Targeting of magnolin on ERKs inhibits Ras/ERKs/RSK2-signaling-mediated neoplastic cell transformation

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Mitogen-activated protein kinases play a key role in cell proliferation, cell cycle progression and cell transformation, and activated Ras/extracellular signal-regulated kinases (ERKs)/ribosomal S6 kinase 2 (RSK2) signaling pathways have been widely identified in many solid tumors. In this study, we found that magnolin, a compound found in the Magnolia species, directly targeted and inhibited ERK1 and ERK2 kinase activities with IC50 values of 87 and 16.5 nM by competing with adenosine triphosphate in an active pocket. Further, we demonstrated that magnolin inhibited epidermal growth factor (EGF)-induced p90RSKs phosphorylation at Thr359/Ser363, but not ERKs phosphorylation at Thr202/Tyr204, and this resulted in inhibition of cell proliferation by suppression of the G1/S cell cycle transition. Additionally, p38 kinases, Jun N-terminal kinases and Akts were not involved in the magnolin-mediated inhibitory signaling. Magnolin targeting of ERK1 and 2 activities suppressed the phosphorylation of RSK2 and downstream target proteins including ATF1 and c-Jun.

Introduction

The Magnolia species has been traditionally used as an oriental medicine to treat nasal congestion associated with headache, sinusitis, anti-inflammation and allergic rhinitis (1). Topical application of the Magnolia flos (flocculus: a small budding flower) extract inhibits passive cutaneous anaphylaxis induced by anti-dinitrophe- nyl IgE in rats (2). Magnolin is an ingredient found in the Magnolia species (2,3), and it has been widely used in oriental medicine to treat human diseases including empyema, nasal congestion, sinusitis and inflammation and has been shown to inhibit the production of tumor necrosis factor-α (3). Recent studies have shown that magnolin influences nitric oxide (NO) and prostaglandin E2 production by inhibiting extracellular signal-regulated kinase (ERK) (4), a key signaling pathway that regulates cell proliferation, transformation and cancer development (5,6). However, the biological effects of magnolin on cell proliferation and transformation have not been elucidated.

Mitogen-activated protein kinases (MAPKs), ERKs, p38 kinase and c-Jun N-terminal kinases (JNKs) are key regulators of cell proliferation and oncopogenesis (7,8). ERKs mediate mitogen-induced activation signals to the nucleus through phosphorylation of 90 kDa ribosomal S6 kinases (p90RSKs) (9,10), which are a family of serine/threonine kinases that respond to many growth factors, peptide hormones and neurotransmitters (11,12). The correlation between activation of the ERK signaling pathway and cell transformation has been established. For example, many human solid cancers contain an activated Ras mutation (RasG12V; constitutively active Ras) with high occurrences observed in colon cancer (45%), pancreatic cancer (90%), non-small-cell lung cancer (35%) and melanomas (15%) (13). Our previous results demonstrated that epidermal growth factor (EGF) stimulation induces the ERKs signaling pathways including ERKs/RSK2/histone H3 (5) and the ERKs/RSK2/cAMP-dependent transcription factor 1 (ATF1) signaling axis (14). These signaling pathways closely regulate cell proliferation and cell cycle transition from the G1 to S phase induced by EGF (5,14). Notably, our recent studies have demonstrated that phosphorylation of c-Jun at Ser63 and 73 plays an important role in cell proliferation and cell transformation induced by tumor promoters, such as EGF (15). The collective results support the view that the ERKs/RSK signaling pathway plays an important role in cell proliferation and transformation. However, although ERKs are an important target molecule of anticancer drugs or chemopreventive agents, the identification or development of small molecules as ERKs inhibitors has been barely studied.

Inhibitors of the ERK signaling pathway can be classified into two different categories: indirect and direct inhibitors. Indirect inhibitors of the ERK signaling pathway cover upstream kinases including Raf and MEKs. Because constitutive active Ras mutations such as the RasG12V and Raf mutations have been identified in a higher percentage in human solid cancers, the ERKs signaling pathway has become an important target in the development of novel chemotherapeutic agents and is desirable particularly due to its converging functions in cancer development (16). Recently, several ERK-specific inhibitors have been found by high-throughput phosphorylation assays with an in-house chemical library. For example, small molecule FR180204 (17) is an inhibitor of ERK1 and ERK2 with IC50 values of 0.51 and 0.33 μM, respectively (18). More recently, FR148083 was identified as an ERK2 inhibitor and it suppressed ERK2 activity with an IC50 value of 80 nM on an enzyme assay screening. However, these compounds also showed inhibition of other enzymes such as transforming growth factor-β-induced AP-1-dependent luciferase expression with an IC50 value of 50 nM (16). These results suggest that although identification of specific inhibitors against the ERK1 and/or ERK2 has been improving, more studies are necessary to reach the point where these agents can cure many human solid cancers or prevent cancer development.

