA; and P27/Kip1-NT, P27/Kip1 nontargeting RNA.

vascularized glioblastoma multiforme.[25] In the present study, our data demonstrated that magnolol (100 μM) increased the protein levels of p-ERK, p-p38 MAPK, and p-AKT (Figure 3A) but not p-JNK (Figure 3B). Pretreatment of U373 with an ERK inhibitor (PD9805), a PI3K inhibitor (LY294002), or a p38 MAPK inhibitor (SB203580) but not a JNK inhibitor (SP600125) reduced the magnolol-induced upregulation of p27/Kip1 and apoptosis (parts A and B of Figure 4). These data suggest that magnolol induced apoptosis in U373 through upregulation of p27/Kip1 mediated by activating the ERK, AKT, and p38 MAPK pathways. Previously, it has been suggested that cSrc might be the upstream molecule regulating the activity of Ras-MAPK kinase, p38 MAPK, and PI3K.[26] Recently, we also demonstrated that cSrc is involved in the folic-acid-induced antiproliferation[27] and antimigration[28] in vascular endothelial cells. In this study, our data showed that treatment with magnolol (100 μM) for 2 min activated cSrc in U373 (Figure 5A). Moreover, pretreatment with a cSrc inhibitor, PP2, reduced the magnolol-induced activations of ERK, p38 MAPK, and AKT in U373, suggesting that cSrc is the upstream molecule of ERK, p38 MAPK, and AKT. To map the signaling pathway involved in the magnolol-induced p27/Kip1 upregulation, we found that pretreatment of an ERK inhibitor, PD98059, reduced the magnolol-induced activation of p38 MAPK and upregulation of p27/Kip1. However, the magnolol-induced activation of AKT was not significantly affected by PD98059, suggesting that p38 MAPK but not AKT was the downstream molecule of ERK. Taken together, our data suggested that cSrc might be the key molecule in regulating the magnolol-induced apoptosis in U373 and could be a promising

molecular target for new drug development for treating glioblastmoma.

In this study, we found that p27/Kip1 was upregulated by magnolol treatment through activation of MAPK signaling proteins, such as p38 MAPK, and ERK, as well as an AKT-mediated pathway. Inhibition of these kinases by specific inhibitors downregulated the p27/Kip1 expression, which, in turn, attenuated the occurrence of apoptosis in U373 cells (Figures 4 and 5). On the other hand, it has been reported that reduced p27/Kip1 levels are associated with a poor prognosis in many human gliomas.[29,30] These findings imply that increased expression of the p27/Kip1 protein in response to many chemotherapeutic drugs should be important for killing human tumor cells by altering the proliferation/apoptosis balance.[31,32] On the basis of the results from the present study, we propose a model of the molecular mechanism underlying magnolol-induced migration inhibition in U373, as shown in Figure 7. In this study, our data suggested that p27/Kip1 might play a central role in magnolol-induced apoptosis in U373 and regulation of signaling pathways involved in the upregulation of the p27/Kip1 protein might alter the outcome of therapy in U373 cancers.

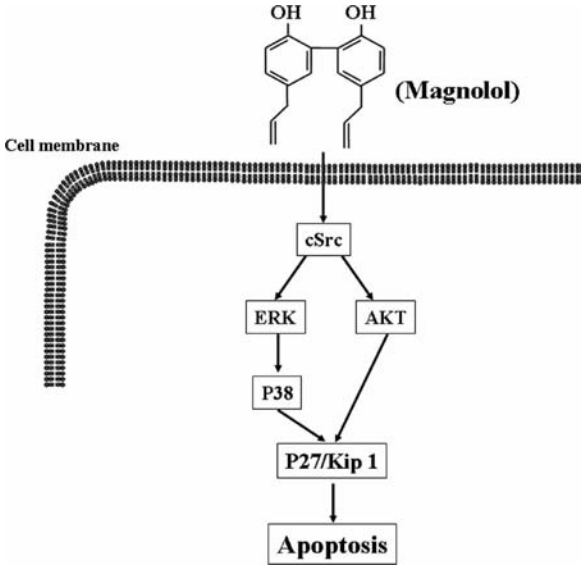

Figure 7. Model for magnolol-induced apoptosis in U373. Magnolol at a concentration of 100 μM activated cSrc, which, in turn, activated AKT and ERK-p38 signaling pathways, subsequently increased the level of p27/Kip1 protein, and finally, induced apoptosis in U373.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-2-27361661, ext. 3221. Fax: +886-2-23778620. E-mail: wslee@tmu.edu.tw.

