Licochalcone A, a Natural Inhibitor of c-Jun N-Terminal Kinase 1

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Abstract

The c-Jun N-terminal kinases (JNK) play an important role in many physiologic processes induced by numerous stress signals. Each JNK protein appears to have a distinct function in cancer, diabetes, or Parkinson’s disease. Herein, we found that licochalcone A, a major phenolic constituent isolated from licorice root, suppressed JNK1 activity but had little effect on JNK2 in vitro activity. Although licochalcone A binds with JIP1 competitively with either JNK1 or JNK2, a computer simulation model showed that after licochalcone A binding, the ATP-binding cleft of JNK1 was distorted more substantially than that of JNK2. This could reduce the affinity of JNK1 more than JNK2 for ATP binding. Furthermore, licochalcone A inhibited JNK1-mediated, but not JNK2-mediated, c-Jun phosphorylation in both ex vivo and in vitro systems. We also observed that in colon and pancreatic cancer cell lines, JNK1 is highly expressed compared with normal cell lines. In cancer cell lines, treatment with licochalcone A or knocking down JNK1 expression suppressed colon and pancreatic cancer cell proliferation and colony formation. The inhibition resulted in G1 phase arrest and apoptosis. Moreover, an in vivo xenograft mouse study showed that licochalcone A treatment effectively suppressed the growth of HCT116 xenografts, without affecting the body weight of mice. These results show that licochalcone A is a selective JNK1 inhibitor. Therefore, we suggest that because of the critical role of JNK1 in colon cancer and pancreatic carcinogenesis, licochalcone A might have preventive or therapeutic potential against these devastating diseases. Cancer Prev Res; 7(1); 1–11. ©2013 AACR.

Introduction

Mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPKs, function as regulatory effectors in cell proliferation, survival, and death. JNKs have been shown to phosphorylate c-Jun (Ser63/73) in response to ultraviolet (UV) irradiation (1–3). Activated JNKs phosphorylate numerous substrates, including transcription factors like activator protein-1 (AP-1), thereby affecting gene expression and subsequent cellular functions (4). Some kinases form signaling complexes mediated by scaffold proteins, such as JNK-interacting protein 1 (JIP1). JIP1 physically associates with JNKs, MKK7, and members of the mixed lineage kinases (MLK) to stimulate JNKs activity (5). Human small cell lung cell transformation by H-Ras requires JNK1 (6). JNK1 expression is increased in breast cancer (7). JNK1, not JNK2, is activated in colon cancer (8, 9). Enhanced JNK1 activation is associated with increased human hepatocellular carcinoma (HCC) tumor size and deficient tumor encapsulation (10). Disruption of JNK1 in mice causes defective transformation of pre-B cells by BCR-ABL (11). These studies suggest an oncogenic function of JNK1 in certain cancers. However, selective inhibitors for JNK1 have not identified.

Many anticancer drugs exert adverse side effects, which can be severe and life-threatening. Thus, identification of novel anticancer compounds from natural products might be a safer alternative and a promising strategy for cancer prevention or treatment. Many traditional herbal medicines and certain food constituents exhibit anti-inflammatory and antioxidative effects, suggesting the potential of herbal medicines as chemopreventive agents. Licorice root is a common source of licorice used in traditional and herbal medicines (12). The pharmacologic activities of licorice in treatment of human diseases such as cancer, gastric ulcers, bacterial infections, and immunodeficiency have gained a great deal of interest. Licochalcone A is a major phenolic constituent of licorice exhibiting antiproliferative and anti-inflammatory properties in human and murine cells (13). Despite its anticancer activity against human cancers, the
molecular mechanism by which licochalcone A exerts its effects is not understood. Identification of cellular targets associated with the suppression of malignancy will provide a better understanding of anticancer mechanisms. Therefore, the delineation of the molecular mechanism of licochalcone A requires further investigation.

Here, we observed that licochalcone A inhibited JNK1, but not JNK2, activity. Licochalcone A competed with JIP1 for binding with JNK1. Its binding distorted the ATP-binding cleft, which reduced the affinity of JNK1 for ATP. Licochalcone A suppressed JNK1-mediated c-Jun phosphorylation. JNK1 was highly expressed in colon and pancreatic cancer cells, and licochalcone A suppressed proliferation and anchorage-independent growth of these cancer cells. The specific targeting of JNK1 in cancer cells might provide opportunities for tumor therapy or prevention based on the use of small molecules that inhibit JNK1.