Oriental medicinal herbs and dietary foods contain many useful natural compounds and have been widely used to identify novel compounds that may have therapeutic value in the treatment of human diseases. Some of these compounds, such as myricetin, quercetin from dietary foods and epigallocatechin gallate from green tea, inhibit cell proliferation and transformation (19), highlighting the importance of efforts to identify natural compound(s) that inhibit the ERKs/RSK...

Abbreviations: ATP, adenosine triphosphate; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinases; MEM, modified Eagle’s medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; PDB, Protein Data Bank; RSK2, ribosomal S6 kinase 2; SDS, sodium dodecyl sulfate.
signaling pathway while suppressing the MAPK pathway in a non-toxic manner (13). The present study found that magnolin is capable of suppressing cell proliferation and inhibiting the ERKs/RSK2 signaling pathway by targeting ERK1 and ERK2 activities.

Materials and methods

Reagents and antibodies

Tris, NaCl, sodium dodecyl sulfate (SDS) and buffer preparations were purchased from Sigma-Aldrich (St Louis, MO). Cell culture medium and other supplements were purchased from Life Science Technologies (Rockville, MD). Antibodies for the western blot analysis were purchased from Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) or Upstate Biotechnology (Lake Placid, NY). Human recombinant EGF was purchased from BD Sciences (San Jose, CA). Magnolin extracted from the dried flower buds of Magnolia fargesii (4) was generously provided by S.-R.O. of the Korea Research Institute of Bioscience and Biotechnology. Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich Co. LLC.

Magnolin treatment

Magnolin (>99.9% high-performance liquid chromatography purity) was prepared with a stock solution (100 mM: 1000x) by dissolving it in DMSO (99.7%), after which it was aliquoted and stored at -20°C. The magnolin was freshly diluted in DMSO (99.7%) with 100x concentrations before utilization and treatment of cells by medium exchange with magnolin pre-mixed cell culture medium, in which the DMSO did consist of >0.1% of the total medium volume.

Cell culture and transfection

RSK2-1 and RSK2-2 mouse embryonic fibroblasts were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic at 37°C in a 5% CO2 incubator. JB6 Cl41 mouse epidermal cells and NIH3T3 cells were cultured in 5% FBS-modified Eagle’s medium (MEM) and 10% calf serum–DMEM, respectively. A549 human lung cancer cells harboring RasG12V constitutive active mutation and H226 human lung cancer cells harboring wild-type Ras were cultured in 10% FBS–F-12 and 10% FBS–RPMI-1640. The cells were maintained by splitting at 80–90% confluence and the media were changed every 2 or 3 days. When the cells reached 50–60% confluence, transfection of the expression vectors was done using jetPEI (Polyplus-Transfection, New York, NY) according to the manufacturer’s instructions.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay

To measure cell proliferation, JB6 Cl41 cells (1 × 10^5) were seeded into 48-well plates in 100 µl of 5% FBS–MEM and incubated for 24 h at 37°C in a 5% CO2 incubator, and then at 0h, the absorbance was measured at OD 492 and 690 nm using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based CellTiter 96 Aqueous One Solution according to manufacturer’s instructions (Promega, Madison, WI). Briefly, 20 µl of the MTS solution was added to the cells in each well, and then the cells were incubated for 1 h at 37°C in a 5% CO2 incubator. The reaction was stopped by adding 25 µl of 10% SDS solution to each well and then the absorbance was measured immediately at 492 and 690 nm. The inhibition of cell proliferation by magnolin was evaluated by comparing the absorbance of a vehicle-treated control group over 96h at 24-hour intervals. To examine the cytotoxicity of magnolin, the cells (2 × 10^5) were seeded into 96-well plates in 100 µl of 5% FBS–MEM, were cultured overnight and were treated with DMSO (0.1% of final concentration) or 100 µM of magnolin. The cytotoxicity of magnolin was determined by measuring OD at 492 and 690 nm for 48 h at 24-hour intervals using MTS-based CellTiter 96 Aqueous One Solution according to the manufacturer’s instructions as described above.

Immunocytofluorescence

EFG-induced cell transformation was investigated in the JB6 Cl41 cells. Briefly, cells (8 × 10^4/ml) were exposed to EFG (0.1–10ng/ml) in 1 ml of 0.3% Basal Medium Eagle agar containing 10% FBS. The cultures were maintained in a 37°C, 5% CO2 incubator for 10 days and the cells colonies were scored using an ECLIPSE Ti inverted fluorescence microscope and the NIS-Elements AR (V. 4.0) computer software program (NIKON Instruments Korea, Gangnam, Seoul, Korea) as described previously (20).

