Isoangustone A, A Novel Licorice Compound, Inhibits Cell Proliferation by Targeting PI3K, MKK4, and MKK7 in Human Melanoma

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Introduction

The root of the licorice (Glycyrrhiza) plant species has been used in herbal medicine. Multiple lines of evidence have shown that licorice root possesses antioxidant, anti-inflammatory, antiviral, antitumor, hepatoprotective, and cardioprotective bioactivities (1–4). Glycyrrhizin, a triterpene compound, is considered as the principal biologically active component of licorice (4). Therefore, many studies have focused on the bioactivity of glycyrrhizin. Previous studies demonstrated that licorice and glycyrrhizin can attenuate inflammation, melanoma cell proliferation, and metastasis both in vitro and in vivo (5–7). However, long-term consumption of glycyrrhizin results in undesirable mineralocorticoid excess, hypertension, and hypokalemia. Hypertension is caused by reduction in the activity of 11β-hydroxysteroid dehydrogenase type II by glycyrrhetic acid, a metabolite of glycyrrhizin generated in the intestine (4, 8, 9). Therefore, finding additional active anticancer compounds in licorice other than glycyrrhizin is appropriate.

Uncontrolled proliferation is one of the most critical characteristics of cancer cells (10). Deregulation of cell cycle leads to increased proliferation and carcinogenesis (11). Passage through the cell cycle is strictly controlled by cyclin/cyclin-dependent kinase (CDK) complexes (12). Cyclins D and E bind to CDK4/6 and CDK2, respectively, and sequentially phosphorylate the retinoblastoma (Rb) protein. This facilitates the transition from G1 to S phase (13). Amplification of the cyclin D1 gene and overexpression of the cyclin D1 protein are found in several cancer types, including parathyroid adenoma, breast, colon, lymphoma, prostate, and melanoma (14–16). Cyclin D1 plays a prominent role in cell cycle progression and is a target of multiple cell cycle regulatory proteins, including cyclins D1 and E in the SK-MEL-28 human melanoma cell line. IAA suppressed the phosphorylation of Akt, GSK-3β, and JNK1/2. IAA also bound to phosphoinositide 3-kinase (PI3K), MKK4, and MKK7, strongly inhibiting their kinase activities in an ATP-competitive manner. Moreover, in a xenograft mouse model, IAA significantly decreased tumor growth, volume, and weight of SK-MEL-28 xenografts. Collectively, these results suggest that PI3K, MKK4, and MKK7 are the primary molecular targets of IAA in the suppression of cell proliferation. This insight into the biologic actions of IAA provides a molecular basis for the potential development of a new chemotherapeutic agent. Cancer Prev Res; 6(12); 1293–303. ©2013 AACR.
in driving tumorigenesis (17). Melanoma shows mutations and/or amplification of receptor tyrosine kinases and amplification of the cyclin D1 and cdk4 genes, which regulate cell-cycle progression (18, 19). Thus, inhibition of cyclin D1 expression could be a promising anticancer strategy for melanoma.

Various signaling pathways regulating cell-cycle progression have been reported. The mitogen-activated protein kinase (MAPK) signaling pathways play important roles in many biologic processes, including cell-cycle progression, proliferation, inflammation, apoptosis, and differentiation. The MAPKs include extracellular signal–regulated kinases (ERK), c-jun—NH2—kinases (JNK), and p38. They are activated by specific MAPK kinases (MAPKK), including MAP–ERK kinase (MEK) 1/2, MAPKK4/7, and MAPKK3/6 (20). The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is also important for cell survival and growth and plays a pivotal role in tumorigenesis (21). Elevated expression or excessive activation of PI3K has been observed in many tumor cells (22, 23). Glycogen synthase kinase (GSK)-3b, a downstream target of the PI3K pathway, causes cyclin D1 degradation in response to mitogenic signals (24, 25). PI3K-dependent phosphorylation of GSK-3β at Ser9 inhibits activation of GSK-3β and thus stabilizes cyclin D1 (25). Therefore, regulation of the PI3K/Akt/GSK-3β pathway is also important for cell survival and growth.