Materials and Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and FBS were from Life Technologies, Inc. Licochalcone A and antibodies against JNK1 and p21 were from Santa Cruz Biotechnology, Inc. Antibodies against JNK2, c-Jun, p-c-Jun, and p53 and 10× kinase buffer were from Cell Signaling Technology, Inc. Active JNK1 and JNK2 were from Upstate Biotechnology, Inc. Licochalcone A was synthesized and purchased from Calbiochem (EMD Millipore, CAS 58749-96-0). Reagents were from Schrödinger, Inc. Antibodies against JNK1 and p21 were purchased from Cell Signaling Technology, Inc. Antibodies against JNK2, c-Jun, p-c-Jun, and p53 were from Santa Cruz Biotechnology, Inc. Active JNK1 and JNK2 were from Upstate Biotechnology, Inc. Licochalcone A was synthesized and purchased from Calbiochem (EMD Millipore, CAS 58749-96-0) and the purity was >96%.

Kinase assay

His-c-Jun was used for an in vitro kinase assay with active JNK1 and JNK2. Reactions were conducted at 30°C for 30 minutes in a mixture containing 20 ng active kinase, 2 µg His-c-Jun protein, 50 µmol/L unlabeled ATP, and 10 µCi [γ-32P] ATP. Reactions were stopped with 6× SDS sample buffer. Samples were boiled, separated by 12% SDS-PAGE, and visualized by autoradiography.

Cell culture

JNK1−/−, JNK2−/−, or JNK2−/− mouse embryonic fibroblasts (MEF) or Panc1 pancreatic cancer cells were cultured in DMEM with 10% FBS. HCT116 colon cancer or SK-BR-3 breast cancer cells were cultured in McCoy’s 5A Medium with 10% FBS. HepG2 liver cancer cells were cultured in minimum essential medium (MEM) and 10% heat-inactivated FBS. HEK 293 cells are a human kidney epithelial cell line with high transfection ability and were cultured in DMEM with 10% FBS. Cells were cytogenetically tested and authenticated before freezing. Each vial of frozen cells was thawed and maintained for a maximum of 8 weeks.

Pull-down assays

Licochalcone A (2.5 mg) was coupled to CNBr-activated Sepharose 4B (GE Healthcare Biosciences) matrix beads (0.5 g) in 0.5 mol/L NaCl and 40% dimethyl sulfoxide (DMSO; pH 8.3) overnight at 4°C, according to the manufacturer’s instructions. Active JNK1 and JNK2 or JNKwt, JNK1−/−, and JNK2−/− cell lysates (500 µg) were mixed with licochalcone A–conjugated Sepharose 4B beads or with Sepharose 4B beads alone as control (30 µL, 50% suspension). Binding was determined by Western blot analysis.

Western blotting and immunoprecipitation

JNKwt, JNK1−/−, or JNK2−/− MEFs (5 × 105) were cultured in 10-cm dishes and then starved in serum-free medium for 24 hours. The cells were treated for 1 hour with various concentrations of licochalcone A (0, 5, 10, or 25 µmol/L) dissolved in serum-free media before exposure to UVB (4 kJ/m²). The licochalcone A stock solution was in DMSO and the final concentration of DMSO in the media was less than 0.1%. Cells were harvested and proteins extracted in an NP-40 lysis buffer. Protein concentration for Western blotting was measured using a protein assay kit (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride membranes and hybridized with appropriate antibodies. Anti-V5 (JNK1) or anti-Xpress (JNK2) was used for immunoprecipitation of HEK 293 cell lysates (1 mg). Antibody binding was conducted at 4°C overnight and proteins were visualized by Western blotting using antibody (JIP1).

Molecular modeling

Modeling of licochalcone A with JNK1 and JNK2 was conducted using Schrödinger Suite 2011 software programs (14). X-ray diffraction structures of JNK1 (PDB ID:3ELJ; ref. 15) and JNK2 (PDB ID:3NPC; ref. 16) were obtained from the Protein Data Bank (17). Structures were prepared with the Protein Preparation Wizard (Schrödinger). Hydrogen atoms were added and water molecules removed. A JIP1-binding site–based receptor grid was generated for docking with each JNK. Licochalcone A was prepared for docking by default parameters using LigPrep (Schrödinger). Licochalcone A protein docking was accomplished using Glide and default parameters under the extra precision (XP) mode to obtain the best-docked representative structure.