Cell cycle analysis

JB6 Cl41 cells (2 × 10^5) were seeded into 60 mm diameter dishes and cultured overnight at 37°C in a 5% CO2 incubator. To examine the cell cycle under normal cell culture conditions, JB6 Cl41 cells were treated with the indicated concentrations of magnolin in complete cell culture medium for 12h. To explore the effect of magnolin on the cell cycle transition induced by EGF, JB6 Cl41 cells were pretreated with the indicated concentration of magnolin for 30 min and then stimulated with EGF (1 ng/ml) for 12h in the presence of the indicated concentrations of magnolin. The cells were harvested, fixed and then stained with propidium iodide (20 µg/ml) for 15 min at 4°C. The cell cycle distribution was measured by fluorescence-activated cell sorting (FACS) flow cytometry (BD FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, NJ).

Western blotting

Samples containing equal amounts of protein were resolved by 8–10% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were incubated in a blocking buffer containing 5% skim milk and then were probed with phospho-specific antibodies against phospho-ERK, total ERK, phospho-RSK, total RSK, phospho-Akt, total Akt, phospho-c-Jun and total c-Jun (all were obtained from Cell Signaling Technology). The antibodies against total ATF1, cyclooxygenase-2 and β-actin were purchased from Santa Cruz Biotechnology and phospho-ATF1 antibody was obtained from Abcam (Cambridge, MA). Western blots were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) using a Chemidoc XRS imager system (Bio-Rad Laboratories, Hercules, CA).

Computational docking of magnolin

The crystal structures of two proteins were obtained from Protein Data Bank, PDB entries: ZZQ (ERK1) and 1WZY (ERK2). The crystal structure was prepared using the Protein Preparation Wizard in Maestro v9.2. Hydrogens were added consistent with a pH of 7. All water molecules were removed. Then the structure was minimized with an root-mean-square deviation cutoff value of 0.3 Å. The program Glide v5.7 was used for ligand docking. The receptor grid was created with the centroid of the crystal ligand as the center of the grid. Flexible docking was performed with the standard precision mode. The number of poses per ligand was set to 10 in the postdocking minimization, and at most 5 poses would be the output. The other parameters were kept in the default.

Reporter gene assay

For the AP-1 luciferase assay, JB6 Cl41 cells stably transfected with AP-1 luciferase reporter plasmid were cultured in 24-well plates and then starved in serum-free medium for 16 h. The cells were pretreated with the indicated concentration of magnolin for 30 min and then cotreated with EGF (10 ng/ml) and the indicated concentration of magnolin for 12 h. JB6 Cl41 cells (2 × 10^4) were seeded into 24-well plates and cultured for 24 h. For the ATF1 transactivation activity assay, the cells were cotransfected with pGAL4-ATF1 expression and p5Glu/kb luciferase reporter plasmids together with 20 pg of pHRL-SV40 Renilla luciferase reporter plasmids and then cultured for 12 h. The cells were starved for overnight, pretreated with the indicated concentration of magnolin for 30 min and then cotreated with EGF (10 ng/ml) and the indicated concentration of magnolin for 6 h. The cells were disrupted, and the firefly luciferase activity was measured using a VX200 (PerkinElmer Inc, Waltham, MA). The firefly luciferase activity was normalized by Renilla luciferase activity to equalize the transfection efficiency.

In vitro kinase assay

The His-RSK2-328-740 truncated fusion proteins were purified from BL21 using nickel–nitrilotriacetic acid agarose beads (Qiagen Korea Ltd, Seoul, Korea). Active ERK1 or ERK2 (20ng), RSK2 (100ng) and 100 µM of cold adenosine triphosphate (ATP) were combined with the indicated concentration of magnolin in 20 µl of the reaction mixture. The kinase reaction was carried out at 30°C for 30 min and stopped by adding 6x SDS sample buffer and boiling. The proteins were resolved in SDS–polyacrylamide gel electrophoresis and visualized by western blotting using the indicated specific antibodies. The band intensity was measured by the NIH Image J (ver. 1.37) computer program, and the IC50 levels of magnolin on the ERK1 and ERK2 activities were calculated based on the band intensities.
Results

Magnolin suppresses cell proliferation by inhibition of G1/S cell cycle transition in JB6 Cl41

