Caffeic Acid Directly Targets ERK1/2 to Attenuate Solar UV-Induced Skin Carcinogenesis

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Abstract
Caffeic acid (3,4-dihydroxy cinnamic acid) is a well-known phenolic phytochemical present in coffee and reportedly has anticancer activities. However, the underlying molecular mechanisms and targeted proteins involved in the suppression of carcinogenesis by caffeic acid are not fully understood. In this study, we report that caffeic acid significantly inhibits colony formation of human skin cancer cells and EGF-induced neoplastic transformation of HaCAt cells dose-dependently. Caffeic acid topically applied to dorsal mouse skin significantly suppressed tumor incidence and volume in a solar UV (SUV)-induced skin carcinogenesis mouse model. A substantial reduction of phosphorylation in mitogen-activated protein kinase signaling was observed in mice treated with caffeic acid either before or after SUV exposure. Caffeic acid directly interacted with ERK1/2 and inhibited ERK1/2 activities in vitro. Importantly, we resolved the cocrystal structure of ERK2 complexed with caffeic acid. Caffeic acid interacted directly with ERK2 at amino acid residues Q105, D106, and M108. Moreover, A431 cells expressing knockdown of ERK2 lost sensitivity to caffeic acid in a skin cancer xenograft mouse model. Taken together, our results suggest that caffeic acid exerts chemopreventive activity against SUV-induced skin carcinogenesis by targeting ERK1 and 2. Cancer Prev Res; 7(10); 1056–66. ©2014 AACR.

Introduction
Coffee is among the most widely consumed beverages in the world. More than 50% of Americans drink coffee daily. The consumption of around 4 cups of coffee a day is associated with a 38% lower risk of breast cancer in premenopausal women (1). Consumption of at least 5 cups of coffee a day is associated with a 40% lower risk of brain tumors in humans compared with non-coffee drinkers (2). Recent results also show that daily consumption of 6 or more cups of coffee is associated with a 30% reduced prevalence of nonmelanoma skin cancer (3). Skin cancer is the most common cancer in the United States with more than 1 million new cases reported each year, comprising approximately 40% of all diagnosed cancers (4). Epidemiologic evidence suggests that solar ultraviolet (SUV; i.e., sunlight) irradiation is the most important risk factor for skin carcinogenesis (5, 6).

Caffeic acid (3,4-dihydroxycinnamic acid) is the major phenolic compound naturally found in coffee. A 200 mL cup of coffee provides about 35 to 175 mg of caffeic acid. Caffeic acid reportedly exerts a broad spectrum of pharmacologic activities, including anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective activities (7–9). Recent studies suggest that caffeic acid has anticancer effects against human renal carcinoma (10) and colon cancer metastasis (11). However, the underlying molecular mechanisms and targeted proteins involved in the suppression of carcinogenesis by caffeic acid are not fully understood.

The mitogen-activated protein kinase (MAPK) pathway encompasses different signaling cascades of which the Ras–Raf–MEK–extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway is one of the most commonly deregulated in human cancer. This pathway mediates multiple cellular functions, including proliferation, growth, and senescence (12). Abnormalities in ERKs signaling were reported in approximately 1 of 3 of all human cancers, including lung, liver, and melanoma (13). SUV irradiation activates ERKs in human skin (14) and compared with actinic keratosis, squamous cell carcinomas show higher ERKs phosphorylation (15). Hyperactivation of ERKs
results in deregulated cell proliferation in several human cancers, including skin cancer (16, 17), suggesting that inhibition of ERKs signaling represents a potential approach for SIV-induced skin cancer prevention.

Here, we report the cocrystal structure of caffeic acid with ERK2 and show that caffeic acid directly inhibited SIV-induced activation of ERK1/2 and suppressed ERKs signaling. Treatment of dorsal mouse skin with caffeic acid either before or after SIV exposure strongly reduced skin tumor number and size in mice.