In the present study, we compared the anticancer activity of isoangustone A (IAA; Fig. 1A), a novel licorice compound, and glycyrrhizin (Fig. 1B), in SK-MEL-28 cells. In a xenograft model, we found that IAA significantly reduced the size and weight of tumors in nude mice. We also demonstrated that IAA was more effective than glycyrrhizin on anchorage-independent cell growth in SK-MEL-28 cells (Fig. 1C).

Materials and Methods

Materials

IAA was obtained as described previously (26). Glycyrhizina was purchased from Sigma-Aldrich. Dulbecco’s modified Eagle medium (DMEM) and FBS were from Gibco BRL. Antibodies against cyclin D1, cyclin E, CDK4, CDK2, phosphorylated Rb (Ser807/811), Akt (Thr308 and Ser473), GSK-3β (Ser9), JNK1/2 (Thr183/Tyr185), p38 (Thr180/Tyr182), MKK4 (Ser257), MKK7 (Ser271/Thr275), and total Rb, Akt, GSK-3β, JNK1/2, p38, MKK4, and MKK7 were purchased from Cell Signaling Technology. Phosphorylated (Thr202/Tyr204) and total ERK1/2 antibodies were from Santa Cruz Biotechnology. Anti-β-actin was from Sigma-Aldrich. CNBr-Sepharose 4B and [γ-32P]ATP were from GE Healthcare.

Cell culture

SK-MEL-28 and SK-MEL-5 cells were from the American Type Culture Collection, and SK-MEL-2 and WM-266-4 cells were from Korean Cell Line Bank at Seoul National University (Seoul, Republic of Korea). Human melanoma cells were cultured in monolayers in 10% FBS in DMEM containing 1,000 units of penicillin and 1 mg/ml streptomycin at 37°C under 5% CO2.

Anchorage-independent cell growth assay

The effects of IAA and glycyrrhizin on anchorage-independent growth were investigated in SK-MEL-28 cells. Cells (8 × 10^3/mL) were incubated in with or without IAA or glycyrrhizin (0–20 μmol/L) in 1 mL 0.33% basal medium Eagle agar containing 10% FBS, or in 3.5 mL 0.5% basal medium Eagle agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO2 incubator for 10 days, and cell colonies were counted under a microscope using the Image-Pro Plus software program (Media Cybernetics).

Cell proliferation assay

Cells were cultured overnight in 96-well plates (3,000 cells/well) in 10% FBS/DMEM, and then treated with IAA or glycyrrhizin at the indicated concentration for 24, 48, or 72 hours and 20 μL MTTR or MTTS reagent were added to each well. For the MTTR assay, the media were removed and 200 μL of DMSO were added to each well and absorbance was read at 570 nm. The reduction in MTTR was measured 1 hour later by spectrophotometry at 492 and 690 nm as background (Multiskan MS, Labsystems). Three independent experiments were performed.

Cell-cycle analysis

SK-MEL-28 cells were seeded in 60-mm dishes (1.5 × 10^5 cells/dish) and cultured for 24 hours in 10% FBS/DMEM. The cells were treated with IAA or glycyrrhizin at various concentrations (0–20 μmol/L) and trypsinized 48 hours later, washed with ice-cold Dulbecco’s PBS (DPBS) and fixed in ice-cold 70% ethanol at –20°C overnight. Then the cells were washed twice with 20 μg/mL RNase A and 200 μg/mL propidium iodine in DPBS at room temperature for 30 minutes in the dark. The cell-cycle phase was determined by FACSCalibur (BD Biosciences). Data were gathered using ModFit LT (Verity Software House, Inc.).