Based on the effect of magnolin on ERK inhibition (4) and the importance of the Ras/ERK/RSK2 signaling pathway in cell transformation (5), we hypothesized that magnolin might be a useful chemopreventive agent. We treated magnolin in JB6 Cl41 cells and found that 30 and 60 μM of magnolin inhibited ~40 and 70% of the cell proliferation compared with DMSO (0.1% of final concentration; vehicle)-treated control cells (Figure 1B). Additionally, magnolin did not show cytotoxicity in JB6 Cl41 cells in an ex vivo cell culture system, even at concentrations exceeding 100 μM (Supplementary Figure 1A, available at Carcinogenesis Online). The MAPK cascades have been implicated in the regulation of cell proliferation, survival, growth and motility (13,21,22) as well as tumorigenesis (5,23,24). The hypothesis that magnolin might suppress cell cycle progression was proven by the cell cycle distribution analysis using flow cytometry. We found that magnolin increased the population of G1/G0 cell cycle phase to ~55% with the concentration of 30 μM and ~60% with the concentration of 60 μM, compared with ~42% of vehicle-treated control cells (Figure 1C). However, G2/M cell cycle phase of cells was marginally increased compared with the vehicle-treated control cells (Figure 1C; Supplementary Figure 1B, available at Carcinogenesis Online). Further studies on the G1/S cell cycle transition by synchronization and EGF-induced cell cycle release demonstrated that the induction of S cell cycle phase and reduction of G1/G0 cell cycle phase by EGF (Figure 1D; Supplementary Figure 2, available at Carcinogenesis Online) was abrogated by magnolin treatment in a concentration-dependent manner and the 60 μM of magnolin treatment totally abolished the EGF stimulatory effect of the cell cycle progression (Figure 1D; Supplementary Figure 2, available at Carcinogenesis Online). In addition, final concentration of 0.1% DMSO did not affect the EGF-induced cell cycle progression (Figure 1D, column 1 versus column 2), and treatment with 10ng/ml of the EGF produced similar results as with treatment with 1 ng/ml of EGF stimulation.

Fig. 1. Magnolin suppresses cell proliferation by inhibition of G1/S cell cycle transition in JB6 Cl41. (A) Chemical structure of magnolin. (B) JB6 Cl41 cells (1 × 10^3) were seeded into 96-well cell culture plates and cell proliferation was measured at 24 h interval over 96h. Data are presented as the mean ± SD of values from hexaplicate experiments, and the statistical significance was determined using Student’s t-test (*P < 0.001). (C) JB6 Cl41 cells (2 × 10^5) were seeded into 60 mm cell culture dishes and cultured overnight. The cells were treated with the indicated concentrations of magnolin for 12 h and then the cell cycle population was measured by FACS analysis. Data are presented as the mean ± SD of values from triplicate experiments, and statistical significance was determined using Student’s t-test (*P < 0.05; **P < 0.005). (D) JB6 Cl41 cells (2 × 10^5) were seeded into 60 mm cell culture dishes and cultured overnight. They were starved for 24 h supplemented with 0.1% FBS–MEM; the cells were then pretreated with the indicated concentration of magnolin for 30 min with the indicated concentration of magnolin and then treated with EGF and each indicated concentration of magnolin for 12h. The cell cycle population was measured by FACS. Data are presented as the mean ± SD of values from triplicate experiments, and statistical significance was determined using Student’s t-test (*P < 0.05; **P < 0.005).
These results demonstrated that magnolin inhibited cell proliferation induced by tumor promoters such as EGF through inhibition of the G1/S cell cycle transition.

**Magnolin inhibits selectively the ERKs/RSKs signaling pathway but not JNKs, p38 kinases and AKTs signaling pathways**

To decipher the inhibitory signaling pathway(s) of cell proliferation by magnolin, we hypothesized that magnolin might suppress the ERKs/RSKs signaling pathway because magnolin inhibits cell proliferation and EGF-induced G1/S cell cycle progression (Figure 1B and D). To examine the hypothesis, we analyzed phosphorylation protein profiles by treatment with EGF with or without magnolin. Magnolin inhibited the phosphorylation of RSKs at Thr359/Ser363 (Figure 2A; Supplementary Figure 3, available at Carcinogenesis Online). Interestingly, we found that magnolin did not modulate the phosphorylation of MEKs and Akts (Figure 2A and B). In addition, phosphorylations of p38 kinases and JNKs were not detected with EGF stimulation as shown in our previous publication (14), and magnolin did not alter phosphorylation of p38 kinases and JNKs (Figure 2C). To confirm whether the inhibitory effect of magnolin on RSKs phosphorylation at Thr359/Ser363 was selective in the MEKs/ERKs signaling axis or not, we compared phosphorylation of ERKs and RSKs by treatment with an MEK1/2 inhibitor (PD98059), an ERKs upstream kinase inhibitor and magnolin. We found that PD98059 inhibited ERK1/2 phosphorylation at Thr202/Tyr204 (Figure 2D). In contrast, ERK1/2 phosphorylation at Thr202/Tyr204 was not altered by magnolin treatment (Figure 2D). Importantly, phosphorylation of RSKs at Thr359/Ser363 was equally inhibited by magnolin and PD98059 (Figure 2D). Notably, cell proliferation of JB6 Cl41 cells was inhibited by treatment with magnolin or PD98059 in a concentration-dependent manner (Figure 2E). These results demonstrated that magnolin selectively inhibited ERKs activities.