Energy minimization and molecular dynamics simulation

To study JNK1 and JNK2 crystal structure distortion after licochalcone A binding at the JIP1-binding sites, a loop energy minimization and dynamics simulation were conducted in 100 ps by default parameters using the Impact software program from Schrödinger. Each crystal structure before and after MD was aligned and compared.

MTS assay

HCT116, Panc1, SK-BR-3, or HepG2 cells were seeded (1 × 103) into 96-well plates, and then treated with different doses of licochalcone A. After incubation for 24, 48, 72, or 96 hours, CellTiter 96 Aqueous One Solution (20 µL; Promega) was added and cells incubated for 1 hour in a 37°C, 5% CO₂ incubator. Absorbance was read at 492 and 690 nm.
Anchorage-independent cell growth assay

Cells (8 × 10^4 per well) were suspended in 1 mL BME, 10% FBS, and 0.33% agar and plated with various concentrations of licochalcone A on 3 mL of solidified BME containing 10% FBS and 0.5% agar with different concentrations of licochalcone A for 10 days. Colony number was determined by microscope and Image-Pro Plus software (Media Cybernetics, Inc.).

Animal study

JNK<sup>wt</sup>, JNK1<sup>−/−</sup>, or JNK2<sup>−/−</sup> mice (6–8 weeks) each were divided into 3 age-matched groups: vehicle group (n = 5), vehicle/SUV group (n = 5), 1 mg licochalcone A/SUV group (n = 5; Supplementary Table S1). In the vehicle group, acetone (150 µL) was topically applied to mouse dorsal skin with no exposure to SUV. In the vehicle/SUV group, the dorsal skin was topically treated with acetone (150 µL) before SUV irradiation. The mice in the 1 mg licochalcone A/SUV groups received topical application of licochalcone A (1.0 mg) in 150 µL of acetone before SUV irradiation. Mice were irradiated with one dose of solar UV light (142 kJ/m²) and dorsal trunk skin samples were harvested at 24 hours after irradiation and frozen for Western blot analysis.

Cell-cycle and apoptosis analyses

Cells were plated in 60-mm dishes and treated with vehicle or licochalcone A for 48 hours. Cells were fixed in 70% ethanol at 20°C for 24 hours. After staining, cell-cycle distribution or apoptosis was determined by FACScalibur (BD Biosciences).

Reporter gene activity

Cells (6 × 10<sup>4</sup> per well) were seeded into 12-well dishes. Cells were transfected with the AP-1 reporter plasmid (600 ng) and an internal control (Renilla, 10 ng) and incubated for 24 hours. Cells were treated with vehicle or licochalcone A for 12 hours. Firefly and Renilla luciferase activities were measured using substrates provided in the reporter assay system (Promega). Luciferase activity was normalized to Renilla luciferase activity.

Xenograft mouse model

Athymic nude mice [Cr:NIH (S), NIH Swiss nude, 6-week-old] were from Harlan Laboratories and maintained under "specific pathogen-free" conditions based on guidelines established by the University of Minnesota Institutional Animal Care and Use Committee. Mice were divided into groups (n = 10/group). HCT116 colon cancer cells (1.5 × 10<sup>6</sup>/0.1 mL) were injected subcutaneously into the right flank of each mouse. Licochalcone A was freshly prepared every time and protected from light before injection. The first group (10 mice) received 100 µL vehicle only (2% DMSO and 5% Tween-20 in PBS) intraperitoneally (i.p.) 3 times a week for 4 consecutive weeks. The other 2 groups (10 mice per group) were given 100 µL of licochalcone A (dissolved in 2% DMSO and 5% Tween-20 in PBS) i.p. 3 times a week at a dose of 10 or 20 mg/kg body weight (B.W.) for 4 consecutive weeks. Tumor volume (length × width × depth × 0.52) was measured 3x week. Body weights were recorded every week. At the end of the study, xenograft tumors were weighed and frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin.
Immunohistochemistry staining

Tumor tissues were embedded in paraffin and subjected to immunohistochemistry. Tissues were deparaffinized and hydrated then permeabilized with 0.5% Triton X-100/C2 PBS for 10 minutes. Tissues were hybridized with Ki-67 (1:200) or p-c-Jun (1:100) as the primary antibody and biotinylated goat anti-rabbit IgG as the secondary antibody. An ABC kit (Vector Laboratories, Inc.) was used to detect protein targets according to the manufacturer’s instructions. After developing with 3,3'-diaminobenzidine, the sections were counterstained with hematoxylin and observed by microscope (200×) and using the Image-Pro Plus software (v. 6.1) program (Media Cybernetics).