Western blot analysis

SK-MEL-28 cells were cultured for 24 hours in 10% FBS/DMEM and then IAA or glycyrrhizin was added for 24 or 48 hours. Then the cells were scraped, treated with lysis buffer [10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L dithiothreitol (DTT), 0.1 mmol/L phenylmethylsulfonylfluoride (PMSF), 10% glycerol, and 1 μg/mL protease inhibitor cocktail] for 30 minutes on ice and centrifuged at 12,000 × g for 10 minutes. For determination of protein levels in SK-MEL-28 xenografts, tumors from each mouse were lysed with T-PER buffer (Pierce).

The concentration of protein in the supernatant fractions was measured using a dye-binding protein assay kit, as described by the manufacturer (Bio-Rad Laboratories). Lysate aliquots (40 μg protein) were subjected to 10% or 12% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore Corporation). After transfer, the membrane was blocked in 5% fat-free milk for 1 hour at
room temperature and incubated with a specific primary antibody at 4°C overnight. After incubation with a horse-radish peroxidase–conjugated secondary antibody, protein bands were detected using an enhanced chemiluminescence detection kit (GE Healthcare).

**PI3K assay**

An active PI3K protein (100 ng) was incubated with IAA or LY294002 for 10 minutes at 30°C. The mixture was incubated with 20 μL of phosphatidylinositol (0.5 mg/mL; Avanti Polar Lipids) for 5 minutes at room temperature and then incubated in reaction buffer (100 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.6, 50 mmol/L MgCl₂, 250 μmol/L ATP containing 10 μCi [γ-32P] ATP) for an additional 10 minutes at 30°C. The reaction was stopped by adding 15 μL HCl (4 N) and 130 μL chloroform: methanol (1:1). After vortexing, 30 μL of the lower chloroform phase was spotted onto a 1% potassium oxalate–coated silica gel plate, which was previously activated for 1 hour at 110°C. The resulting [32P]-labeled phosphatidylinositol-3-phosphate was separated by thin-layer chromatography, and the radiolabeled spots were visualized by autoradiography. Relative kinase activity was quantified using ImageJ.

**MKK4 and MKK7 kinase assays**

The in vitro MKK4 and MKK7 kinase assays were performed following the instructions provided by Upstate Biotechnology. Briefly, 40 ng active MKK4 or MKK7 recombinant proteins were reacted with IAA (0, 10, or 20 μmol/L) at 30°C for 10 minutes. For each reaction, 5 μL of 5× kinase buffer (250 mmol/L Tris–HCl, pH 7.5, 0.5 mmol/L EGTA,
and 0.5% 2-mercaptoethanol), 5 μL ATP (500 μmol/L), and 2.25 μg inactive INK1 were added. The mixtures were incubated at 30°C for 15 minutes. A 5 μL aliquot was removed from the reaction mixture containing 10 μL of ATP-2 substrate peptide (2 mg/mL), 5 μL of the 5× kinase buffer, and 5 μL of [γ-32P]-ATP (0.16 μCi/μL) solution, and was incubated at 30°C for 15 minutes. Next, 20 μL aliquots were transferred to p81 filter paper and washed three times with 1% phosphoric acid for 5 minutes per wash and once with acetone for 5 minutes. Radioactive incorporation was determined using a scintillation counter (LS6500; Beckman Coulter). Each experiment was performed three times.

Pull-down assays

Recombinant PI3K (100 ng), MKK4 (200 ng), MKK7 (200 ng), or tumor lysates (500 μg) was incubated with IAA-conjugated Sepharose 4B (or Sepharose 4B as a negative control) beads (100 μL, 50% slurry) in an immunoprecipitation reaction buffer (50 mmol/L Tris–HCl, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% Nonidet P-40, and 0.02 mmol/L PMSF) containing 2 μg/mL bovine serum albumin and a 1× protease inhibitor mixture at 4°C overnight with gentle rocking. The beads were washed five times with immunoprecipitation reaction buffer and the protein-bound beads were analyzed by immunoblotting.