**Fig. 2.** Magnolin inhibits selectively the ERKs/RSKs signaling pathway but not JNKs, p38 kinases and the Akts signaling pathways. (A) JB6 Cl41 cells (1 × 106) were seeded in 100 mm cell culture dishes and cultured overnight. The cells were starved for 24 h with 0.1% FBS–MEM, pretreated with the indicated concentration of magnolin for 30 min and then cotreated with EGF (10 ng/ml) for 30 min. The specific proteins were visualized by western blotting using specific antibodies as indicated. β-Actin was used for the internal control to verify equal protein loading. (B) The JB6 Cl41 cells were prepared as described in (A). The proteins were extracted and phospho-Akt and total Akt were visualized by western blotting using specific antibodies as indicated. β-Actin was used for the internal control to verify equal protein loading. (C) The JB6 Cl41 cells were prepared as described in (A). The proteins were extracted and phospho-p38 MAPKs and total p38 MAPKs and total JNKs were visualized by western blotting using specific antibodies as indicated. β-Actin was used for the internal control to verify equal protein loading. (D) JB6 Cl41 cells (1 × 106) were seeded in 100 mm cell culture dishes and cultured overnight. The cells were starved for 24 h, pretreated with the indicated concentration of magnolin or PD98059 for 30 min and then cotreated with EGF (10 ng/ml) and then the indicated concentration of magnolin or PD98059 for 30 min. The proteins were extracted and visualized by western blotting using specific antibodies as indicated. β-Actin was used for the internal control to verify equal protein loading. (E) JB6 Cl41 cells (1 × 106) were seeded into 96-well cell culture plates and cell proliferation was measured at 24 h interval over 96 h with the indicated concentration of magnolin and PD98059. Data are presented as the mean ± SD of values from hexaplicate experiments and statistical significance was determined using Student’s t-test (*P < 0.001).
Magnololin targets ERK1 and ERK2

To explore in more detail whether magnololin inhibits ERK1 and 2 activities, we partially purified truncated RSK2 protein containing 328–740 amino acids (His-RSK2-328-740) (Figure 3A, left panel) and conducted an in vitro kinase assay with active ERK1 and RSK2-328-740 protein. We found that ERK1-mediated phosphorylation of RSK2 at Thr359/Ser363 and Thr577 was inhibited >90% by magnololin (3.75 µM) (Figure 3A, right panel). Taken together with Figure 2, these results demonstrated that magnololin inhibited ERK1 and 2 activities. Thus, we conducted computational docking of magnololin with ERK1 and 2. For flexible docking of magnololin and ERK1 or 2 by the standard precision mode, the crystal structures of ERK1 (2ZQO) and ERK2 (1WZY) were obtained from the PDB (http://www.rcsb.org/pdb/home/home.do). We found that magnololin formed hydrogen bonds with Lys168 of ERK1 (Figure 3B, left panel) and Met108 and Lys54 of ERK2 (Figure 3B, right panel), respectively. Magnololin in the active pocket of ERK1 or 2 was completely different in molecular structure and binding angle (Figure 3B, left right panels). The docking scores of magnololin in the active pocket of ERK1 and 2 were ~7.0 and ~6.68, respectively. To measure the IC50 of magnololin on ERK1 or 2 activity, we reconducted an in vitro kinase assay with a nanomolar concentration of magnololin. We found that the IC50 value of magnololin on ERK1 activity was about 87 nM (Figure 3C) and 16.5 nM on ERK2 activity (Figure 3D). To confirm whether magnololin was competitive with ATP or not, we conducted a magnololin competition assay with ATP–agarose beads. We found that an increase in magnololin decreased ERK2 binding in ATP–agarose beads in a concentration-dependent manner (Figure 3E). These results indicated that magnololin targeted the active pocket of ERK1 and 2 by competition with ATP, resulting in the inhibition of cell proliferation through inhibition of the ERKs/RSK2 signaling pathway.

Magnololin inhibits ATF1 and AP-1 transactivation activities

Our previous study demonstrated that the ERKs/RSK2/ATF1 signaling axis and AP-1 signaling pathways play a key role in cell proliferation and transformation (14,15). Thus, we confirmed whether magnololin inhibited phosphorylations of ATF1 and c-Jun, a critical component of the AP-1 complex, nuclear localization of phospho-ATF1 and phospho-c-Jun and transactivation activities of ATF1 and AP-1 complex. Our results demonstrated that magnololin inhibited the EGF-induced ATF1 phosphorylation at Ser63 in a concentration-dependent manner (Figure 4A, upper panel). Moreover, the ATF1 transactivation activity that had been increased by EGF stimulation was suppressed by cotreatment with EGF and magnololin in the same way as the inhibition of ATF1 phosphorylation by magnololin in a concentration-dependent manner (Figure 4A, graph). Importantly, the nuclear-phospho-ATF1 protein level induced by EGF was abrogated by cotreatment with EGF and magnololin in western blotting (Figure 4B, upper panel) and immunocytofluorescence assay (Figure 4B, bottom panel) in a concentration-dependent manner. Notably, EGF-induced c-Jun phosphorylation at Ser63 and Ser73 and AP-1 transactivation activity was inhibited by cotreatment with EGF and magnololin in a concentration-dependent manner (Figure 4C). Similarly, nuclear phospho-c-Jun was increased by EGF stimulation, and magnololin suppressed EGF-induced nuclear c-Jun phosphorylation in a concentration-dependent manner (Figure 4D). Taken together, these results demonstrated that the magnololin-mediated inhibition of ERKs activity results in inhibition of cell proliferation through suppression of ATF1 and AP-1 transactivation activities.