Statistical analysis

All quantitative data are expressed as mean values ±SD or ±SE, and significant differences were determined by Student t test, factorial ANOVA (Scheffe post hoc test) or 2-way ANOVA as indicated. P < 0.05 was used as the criterion for statistical significance.

Results

Licochalcone A binds with JNK1 or JNK2

Licochalcone A reportedly inhibits AP-1 activity (18). However, the molecular mechanism explaining the inhibitory effect of licochalcone A has not been elucidated. c-Jun is the most well-recognized JNK substrate and JNK might be
potential target protein of licochalcone A (Fig. 1A). To examine this idea, we conjugated licochalcone A with CNBr-Sepharose 4B beads and conducted a pull-down assay using MEFs isolated from JNK\(^{wt}\), JNK\(^{1/-}\) or JNK\(^{2/-}\) embryos (E12.5). Results revealed that licochalcone A binds with JNK1 or JNK2 in all 3 MEF types (Fig. 1B). We also observed that licochalcone A does not bind with p38 or ERKs in JNK\(^{wt}\) MEFs (Supplementary Fig. S1A). We confirmed that active JNK1 or JNK2 (100 ng) binds with licochalcone A-Sepharose 4B beads, but not with Sepharose 4B beads alone (Fig. 1C). Furthermore, we conducted a pull-down assay with JNK1 protein fragments. Results indicated that the each truncated JNK1 protein bound with licochalcone A (Supplementary Fig. S1B).

**Licochalcone A inhibits JNK1, but not JNK2, activity**

Next, we determined whether licochalcone A affects the kinase activity of JNK by conducting in vitro kinase assays with active JNK1 and JNK2, His-c-Jun as substrate, and ATP. JNK1 activity was suppressed about 55% by licochalcone A (Fig. 2A). Unexpectedly, licochalcone A had only a small (7%) inhibitory effect on JNK2 activity (Fig. 2B). These results indicate that licochalcone A might be a selective inhibitor of JNK1 with little effect on JNK2. We confirmed that JNK1 activity was inhibited in a dose-dependent manner by licochalcone A (Fig. 2C).

JNK inhibitors are reportedly classified as binding to the JIP site, ATP active site, or both sites (20, 21). The crystal structure of JNK1 complex with pepJIP1 showed that pep-JIP1 distorts the ATP-binding cleft, reducing the affinity of this kinase for ATP (22). This study provided us with a basis for determining the specificity of licochalcone A for JNK1 or JNK2. We co-transfected JIP1 and pcDNA5-V5-JNK1 or pcDNA4-HA-JNK2 plasmids into HEK 293 cells and incubated the cells for 24 hours with or without licochalcone A. The interaction between JNK1 and JIP1 was significantly displaced by co-incubation with licochalcone A (Fig. 3A). However, the JNK2/JIP1 interaction was only slightly changed with licochalcone A (Fig. 3B).

To understand the interaction of licochalcone A with JNK1 and JNK2, we constructed a computational docking model.
Docked models showed that licochalcone A binds at the JIP1-binding pocket of either JNK1 or JNK2 (Supplementary Fig. S2A and S2B). Then we conducted an energy minimization and molecular dynamics (MD) simulation. The MD results indicated that the ATP-binding pocket of JNK1 was distorted more dramatically than that of JNK2 (Supplementary Video S1 and S2) when binding licochalcone. We then compared the changes in the distance of specific residues around the ATP-binding pocket of JNK1 and JNK2. Results indicated that after licochalcone A binding, the ATP pocket of JNK was more dramatically distorted compared with JNK2 (Supplementary Video S1 and S2). Overall, computational simulation results show that licochalcone A binds at the JIP1-binding pocket of either JNK1 or JNK2. However, the binding of licochalcone A with JNK1 reduces the affinity of JNK1 for ATP more than it affects the affinity of JNK2 for ATP.