ATP and IAA competition assay

Briefly, 100 ng active PI3K or 200 ng active MKK4 and MKK7 were incubated overnight at 4°C with 0, 10, or 100 μmol/L ATP. Next, 100 μL IAA-Sepharose 4B or 100 μL Sepharose 4B beads were added and incubated overnight at 4°C. Then the samples were washed and the proteins were detected by Western blotting.

Xenograft model

SK-MEL-28 cells (5 × 10^6 cells/mouse) with 50% Matrigel (BD Bioscience) were injected into male Balb/c nu/nu mice and then randomly divided into three groups. On the basis of a previous study (27), vehicle or 2 or 10 mg/kg body weight of IAA were administered by intraperitoneal injection daily. Overall body weight was recorded every week and tumor volumes were measured with calipers and calculated using the formula: [π/6 (length) × (width) × (height)] until the end of the experiment (5 weeks).

Molecular modeling

Insight II (Accelrys Inc.) was used for the docking study and structure analysis with the crystal coordinates of PI3K, MKK4, or MKK7 in complex with ATP or IAA (accession codes 1E8X or 1E90) and are available in the Protein Data Bank (28).

Statistical analysis

When applicable, data are expressed as mean ± SD. The Student t test was used for single statistical comparisons. A probability value of P < 0.05 was used as the criterion for statistical significance.

Results

IAA is more effective than glycyrrhizin in attenuating growth of human melanoma cells

To study the effect of IAA on cancer cell growth, we used several human melanoma cell lines, including SK-MEL-2, 5, 28, and WM-266-4. IAA significantly suppressed the growth of all melanoma cells tested (Fig. 1A). We next examined the effect of IAA or glycyrrhizin on anchorage-independent growth of SK-MEL-28 cells and IAA significantly suppressed growth in a dose-dependent manner (Fig. 1B, a–c). Treatment with 20 μmol/L of IAA inhibited the growth of SK-MEL-28 cells up to 67% as compared with untreated control cells (Fig. 1B, b–d). However, glycyrrhizin had no effect on anchorage-independent growth at concentrations up to 20 μmol/L (Fig. 1B, d–f).

IAA suppresses proliferation and induces G1-phase cell-cycle arrest in SK-MEL-28 cells

Next, we examined the effect of IAA or glycyrrhizin on proliferation of SK-MEL-28 cells. Compared with glycyrrhizin, IAA more effectively inhibited proliferation in a dose- and time-dependent manner (Fig. 2A and B). On the basis of the growth response of SK-MEL-28 cells to IAA, we next examined its effect on cell-cycle progression and results revealed that IAA caused cell-cycle arrest at G1-phase (Fig. 2C), but treatment with glycyrrhizin had no effect on cell cycle (Fig. 2D). Because treatment with IAA did not affect the number of cells in sub-G1 (data not shown), IAA-induced growth inhibition was not attributable to apoptosis.

IAA decreases the abundance of G1-phase–related proteins mediated through the Akt/GSK-3β and MKK4/MKK7/JNKs signaling pathways

To determine the mechanism responsible for IAA-induced G1-phase cell-cycle arrest, we evaluated the expression levels of the various cyclins and CDKs involved in the G1→S phase progression. IAA inhibited the expression of cyclins D1 and E in SK-MEL-28 cells, whereas glycyrrhizin had no effect (data not shown). Because phosphorylation of Rb is crucial for S-phase transition and is the most recognized substrate of cyclin D–dependent kinases, we determined the effect of IAA or glycyrrhizin on Rb phosphorylation at Ser807/Ser811, the known sites of Rb phosphorylation by CDK4 (Fig. 3A). IAA substantially suppressed phosphorylation of Rb in a dose-dependent manner in SK-MEL-28 cells. Because the degradation of cyclin D1 is regulated by the PI3K/Akt/GSK-3β signaling cascade (24, 25), we examined the effect of IAA on the phosphorylation of Akt and GSK-3β (24). Results showed that IAA inhibited the phosphorylation of Akt (Ser473, Thr308) and GSK-3β (Ser9; Fig. 3B). In addition, IAA suppressed the phosphorylation of JNK1/2, but had no effect on ERK1/2 or p38 (Fig. 3C). Next, we examined the effect of IAA on the phosphorylation of MKK4 and MKK7, the upstream kinases of JNK1/2. IAA had no effect on MKK4 or MKK7 phosphorylation (Fig. 3D). These results suggested that an
upstream Akt kinase, PI3K, MKK4, or MKK7 might be potential targets of IAA.