Funding

This work was supported by a research grant from the National Science Council of Taiwan (NSC-101-2320-B-038-005).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

FBS, fetal bovine serum; MAG, magnolol; NT, nontarget; PBS, phosphate-buffered saline; siRNA, small interfering RNA

REFERENCES

(1) Van Meir, E. G.; Hadjipanayis, C. G.; Norden, A. D.; Shu, H. K.; Wen, P. Y.; Olson, J. J. Exciting new advances in neuro-oncology: The avenue to a cure for malignant glioma. *Ca-Cancer J Clin* **2010**, *60*, 166−193.

(2) Chen, L. C.; Liu, Y. C.; Liang, Y. C.; Ho, Y. S.; Lee, W. S. Magnolol inhibits human glioblastoma cell proliferation through upregulation of p21/Cip1. *J. Agric. Food Chem.* **2009**, *57*, 7331−7337.

(3) Ruan, J.; Zhang, L.; Yan, L.; Liu, Y.; Yue, Z.; Chen, L.; Wang, A. Y.; Chen, W.; Zheng, S.; Wang, S.; Lu, Y. Inhibition of hypoxia-induced epithelial mesenchymal transition by luteolin in non-small cell lung cancer cells. *Mol. Med. Rep.* **2012**, *6*, 232−238.

(4) Hsu, Y. F.; Lee, T. S.; Lin, S. Y.; Hsu, S. P.; Juan, S. H.; Hsu, Y. H.; Zhong, W. B.; Lee, W. S. Involvement of Ras/Raf-1/ERK actions in the magnolol-induced upregulation of p21 and cell-cycle arrest in colon cancer cells. *Mol. Carcinog.* **2007**, *46*, 275−283.

(5) Lin, S. Y.; Chang, Y. T.; Liu, J. D.; Yu, C. H.; Ho, Y. S.; Lee, Y. H.; Lee, W. S. Molecular mechanisms of apoptosis induced by magnolol in colon and liver cancer cells. *Mol. Carcinog.* **2001**, *32*, 73−83.

(6) Lin, S. Y.; Liu, J. D.; Chang, H. C.; Yeh, S. D.; Lin, C. H.; Lee, W. S. Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis. *J. Cell. Biochem.* **2002**, *84*, 532−544.

(7) Zhong, W. B.; Wang, C. Y.; Ho, K. J.; Lu, F. J.; Chang, T. C.; Lee, W. S. Magnolol induces apoptosis in human leukemia cells via cytochrome *c* release and caspase activation. *Anticancer Drugs* **2003**, *14*, 211−217.

(8) Li, L. F.; Lu, J.; Li, X. M.; Xu, C. L.; Deng, J. M.; Qu, R.; Ma, S. P. Antidepressant-like effect of magnolol on BDNF up-regulation and serotonergic system activity in unpredictable chronic mild stress treated rats. *Phytother. Res.* **2012**, *26*, 1189−1194.

(9) Tsai, T. H.; Chou, C. J.; Chen, C. F. Pharmacokinetics and brain distribution of magnolol in the rat after intravenous bolus injection. *J. Pharm. Pharmacol.* **1996**, *48*, 57−59.

(10) Lin, Y. R.; Chen, H. H.; Ko, C. H.; Chan, M. H. Differential inhibitory effects of honokiol and magnolol on excitatory amino acid-evoked cation signals and NMDA-induced seizures. *Neuropharmacology* **2005**, *49*, 542−550.