Licochalcone A inhibits JNK1-, not JNK2-mediated, c-Jun phosphorylation

JNK1 and JNK2 are activated in response to UV, γ-irradiation, and osmotic shock. JNKs are best known for their role in the activation of the c-Jun/AP-1 transcription factor complex. JNKwt, JNK1−/−, and JNK2−/− MEFs were used as a model to examine the selective specificity of licochalcone A. These MEFs were exposed to UVB (4 kJ/m2) and UVB induced increased phosphorylation of c-Jun (Ser73) at 15 to 120 minutes after UV exposure in each cell type (Supplementary Fig. S3). Each respective MEF type was treated with licochalcone A at various doses for 30 minutes followed by treatment with UVB (4 kJ/m2) in the same medium containing licochalcone A. We found that both c-Jun phosphorylation and total c-Jun protein levels induced by UVB were suppressed by licochalcone A in a dose-dependent manner in JNK2−/− MEFs, which only express JNK1 (Fig. 4A, right). Both c-Jun phosphorylation and total c-Jun protein expression induced by UVB stimulation were partially suppressed by treatment with licochalcone A (25 μmol/L) in JNKwt MEFs, which express both JNK1 and JNK2 (Fig. 4A, left). However, neither UV-induced c-Jun phosphorylation or total c-Jun protein level were affected by licochalcone A in JNK1−/− MEFs, which only express JNK2 (Fig. 4A, left).

Figure 4. Licochalcone A inhibits JNK1-mediated, but not JNK2-mediated, c-Jun phosphorylation.

A, licochalcone A inhibits c-Jun phosphorylation in JNKwt and JNK2−/− MEFs. MEFs were treated with different doses of licochalcone A and stimulated with UVB (4 kJ/m2). Cells were harvested at the indicated times, and protein levels of phosphorylated c-Jun and total c-Jun were visualized by Western blotting. β-Actin verified equal protein loading. B, effect of licochalcone A on solar UV (SUV)-induced c-Jun phosphorylation in JNKwt, JNK1−/−, and JNK2−/− mouse skin tissue. Representative protein samples from each group harvested at the same time point were analyzed by Western blotting to detect phosphorylated c-Jun and total c-Jun. β-Actin verified equal protein loading. For A and B, each assay was conducted 3 times and similar results were obtained. Representative Western blot analyses are shown, and data were quantified using ImageJ density analysis. Significant differences were evaluated using factorial ANOVA (with Scheffe F post hoc test), and the asterisks indicate a significant decrease in c-Jun phosphorylation (*, P < 0.01).
middle). These results showed that licochalcone A specifically inhibits JNK1-mediated c-Jun phosphorylation.

To further explore the inhibitory effect of licochalcone A, adult JNK\(^{\text{wt}}\), JNK1\(^{-/-}\) and JNK2\(^{-/-}\) mice were irradiated with one dose of solar UV light (142 kl/m\(^2\)) with or without licochalcone A treatment and dorsal skin samples were harvested 24 hours later. We examined protein samples from mouse skin by Western blotting. Both c-Jun phosphorylation and total c-Jun protein levels were upregulated by solar UV in JNK\(^{\text{wt}}\), JNK1\(^{-/-}\), and JNK2\(^{-/-}\) mice compared with untreated mice (Fig. 4B). In mice treated with licochalcone A, both UV-induced c-Jun (Ser73) phosphorylation and total c-Jun level were decreased in JNK2\(^{-/-}\) mice, but the decrease was much less in JNK1\(^{-/-}\) mice.
Collectively, these results suggest that licochalcone A protects mouse skin from solar UV light–induced c-Jun activation by inhibiting JNK1 signaling.