**IAA suppresses PI3K, MKK4, and MKK7 kinase activities by directly binding in an ATP-competitive manner**

To investigate whether PI3K, MKK4, or MKK7 is a molecular target of IAA, we determined the effect of IAA on the in vitro kinase activities of these proteins. Treatment with IAA markedly suppressed PI3K activity in a dose-dependent manner (Fig. 4A). LY294002, a well-known commercially available inhibitor of PI3K, was used as a positive control. We also found that IAA strongly suppressed MKK4 activity (Fig. 4B) and attenuated MKK7 activity (Fig. 4C) in a dose-dependent fashion. These data indicate that PI3K, MKK4, and MKK7 are important molecular targets of IAA in the suppression of SK-MEL-28 cell proliferation. Next, we determined whether IAA directly interacts with PI3K, MKK4, or MKK7 to inhibit their respective kinase activity. Pull-down assay results indicated that PI3K, MKK4, or MKK7 precipitated with IAA-conjugated Sepharose 4B beads, but not with Sepharose 4B beads only (Fig. 4A–C). These results demonstrated that IAA directly binds to PI3K, MKK4, or MKK7. ATP treatment blocked the binding ability of IAA with each of these kinases in a dose-dependent manner, suggesting that IAA competes with ATP to bind to each kinase (Fig. 4A–C). These results suggest that inhibition of PI3K, MKK4, and MKK7 activities by IAA occurs through direct binding of IAA in an ATP-competitive manner.

**IAA suppresses xenograft growth of SK-MEL-28 cells**

Because IAA effectively suppressed the proliferation of SK-MEL-28 cells, we examined the effects of IAA in an in vivo xenograft mouse model. The average volume of tumors in vehicle-injected mice reached 1,312 mm³ at 5 weeks post-injection. However, at this time, the average tumor volume was only 814 or 541 mm³ in mice treated with 2 or 10 mg/kg IAA, respectively (Fig. 5A). At the end of the study, tumors from each group were removed and weighed. Treatment with IAA (2 or 10 mg/kg) significantly suppressed tumor weight compared with the control group (Supplementary Fig. S1). IAA treatment did not cause any loss in overall body weight, indicating that the dosages used were not toxic to the animals (Fig. 5B). Furthermore, IAA markedly inhibited the expression of proliferating cell nuclear antigen (PCNA), indicating that IAA could reduce proliferation of SK-MEL-28 cells and tumor growth in vivo (Fig. 5C). Tumor lysates from mice treated with 2 or 10 mg/kg of IAA showed decreased phosphorylation levels of Akt (Fig. 5C). Pull-down assays provided evidence that IAA could bind to the PI3K, MKK4, and MKK7 in SK-MEL-28 xenograft tumors (Fig. 5D). Collectively, these results suggest that IAA could serve as an effective anticancer agent with the potential to inhibit or delay the tumorigenicity of SK-MEL-28 cells in an in vivo system.