Magnololin inhibits EGF-induced cell transformation

Our previous studies demonstrated that RSK2 deficiency suppresses cell proliferation by the impairment of G1/S cell cycle transition (5), and that the blocking of MEKs/ERKs signaling can suppress EGF-induced cell transformation in HaCaT human keratinocytes (25). Furthermore, our results showed that magnololin inhibited ATF1 and AP-1 (c-Fos/c-Jun dimer) transactivation activities (Figure 4). Therefore, we conducted an anchorage-independent cell transformation assay using a tumor promoter such as EGF. We found that magnololin inhibited anchorage-independent cell transformation in JB6 Cl141 cells by ~40% at 30 µM and 75% at 60 µM according to colony numbers and size (Figure 5A). To examine the magnololin sensitivity of Ras/ERKs signaling on cell proliferation and transformation, we utilized A549, which has a constitutive active mutation of Ras (RasG12V), and H226, which contains wild-type Ras (Ras-wt). We found that magnololin inhibited cell proliferation in A549 cells more compared with H229 cells, in a concentration-dependent manner (Figure 5B). Moreover, we found that the anchorage-independent colony growth of A549 cells was inhibited by magnololin treatment in a concentration-dependent manner (Figure 5C; Supplementary Figure 4B, available at Carcinogenesis Online). However, H226 cells did not grow in soft agar (Figure 5C, left graph; Supplementary Figure 4A, available at Carcinogenesis Online). To confirm the findings more precisely, we utilized NIH3T3 cells stably expressing mock or RasG12V (Figure 5D) and confirmed that magnololin inhibited cell proliferation in RasG12V-expressing NIH3T3 cells more sensitively compared with mock expressing control cells (Figure 5E). Moreover, NIH3T3 cells stably expressing RasG12V obtained their ability to show anchorage-independent colony growth, indicating that the cells were transformed, but this was not observed in mock stably NIH3T3 cells (Figure 5F). Importantly, magnololin treatment suppressed anchorage-independent colony growth of the NIH3T3 cells stably expressing RasG12V (Figure 5F, panel and graph). Taken together, these results demonstrated that the magnololin targeting of ERK1 and 2 activities suppressed the Ras/ERK/RSK2-mediated downstream signaling pathways including ATF1 and AP-1, resulting in inhibition of cell proliferation and transformation induced by tumor promoters such as EGF.

Discussion

Identifying natural compounds harboring chemopreventive and/or anticancer activity could prove highly valuable for the treatment of cancer patients after surgery. Our previous studies have demonstrated that many different natural compounds inhibit different signaling pathways. For example, kaempferol, a natural compound abundantly found in the leaves of green onion, inhibits RSK2 N-terminal kinase activity, which, in turn, inhibits anchorage-independent cell transformation (5). Eriodictyol, a flavonoid extracted from Yerba Santa (Eriodictyon californicum), also inhibits RSK2 activity and cell transformation by preventing ATF1 activation (14). In this study, we found that magnololin targeting on the active pocket of ERK1 and ERK2 inhibits the ERKs/RSK signaling pathway and results in suppression of cell proliferation and transformation (2). Thus, magnololin is a useful natural compound for inhibiting ERK1 and 2 activities.

Generally, the initiation of cancer development alters the properties of the affected cell including fasting cell proliferation and cell cycle progression by mutations (26). EGF and 12-O-tetradecanoylphorbol-13-acetate have widely been used as tumor promoters and have been shown to induce anchorage-independent cell transformation in JB6 Cl41 mouse skin epidermal cells and HaCaT human skin keratinocytes (23,25). Magnololin (Figure 1A) isolated from the Magnolia species is a major bioactive component found in Shin-I, the dried flower buds of the Magnolia species (2). We found that magnololin inhibited cell proliferation in a concentration-dependent manner (Figure 1). Our previous results demonstrated that the cell proliferation of RSK2– mouse embryonic fibroblasts was inhibited by the impairment of G1/S cell cycle transition (5). We confirmed that magnololin inhibited phosphorylation of RSKs at Thr359/Ser363 and the RSK2 downstream signaling molecules including c-Jun and ATF1 (Figure 2). However, magnololin did not inhibit the phosphorylation of ERK1 and 2 at Thr202 and Tyr204 (Figure 2). The results indicated that magnololin might target kinases between the RSKs upstream and MEK1/2 downstream kinases. The kinases between MEK1/2 and RSKs are ERK1 and 2 in a MAPK signaling cascade. As expected, magnololin inhibited ERK1 and 2 activities with 87 and 16.5 nM of IC50 values. This result is the first report that identifies the natural compound targeting ERK1 and
Magnolol targeting on ERKs