Licochalcone A suppresses colon and pancreatic cancer cell proliferation and colony formation mediated through JNK1

In the ApcMin mouse model of intestinal cancer, phosphorylation of c-Jun by JNKs was required for cancer development (9). This study suggests that JNKs are oncogenic. To investigate whether JNK1 and JNK2 are involved in human carcinogenesis, we examined JNK1 and JNK2 protein abundance by Western blotting in several different human colon and pancreatic cancer lines compared with a normal cell line. Results indicated higher JNK1 protein levels in all of the colon cancer cell lines, including colorectal adenocarcinoma cells and colorectal carcinoma cells. In contrast, JNK2 expression level was similar in all the colon cell lines, including normal cells (Fig. 5A, left). In addition, JNK1 protein levels were consistently higher in pancreatic adenocarcinoma cells compared with normal pancreatic cells. JNK2 expression levels were relatively low (Fig. 5A, right). Thus, JNK1 may play a more important role than JNK2 in colon and pancreatic carcinogenesis. We evaluated the effect of licochalcone A on proliferation of HCT116 colorectal cancer and Panc1 pancreatic cancer cells, which highly express JNK1. Data indicate that licochalcone A treatment significantly inhibits HCT116 or Panc1 cell growth time and dose dependently (Fig. 5B). We also examined the effect of licochalcone A on anchorage-independent growth of HCT116 and Panc1 cells. Data showed licochalcone A treated HCT116 or Panc1 cells showed impaired anchorage-independent growth, leading to a significant dose-dependent reduction in colony formation (Fig. 5C). To further confirm the role of JNK1 in colon carcinogenesis, we used lentivirus to establish stable HCT116 or Panc1 cells expressing sh-mock or sh-JNK1. After JNK1 knockdown, colony formation on soft agar was significantly decreased (Fig. 5D). Similar results were also observed in SK-BR-3 breast cancer and HepG2 liver cancer cells (Supplementary Fig. S4A–S4C). These data confirmed that the inhibition of JNK1 by licochalcone A could suppress colon, pancreatic, breast, or liver tumorigenesis.

Figure 6. Licochalcone A induces G1 phase cell-cycle arrest and apoptosis in HCT116 colon cancer cells. A, licochalcone A inhibits AP-1 luciferase activity in HCT116 colon cancer cells. HCT116 cells were transiently transfected with the AP-1 luciferase reporter gene construct and incubated with licochalcone A (0, 5, 10, or 25 μmol/L). Luciferase activity was measured as described in Materials and Methods. B, licochalcone A increases p53 and p21 expression in HCT116 cells. HCT116 cells were treated with different doses of licochalcone A for 6 hours. Protein levels of p53 and p21 were visualized by Western blotting. β-Actin was used to verify equal protein loading. C, licochalcone A induces G1 arrest in HCT116 colon cancer cells. Cell cycle was analyzed by flow cytometry. D, licochalcone A induces apoptosis in HCT116 colon cancer cells as assessed by flow cytometry. HCT116 cells were incubated with the indicated concentration of licochalcone A for 48 hours and then collected, and apoptosis was detected by Annexin V and PI staining. For A, C, and D, data are shown as mean values ± S.D. obtained from triplicate experiments. Significant differences were evaluated using factorial ANOVA (Scheffe post hoc test), and the asterisks indicate a significant effect (*, P < 0.01).
Licochalcone A induces cell-cycle arrest and apoptosis in HCT116 colon cancer cells

c-Jun represses p53 and p21 expression and deletion of p53 abrogates defects of cells lacking c-Jun (23–25). Licochalcone A inhibits JNK1 activity and c-Jun phosphorylation. We conducted luciferase reporter gene assays in licochalcone A–treated HCT116 cells and found that licochalcone A significantly inhibited the transcriptional activity of AP-1 dose dependently (Fig. 6A). Western blotting results indicated that treatment with licochalcone A induced p53 and p21 expression in HCT116 colon cancer cells (Fig. 6B). The ability to induce G1 cell cycle arrest was examined in HCT116 cells treated or not treated with licochalcone A (Fig. 6C). Exposure of these cells to licochalcone A for 48 hours induced apoptosis as shown by Annexin V/propidium iodide (PI) staining (Fig. 6D). These results showed that licochalcone A induces G1 phase arrest and apoptosis in cancer cells by downregulating c-Jun activity mediated by JNK1.

Licochalcone A suppresses growth of HCT116 xenografts in vivo

We evaluated the effect of licochalcone A on growth of HCT116 colon cancer cell xenografts. Licochalcone A reduced tumor size in the HCT116 colon cancer cell xenograft model dose dependently (Fig. 7A), and no obvious loss of body weight was observed (Fig. 7B), indicating that licochalcone A was well tolerated. Moreover, immunohistochemical analysis of tumors showed that the expression of Ki-67, a cell proliferation marker, and phosphorylation of c-Jun were markedly decreased in licochalcone A–treated tumors compared with controls (Fig. 7C).