**Discussion**

Licorice is extensively cultivated in China, Korea, Japan, India, and Spain. Licorice root is one of the oldest and most frequently used botanicals and is prescribed as treatment in...
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oriental herbal medicine. Glycyrrhizin is considered as the main active compound of licorice (29–32) and is converted by human intestinal bacteria into glycyrrhetic acid (4). Previous studies showed that both glycyrrhizin and glycyrrhetic acid induce apoptosis and inhibit proliferation of melanoma cells (33, 34). Glycyrrhizin also has antimitotic effects on melanoma in vitro and in vivo (7). However, effective concentrations of glycyrrhizin are relatively high, and long-term or large quantities of glycyrrhizin consumption result in side effects such as mineralocorticoid excess, hypertension, and hypokalemia (8, 35). In addition to triterpenoids, about 300 polyphenols are in dried licorice root (35). In the current study, we determined that a novel polyphenol licorice compound, IAA, suppressed both anchorage-independent and -dependent growth of SK-MEL-28 cells better than glycyrrhizin. IAA also inhibited xenograft growth of SK-MEL-28 cells in mice.

Unregulated cell growth is a critical characteristic of cancer cells and a primary requirement for cancer progression (36). Abundant evidence indicates that cell-cycle deregulation plays a crucial role in several types of human cancer (17, 37). Cyclin D1 regulates CDK4 and CDK6 holoenzyme complexes, which phosphorylate and deactivate the tumor suppressor protein Rb. The CDK4 or 6/cyclin D and CDK2/cyclin E complexes regulate progression from G1 to S-phase of the cell cycle (25). We determined that treatment with IAA, but not glycyrrhizin, induced G1-phase arrest in SK-MEL-28 cells. IAA also inhibited G1-phase-related proteins, including cyclins D1 and E, whereas glycyrrhizin had no effect. Our data demonstrate that IAA suppresses proliferation by inducing G1-phase cell-cycle arrest.

PI3K and MAPKs pathways are involved in melanoma development by relaying extracellular signals to regulate diverse cellular processes, including proliferation, cell survival, invasion, and angiogenesis (38, 39). PI3K/Akt-dependent pathways regulate the expression of cyclin D1. Akt phosphorylates GSK-3β at Ser9, thereby decreasing GSK-3β activity and stabilizing cyclin D1 expression (25). Therefore, we determined whether IAA affected the PI3K/Akt/GSK-3β pathway and our data showed that IAA suppresses phosphorylation of both Akt and GSK-3β.

Figure 3. Effects of IAA on expression of G1-phase regulatory proteins and Akt and MAPK signaling pathways in SK-MEL-28 cells. A, IAA inhibits the expression of cyclins D1 and E, and Rb phosphorylation in SK-MEL-28 cells. Cells were treated 48 hours with IAA at the indicated concentrations. The protein level was determined by Western blot analysis using specific antibodies. Data are representative of three independent experiments. Numbers above each band indicate relative density normalized to β-actin (cyclin D1, cyclin E, Rb, CDK4, and CDK2) or total-Rb (p-Rb). B–D, effect of IAA on phosphorylation of Akt and GSK-3β (B), JNK1/2 (C), MKK4 and MKK7 (D) in SK-MEL-28 cells. IAA has no effect on the phosphorylation of ERK1/2 or p38 (C). Cells were treated with IAA (20 μmol/L) and harvested at the indicated times. The level of phosphorylated and total proteins was determined by Western blot analysis. Data are representative of three independent experiments. Numbers above each band show the relative normalized phosphorylation levels versus control at each time point.
We hypothesized that the molecular target of IAA for arresting G1-phase might be upstream kinases of Akt or JNK1/2. We found that IAA can effectively and directly bind to and inhibit PI3K, MKK4, and MKK7 activities, resulting in the attenuation of Akt/GSK-3β and JNK1/2 signaling. Furthermore, IAA competed with ATP for binding, which could explain the reduced activity of these kinases. Collectively, these results suggest that inhibition of cyclin D1 expression by IAA is primarily the result of the direct suppression of PI3K, MKK4, and MKK7 activities.
In light of our experimental finding showing that IAA inhibits the activities of PI3K, MKK4, and MKK7, we performed a molecular docking study to investigate the binding mode of IAA to these proteins. We allowed both the ligand molecule and the amino acids constituting the binding pocket to be flexible. Figure 6A (top) shows the docking conformation of IAA in the ATP-binding pocket of PI3K. The diagram of the PI3K interaction with IAA is shown in Fig. 6A (top). IAA forms hydrogen bonds with the backbone carbonyl oxygen of Ala885 and the side chain carboxylate oxygen of Asp964. It is further stabilized by favorable interactions with polar amino acids Thr887, Thr886, and Asn951, a positively charged residue (Lys833), and a negatively charged residue (Asp836). Lys833 interacts with the α-phosphate of ATP, and this residue is conserved in all PI3Ks. Our docking study also showed that the interaction of IAA with Lys833 helps stabilize the protein–ligand complex. The upper and lower regions, as well as the hydrocarbon chains of the ligand molecules, are stabilized by hydrophobic interactions with several hydrophobic amino acids, including Thr887, Ile881, Met953, Ile831, Ile863, Ile879, Tyr867, Phe965, Leu838, and Leu845. The hydrophobic pocket constituting these amino acids is shown in Fig. 6A (top). Our modeling study showed that IAA binds to the ATP-binding pocket of PI3K.