(11) Chen, C. R.; Tan, R.; Qu, W. M.; Wu, Z.; Wang, Y.; Urade, Y.; Huang, Z. L. Magnolol, a major bioactive constituent of the bark of *Magnolia officinalis*, exerts antiepileptic effects via the GABA/benzodiazepine receptor complex in mice. *Br. J. Pharmacol.* **2011**, *164*, 1534−1546.

(12) Hou, Y. C.; Chao, P. D.; Chen, S. Y. Honokiol and magnolol increased hippocampal acetylcholine release in freely-moving rats. *Am. J. Chin. Med.* **2000**, *28*, 379−384.

(13) Shih, Y. L.; Liu, H. C.; Chen, C. S.; Hsu, C. H.; Pan, M. H.; Chang, H. W.; Chang, C. H.; Chen, F. C.; Ho, C. T.; Yang, Y. Y.; Ho, Y. S. Combination treatment with luteolin and quercetin enhances antiproliferative effects in nicotine-treated MDA-MB-231 cells by down-regulating nicotinic acetylcholine receptors. *J. Agric. Food Chem.* **2010**, *58*, 235−241.

(14) Chen, C. S.; Lee, C. H.; Hsieh, C. D.; Ho, C. T.; Pan, M. H.; Huang, C. S.; Tu, S. H.; Wang, Y. J.; Chen, L. C.; Chang, Y. J.; Wei, P. L.; Yang, Y. Y.; Wu, C. H.; Ho, Y. S. Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins. *Breast Cancer Res. Treat.* **2010**, *125*, 73−87.

(15) Oki, E.; Sakaguchi, Y.; Toh, Y.; Oda, S.; Maehara, Y.; Yamamoto, N.; Sugimachi, K. Induction of apoptosis in human tumour xenografts after oral administration of uracil and tegafur to nude mice bearing tumours. *Br. J. Cancer* **1998**, *78*, 625−630.

(16) Ho, Y. S.; Duh, J. S.; Jeng, J. H.; Wang, Y. J.; Liang, Y. C.; Lin, C. H.; Tseng, C. J.; Yu, C. F.; Chen, R. J.; Lin, J. K. Griseofulvin potentiates antitumorigenesis effects of nocodazole through induction of apoptosis and G2/M cell cycle arrest in human colorectal cancer cells. *Int. J. Cancer* **2001**, *91*, 393−401.