Discussion

Licorice root is one of the most commonly used medicinal plants in traditional Chinese medicine against inflammation. Licorice can be taken as the dried root at a level of 1 to 5 g 3 times per day. Others have reported that 50 g of licorice daily can cause a significant rise in blood pressure.
Licochalcone A is found in relatively large amounts in licorice root. Licochalcone A reportedly reduced cell viability at concentrations >20 μg/ml (26). For our cell studies, we used 25 μmol/l (8.46 μg/ml) as the highest dose, and for our animal study, we used 20 mg/kg as the highest dose. The results showed no obvious loss of body weight, indicating that licochalcone A was well-tolerated (Fig. 7B). Licochalcone A inhibits the production of inflammation-associated mediators by suppressing AP-1 signaling (18). Moreover, licochalcone A protected BALB/c mice from endotoxin shock by inhibiting the production of inflammatory cytokines (27). In addition, studies showed that licochalcone A exerts potent antitumor activity against prostate, breast, and leukemia cells. These attributes suggest that licochalcone A might have therapeutic or preventive potential against malignancies. However, the mechanism and direct targets of licochalcone A have not been elucidated. In this study, we report that licochalcone A directly binds with JNK1 and JNK2 (Fig. 1B) but selectively inhibits only JNK1 kinase activity (Fig. 2A).

The crystal structure of the JNK1 complex with JIP1 revealed the selectivity of licochalcone A for JNK1 over other MAPKs and an allosteric inhibitory mechanism. The JNK1/pepJIP1 complex structure showed that after pepJIP1 binding, the affinity of JNK1 for ATP was reduced by extensive allosteric changes (22). Computational simulation results showed that licochalcone A competes with JIP1 for binding with JNK1. The interaction distorts the ATP binding cleft, which reduces the affinity of JNK1, but not JNK2, for ATP (Fig. 3C). With the aid of supercomputers, the MD simulation could be produced easily (28) and provided more evidence explaining our results.

Evidence suggests that JNK1, not JNK2, plays an important role in the malignant transformation of cells and in tumorigenesis, including lymphoma, lung cancer, breast cancers, and HCCs (11). Mice that are deficient in both Jnk1 and jnk2 exhibit embryonic death at E10.5 (29). Highly selective JNK1 inhibitors could help avoid unwanted side effects and contribute to JNK1-related cancer prevention and therapy. Colorectal cancer, pancreatic cancer, and liver and breast cancers are significant public health problems and the most common cancers worldwide (30). Epidemiologic and biologic data show a clear association between chronic inflammatory conditions and subsequent malignant transformation (31). Colorectal cancer is an inflammatory bowel disease (32), and several studies showed that JNK signaling is critical for pancreatic cancer development (33, 34). Recently, many dietary compounds have been recognized as potential anticancer agents. We examined the effect of licochalcone A on JNK1, which is highly expressed in HCT116 colon cancer and Panc1 pancreatic cancer cells (Fig. 5A). Results showed that licochalcone A suppressed cell growth and colony formation (Fig. 5B and C). Meanwhile, knocking down JNK1 expression in HCT116 and Panc1 cells decreased colony formation (Fig. 5D), indicating that JNK1 plays an important role in the antitumor activity of licochalcone A. Similar results were also observed in human breast and liver cancer cells (Supplementary Fig. S4A–S4C). Moreover, inhibition was associated with increased apoptosis, as well as G1 phase arrest, which is consistent with p53 and p21 up-regulation (Fig. 6B–D). A xenograft study also indicated that licochalcone A effectively suppressed tumor growth without affecting mouse body weight and was accompanied by a decrease in Ki-67 expression (Fig. 7), a marker of proliferation, and also with decreased phosphorylation of c-Jun in tumor tissues. Overall results identified licochalcone A as a chemopreventive and chemotherapeutic agent against colon and pancreatic cancer and suggested that JNK1 is its selective target. Therefore, because of the critical role of JNK1 in certain types of cancer cell growth, licochalcone A might have therapeutic or preventive potential against this devastating disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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