Figure 6A (middle) shows IAA docked to the ATP-binding pocket of MKK4. In the MKK4 protein, the ATP-binding pocket is located at the interface of the N- and C-terminal lobes of the enzyme. The drug molecule forms three hydrogen bonds with the enzyme. Two of the three are within the hinge region of MKK4 and involve the –CO backbone of Glu179 and the –NH backbone of Met181. The third hydrogen bond is with the side chain carboxylate oxygen of Asp186. As shown, Lys260 from the activation loop strongly interacts with one of the hydroxyl groups of IAA. Similarly, the polar amino acids Thr183 and Ser184 favorably interact with this compound. Similar to MKK7 and PI3K, the upper and lower regions of IAA are surrounded by several hydrophobic residues, adding stability to the protein–ligand complex. These results demonstrate that IAA can bind to MKK4 at the ATP-binding pocket.

Figure 6A (bottom) shows the docking model of IAA bound to the ATP-binding pocket of MKK7. The ATP-binding pocket is located at the interface of the N- and C-terminal lobes of MKK7. IAA forms two hydrogen bonds with the hinge region of MKK7, involving the –CO backbone of Glu179 and the –NH backbone of Met181. The third hydrogen bond is with the side chain carboxylate oxygen of Asp186. As shown, Lys260 from the activation loop strongly interacts with one of the hydroxyl groups of IAA. Similarly, the polar amino acids Thr183 and Ser184 favorably interact with this compound. Similar to MKK7 and PI3K, the upper and lower regions of IAA are surrounded by several hydrophobic residues, adding stability to the protein–ligand complex. These results demonstrate that IAA can bind to MKK7 at the ATP-binding pocket.

In light of our experimental finding showing that IAA inhibits the activities of PI3K, MKK4, and MKK7, we performed a molecular docking study to investigate the binding mode of IAA to these proteins. We allowed both the ligand molecule and the amino acids constituting the binding pocket to be flexible. Figure 6A (top) shows the docking conformation of IAA in the ATP-binding pocket of PI3K. The diagram of the PI3K interaction with IAA is shown in Fig. 6A (top). IAA forms hydrogen bonds with the backbone carbonyl oxygen of Ala885 and the side chain carboxylate oxygen of Asp964. It is further stabilized by favorable interactions with polar amino acids Thr887, Thr886, and Asn951, a positively charged residue (Lys833), and a negatively charged residue (Asp836). Lys833 interacts with the α-phosphate of ATP, and this residue is conserved in all PI3Ks. Our docking study also showed that the interaction of IAA with Lys833 helps stabilize the protein–ligand complex. The upper and lower regions, as well as the hydrocarbon chains of the ligand molecules, are stabilized by hydrophobic interactions with several hydrophobic amino acids, including Thr887, Ile881, Met953, Ile831, Ile863, Ile879, Tyr867, Phe965, Leu838, and Leu845. The hydrophobic pocket constituting these amino acids is shown in Fig. 6A (top). Our modeling study showed that IAA binds to the ATP-binding pocket of PI3K.