Fig. 3. Magnolol targets ERK1 and ERK2. (A) Left panel: the His-RSK2-328-740 fusion protein was purified by nickel–nitrilotriacetic acid beads using pHis-RSK2-328-740 bacterial expression plasmid in BL21. The crude extract, washed of the supernatant after bead binding and purified His-RSK2-328-740, was visualized by SDS–polyacrylamide gel electrophoresis and coomassie brilliant blue R-250 staining. Right panel: active ERK1 (20 ng), His-RSK2-328-740 protein (100 ng) and ATP (100 µM of final concentration) were combined with different concentrations of magnolol in 20 µl of reaction mixture as indicated and the reaction was carried out at 30°C for 30 min. The phosphorylated RSK2 at Thr359/ser363 and Thr577 was visualized by western blotting using specific antibodies as indicated. (B) The crystal structures of ERK1 (PDB accession number: 2ZOQ) and ERK2 (PDB accession number: 1WZY) were prepared using the Protein Preparation Wizard in Maestro v9.2. Hydrogens were added until a pH of 7 was reached and minimized with an root-mean-square deviation cutoff value of 0.3 Å. The program Glide v5.7 was used for ligand docking. The receptor grid was created with the centroid of the crystal ligand as the center of the grid. Flexible docking was performed with the standard precision mode. The number of poses per ligand was set to 10 in the postdocking minimization and at most 5 poses for the output. The other parameters were kept the same as the default. (C and D) Optimization of the IC$_{50}$ value of ERK1 and ERK2 activities by magnolol. Active ERK1 or ERK2 were combined with His-RSK2-328-740 protein and ATP, and an in vitro kinase assay was conducted to optimize the IC$_{50}$ values of magnolol on the ERK1 and ERK2 activities as described in (A). The bands intensity was measured using the Image J computer program (ver. 2.0) and the IC$_{50}$ value was calculated based on the band intensities. (E) ATP competition of magnolol in ERK2 active pocket. The active ERK2 protein was combined with ATP-conjugated beads, washed and then competed with magnolol as indicated. The beads were washed and ERK2 bound in ATP-conjugated beads was visualized by western blotting with the ERK1 specific antibody. The band density of ERK2 was measured by Image J (ver. 1.37v) computer program.
Magnolin inhibits ATF1 and AP-1 transactivation activities. (A and C) Magnolin inhibited ATF1 (A) and AP-1 (C) transactivation activity. Upper panels in (A) and (C): JB6 Cl41 cells (1 × 10⁶) were seeded in 100 mm cell culture dishes and cultured overnight. The cells were starved for 24 h, pretreated with the indicated concentration of magnolin for 30 min and then cotreated with EGF (10 ng/ml) and the indicated concentration of magnolin for 30 min. The proteins were extracted and visualized by western blotting using specific antibodies as indicated. β-Actin was used for the internal control to verify equal protein loading. Graphs in (A) and (C): p5xGal4-luciferase reporter plasmid was cotransfected with pGal4-ATF1-wt and phRL-SV40 luciferase activity (an internal control for equal transfection efficiency) into JB6 Cl41 cells and cultured for 12 h. The cells were starved overnight, pretreated with magnolin for 30 min and then cotreated with magnolin and EGF as indicated for 6 h (A). JB6 Cl41 (2 × 10⁴) cells stably transfected with AP-1 luciferase reporter plasmid were seeded into 24-well culture plates and cultured for 24 h. The cells were starved overnight, pretreated with the indicated concentration of magnolin for 30 min and cotreated with magnolin and EG as indicated for 12 h (C). Firefly luciferase activity was measured as described in Materials and methods and normalized with Renilla luciferase activity for equal transfection efficiency (A and C). Data are presented as the mean ± SD of values from triplicate experiments and statistical significance was determined using Student’s t-test (* *P < 0.001). (B and D) Nuclear phosphoprotein levels of ATF1 and c-Jun. Upper panels in (B) and (D): JB6 Cl41 cells (1 × 10⁶) were seeded in 100 mm cell culture dishes and cultured overnight. The cells were starved for 24 h, pretreated with magnolin for 30 min and then cotreated with magnolin and EGF for 30 min as indicated. The cells were fixed, permeabilized, and phospho-ATF1 at Ser63 (B) and phospho-c-Jun at Ser63 (D) were visualized by phospho-specific antibodies and Alexa 568 conjugated secondary antibody. The fluorescence was observed under a fluorescence microscope (×400). 4′,6-Diamidino-2-phenylindole was used for the nuclear staining.
ERK2 activities with the lowest IC_{50} levels. Furthermore, inhibition of ERKs signaling by magnolin-induced G_{1}/G_{0} cycle accumulation through the impairment of the G_{i}/S cell cycle transition (Figure 1C and D). These results are plausible because our previous results indicated that RSK2{^−/−} mouse embryonic fibroblasts cells showed impairment of G_{i}/S cell cycle transition, resulting in the accumulation of G_{i}/G_{0} cell cycle phase (5). These results support the view that magnolin inhibits the progression stage of carcinogenesis.