(17) Griffin, R. J.; Williams, B. W.; Bischof, J. C.; Olin, M.; Johnson, G. L.; Lee, B. W. Use of a fluorescently labeled poly-caspase inhibitor for in vivo detection of apoptosis related to vascular-targeting agent arsenic trioxide for cancer therapy. *Technol. Cancer Res. Treat.* **2007**, *6*, 651−654.
(18) Balducci, M.; Chiesa, S.; Diletto, B.; D'Agostino, G. R.; Mangiola, A.; Manfrida, S.; Mantini, G.; Albanese, A.; Fiorentino, A.; Frascino, V.; De Bari, B.; Micciche, F.; De Rose, F.; Morganti, A. G.; Anile, C.; Valentini, V. Low-dose fractionated radiotherapy and concomitant chemotherapy in glioblastoma multiforme with poor prognosis: A feasibility study. *Neuro-Oncology* **2012**, *14*, 79−86.
(19) Kawamata, N.; Morosetti, R.; Miller, C. W.; Park, D.; Spirin, K. S.; Nakamaki, T.; Takeuchi, S.; Hatta, Y.; Simpson, J.; Wilcyznski, S.; Lee, Y. Y.; Bartram, C. R.; Koeffler, H. P. Molecular analysis of the cyclin-dependent kinase inhibitor gene p27/Kip1 in human malignancies. *Cancer Res.* **1995**, *55*, 2266−2269.
(20) Beniston, R. G.; Campo, M. S. Quercetin elevates p27(Kip1) and arrests both primary and HPV16 E6/E7 transformed human keratinocytes in G1. *Oncogene* **2003**, *22*, 5504−5514.
(21) Philipp, J.; Vo, K.; Gurley, K. E.; Seidel, K.; Kemp, C. J. Tumor suppression by p27Kip1 and p21Cip1 during chemically induced skin carcinogenesis. *Oncogene* **1999**, *18*, 4689−4698.
(22) Huang, C. S.; Ho, W. L.; Lee, W. S.; Sheu, M. T.; Wang, Y. J.; Tu, S. H.; Chen, R. J.; Chu, J. S.; Chen, L. C.; Lee, C. H.; Tseng, H.; Ho, Y. S.; Wu, C. H. SP1-regulated p27/Kip1 gene expression is involved in terbinafine-induced human A431 cancer cell differentiation: an in vitro and in vivo study. *Biochem. Pharmacol.* **2008**, *75*, 1783−1796.
(23) Hiromura, K.; Pippin, J. W.; Fero, M. L.; Roberts, J. M.; Shankland, S. J. Modulation of apoptosis by the cyclin-dependent kinase inhibitor p27(Kip1). *J. Clin. Invest.* **1999**, *103*, 597−604.
(24) von Kriegsheim, A.; Pitt, A.; Grindlay, G. J.; Kolch, W.; Dhillon, A. S. Regulation of the Raf-MEK-ERK pathway by protein phosphatase 5. *Nat. Cell Biol.* **2006**, *8*, 1011−1016.
(25) Mason, W. P.; Belanger, K.; Nicholas, G.; Vallieres, I.; Mathieu, D.; Kavan, P.; Desjardins, A.; Omuro, A.; Reymond, D. A phase II study of the Ras-MAPK signaling pathway inhibitor TLN-4601 in patients with glioblastoma at first progression. *J. Neurooncol.* **2012**, *107*, 343−349.
(26) Ciuffini, L.; Castellani, L.; Salvati, E.; Galletti, S.; Falcone, G.; Alema, S. Delineating v-Src downstream effector pathways in transformed myoblasts. *Oncogene* **2008**, *27*, 528−539.
(27) Lin, S. Y.; Lee, W. R.; Su, Y. F.; Hsu, S. P.; Lin, H. C.; Ho, P. Y.; Hou, T. C.; Chou, Y. P.; Kuo, C. T.; Lee, W. S. Folic acid inhibits endothelial cell proliferation through activating the cSrc/ERK 2/NF-κB/p53 pathway mediated by folic acid receptor. *Angiogenesis* **2012**, *15*, 671−683.
(28) Hou, T. C.; Lin, J. J.; Wen, H. C.; Chen, L. C.; Hsu, S. P.; Lee, W. S. Folic acid inhibits endothelial cell migration through inhibiting the RhoA activity mediated by activating the folic acid receptor/cSrc/p190RhoGAP-signaling pathway. *Biochem. Pharmacol.* **2013**, *85*, 376−384.
(29) Piva, R.; Cancelli, I.; Cavalla, P.; Bortolotto, S.; Dominguez, J.; Draetta, G. F.; Schiffer, D. Proteasome-dependent degradation of p27/kip1 in gliomas. *J. Neuropathol. Exp. Neurol.* **1999**, *58*, 691−696.
(30) Piva, R.; Cavalla, P.; Bortolotto, S.; Cordera, S.; Richiardi, P.; Schiffer, D. p27/kip1 expression in human astrocytic gliomas. *Neurosci. Lett.* **1997**, *234*, 127−130.
(31) Roy, S.; Gu, M.; Ramasamy, K.; Singh, R. P.; Agarwal, C.; Siriwardana, S.; Sclafani, R. A.; Agarwal, R. p21/Cip1 and p27/Kip1 Are essential molecular targets of inositol hexaphosphate for its antitumor efficacy against prostate cancer. *Cancer Res.* **2009**, *69*, 1166−1173.
(32) Shankar, S.; Ganapathy, S.; Chen, Q.; Srivastava, R. K. Curcumin sensitizes TRAIL-resistant xenografts: Molecular mechanisms of apoptosis, metastasis and angiogenesis. *Mol. Cancer* **2008**, *7*, 16.