Figure 6A (middle) shows IAA docked to the ATP-binding pocket of MKK4. In the MKK4 protein, the ATP-binding pocket is located at the interface of the N- and C-terminal lobes of the enzyme. The drug molecule forms three hydrogen bonds with the enzyme. Two of the three are within the hinge region of MKK4 and involve the –CO backbone of Glu179 and the –NH backbone of Met181. The third hydrogen bond is with the side chain carboxylate oxygen of Asp186. As shown, Lys260 from the activation loop strongly interacts with one of the hydroxyl groups of IAA. Similarly, the polar amino acids Thr183 and Ser184 favorably interact with this compound. Similar to MKK7 and PI3K, the upper and lower regions of IAA are surrounded by several hydrophobic residues, adding stability to the protein–ligand complex. These results demonstrate that IAA can bind to MKK4 at the ATP-binding pocket.

Figure 6A (bottom) shows the docking model of IAA bound to the ATP-binding pocket of MKK7. The ATP-binding pocket is located at the interface of the N- and C-terminal lobes of MKK7. IAA forms two hydrogen bonds with the hinge region of MKK7, involving the –CO backbone of
Glu240 and the –NH backbone of Met 242, and a third hydrogen bond with the –CO backbone of Met169. The side chain functional groups of Lys248 and Glu168 interact favorably with the hydroxyl groups of IAA. The upper and lower regions of the ligand molecule are surrounded by a hydrophobic pocket constituting several hydrophobic residues. Our modeling results showed that IAA can bind to MKK7 at the ATP-binding pocket. Overall, these results demonstrate that IAA can bind to the ATP-binding pocket of PI3K, MKK4, and MKK7, which is consistent with the experimental observations.

A recently adopted strategy in anticancer therapeutics is the development of agents that target a variety of kinases. Two multitargeting kinase inhibitors, sunitinib and sorafenib, have been shown to be effective in clinical testing (40, 41). The rationale behind this strategy is that these agents will be more effective by suppressing multiple oncogenic pathways than the highly selective kinase inhibitors administered as single agents (42). In our study, we found that IAA was capable of targeting PI3K, MKK4, and MKK7. IAA markedly inhibits proliferation of human melanoma cells and showed no toxicity in Melan-A normal melanocytes, up to 20 μmol/L (Supplementary Fig. S1). In vivo xenograft results also showed that IAA effectively suppresses SK-MEL-28 tumor growth without loss of weight in mice (Fig. 5B). All of these results suggested that the potency of IAA against melanoma cells could be at least partially attributable to the fact that IAA reduces several proliferation signals rather than only one.

Overall, in the present study, IAA suppressed anchorage-independent and -dependent proliferation of human melanoma cells by substantially blocking cell-cycle progression at the G1-phase. IAA inhibited the expression of G1-phase–related proteins, including cyclins D1 and E, and also attenuated phosphorylation of Rb. IAA bound to PI3K, MKK4, and MKK7. The licorice compound suppressed their respective kinase activity, thereby blocking the Akt/GSK-3β and JNK1/2 signaling pathways, and subsequently reduced cyclin D1 expression. A simplified depiction of our proposed mechanism for the antiproliferative effects of IAA is shown in Fig. 6B. Collectively, these results suggest that PI3K, MKK4, and MKK7 are the primary molecular targets of IAA in the suppression of SK-MEL-28 melanoma cell proliferation. This insight into the biologic actions of IAA might provide a molecular basis for the development of new anticancer agents.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Development of methodology: N.R. Song, M. Montamal, K.W. Lee, Z. Dong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.R. Song, S. Byun, K.W. Lee, Z. Dong
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References
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