The present results of the western blotting analysis indicated that magnolin suppressed phosphorylation of RSK at Thr359/Ser363, but not ERK phosphorylation at Thr202/Tyr204 (Figure 2A), indicating that magnolin targeted ERKs. Furthermore, Akt phosphorylations at Thr308, which is a target amino acid of phosphatidylinositol 3-kinase, and at Ser473, which is a target amino acid of mTORC2, were not significantly altered by magnolin (Figure 2B). In addition, phosphorylation of JNKs and p38 kinases were not induced by EGF treatment (Figure 2C), indicating that magnolin specifically inhibits ERKs/RSK2 signaling, which is a key signaling pathway regulating cell proliferation and transformation (5). This hypothesis was confirmed by the computational docking and in vitro kinase assay, which showed that magnolin targeted ERK1 and 2 (Figure 3). Particularly, because dysregulation of the Ras/Raf/MEK/ERK/RSK signaling pathway plays an important role in cell proliferation, transformation and cancer development in many human solid cancers in vitro and in vivo,
identification of natural and synthetic chemical compounds with a higher specificity that can be used as a chemotherapeutic and/or chemopreventive agent has been desired. However, although there is recognition that imatinib mesylate demonstrated that achievement of kinase specificity is possible, identifying a specific kinase inhibitor is rarely successful. Recently, FR180204 and FR148083 were identified as ERK1- and ERK2-ATP competitive-specific inhibitors, with IC\(_{50}\) values of 50–500 nM (18, 27). In this study, we found that magnolin, a natural compound from *Magnolia flos*, inhibited ERK1 and 2, with IC\(_{50}\) values of 87 and 16.5 nM, respectively (Figure 3C and D). Our computational modeling indicated that Lys168 of ERK1 and Met108 and Lys54 of ERK2 play an important role in the binding with magnolin (Figure 3B). Importantly, the X-ray crystal structure of ERK and FR180204 revealed that the Met108, Gln105, Asp106 and Lys54 in the ATP-binding pocket formed hydrogen bonds (28). Especially, dimethoxyphenyl in magnolin formed a hydrogen bond with Lys168 (1.9 Å) on the ERK1, a bicyclic furan in magnolin formed a hydrogen bond with Met108 (2.1 Å) on the ERK2 and trimethoxyphenyl in magnolin formed a hydrogen bond with Lys54 (2.1 Å) on the ERK2 (Figure 3B). As expected, ERK2 was dissociated from the ATP–agarose beads by competing with magnolin, indicating that magnolin targeted the active pocket of ERK2 (Figure 3E). However, because ERK1 showed higher
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affinity with the ATP-non-conjugated control beads, we failed to show how magnolin competition with ATP–agarose bead in the ERK1 active pocket. To provide more concrete evidence for the interaction between specific amino acids in the ERK active pocket and magnolin, performing point mutagenesis of ERK and magnolin-conjugated bead pull-down assay will be necessary. However, because magnolin does not contain any amine or hydroxyl group, we failed to conjugate magnolin with CNBr–sepharose beads. Alternatively, magnolin binding in the ERK active pocket is measurable with radioisotope-labeled magnolin. Unfortunately, magnolin labeled with 14C or 3H radioisotope was not available. Notably, our results demonstrated that magnolin competed with ATP for ERK2 binding in a concentration-dependent manner (Figure 3E). These results demonstrate that magnolin is a useful natural compound displaying chemopreventive activity. As further evidence of this process, an anchorage-independent cell transformation assay demonstrated that magnolin suppressed the cell transformation of JB6 Cl41 cells in soft agar (Figure 5A) due to the suppression of ATF1 and AP-1 transactivation activities (Figure 4). Inhibition of ERKs/RSK2 signaling suppressed EGF-induced phosphorylation and nuclear localization of ATF1 at Ser63 and c-Jun at Ser63/73 (Figure 4), resulting in cell proliferation (Figure 1B) and transformation (Figure 5A). Taken together, magnolin is a natural compound specifically targeting ERK1 and 2 and is a useful chemotherapeutic and/or chemopreventive agent in human cancer.

Supplementary material

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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References


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