Materials and Methods

Reagents and antibodies

Caffeic acid was purchased from Sigma-Aldrich. Active ERK1 and 2 human recombinant proteins for kinase assays were from Millipore. Antibodies to detect total ERK1 and 2, phosphorylated Elk1 (Ser383), total Elk1, and phosphorylated p90RSK (Thr359/Thr363) were obtained from Cell Signaling Technology. Antibodies against phosphorylated ERK1 and 2 (Thr202/Tyr204), total p90RSK, phosphorylated c-Myc (Thr58/Ser62), total c-Myc and β-actin were from Santa Cruz Biotechnology. The antibody to detect Ki-67 was purchased from Thermo Scientific. CNBr-Sepharose 4B beads were from GE Healthcare.

SUV irradiation system

The SUV resource was comprised of UVA-340 lamps (Q-Lab Corporation), which provide the best possible simulation of sunlight in the critical short wavelength region from 365 nm down to the solar cutoff of 295 nm with a peak emission of 340 nm. The percentage of UVA and UVB of the UVA-340 lamps was measured by a LIV meter as 94.3% and 5.5%, respectively.

Cell culture and transfection

A431 skin cancer cells and HaCat keratinocytes were cultured with DMEM supplemented with 10% FBS and antibiotics. SK-MEL-5 and SK-MEL-28 melanoma cells were cultured with minimum essential medium (MEM) supplemented with 10% FBS and antibiotics. Cells were cytogenetically tested and authenticated before being frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Cells were kept at 37°C in a 5% CO2 incubator. Cells were cultured at 37°C in a 5% CO2 incubator. For transfection experiments, the jetPEI (Qiagen, Inc.) transfection reagent was used following the manufacturer’s instructions.

Primary mouse keratinocyte harvest and culture

SKH1 hairless mice were euthanized and the dorsal skin was removed. All subcutaneous tissue was scraped from the skin until the skin was semitranslucent. The skin (hairy side up) was spread out on a dish and cut into 0.5-× 1-cm strips. Trypsin without EDTA (20 mL, 0.25%) was added to the dish, and the dish was placed in a 37°C incubator for 1.5 hours. DMEM/10% FBS (20 mL) is added and the epidermis scraped off into the medium. The medium was collected and stirred at 100 rpm on a magnetic stirrer for 20 minutes. The medium is filtered through a sterile 70 μm Teflon mesh and the filtrate centrifuged. The keratinocyte growth medium was added to resuspend the cells and cells subsequently maintained at 37°C in a 5% CO2 incubator.

MTS assay

To estimate cytotoxicity, cells were seeded (8 × 10^3 cells/well) in 96-well plates and cultured overnight. Cells were then fed with fresh medium and treated with different doses of caffeic acid. After culturing for various times, the cells were harvested and cytotoxicity of caffeic acid was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazadium) assay kit (Promega) according to the manufacturer’s instructions.

Soft agar assay

Cells (8 × 10^3/mL) were suspended in 1 mL of 0.3% basal medium eagle (BME) top agar containing 10% FBS with various concentrations of caffeic acid and placed over a lower layer of solidified BME, 10% FBS, and 0.5% agar (3 mL) with the same concentrations of caffeic acid. The cultures were maintained at 37°C in a 5% CO2 incubator for 1 to 2 weeks and then colonies were counted under a microscope using the Image-Pro Plus software (v.6) program (Media Cybernetics).

In silico target identification

The PHASE module of the Schrödinger molecular modeling software package (18) is a shape similarity method (19) and was used to search for biologic targets of caffeic acid on the basis of its distinctive structure. The parameter of atom-type for volume was set to MacroModel, which means overlapping volumes are computed only between atoms that have the same MacroModel atom type. The target database was obtained from the Protein Data Bank and our in-house chemical library. To provide more structure orientations for possible alignment, we set the maximum number of conformers per molecule in the library to be generated at 100 while retaining up to 10 conformers per rotatable bond. We filtered out conformers with a similarity score below 0.70. Finally, we obtained a final list of compounds with reported protein targets with a shape similarity score higher than 0.70.

ERK2 purification and crystallization

Purification of the full-length (residues 1-360) human ERK2 was performed as described previously (20). Briefly, a His-tagged ERK2 protein was purified on HisPur Ni-NTA resin (Thermo Scientific); the His-tag removed by incubation with thrombin; and the protein was repurified by FPLC on a Superdex 200 column. Untagged ERK2 was crystallized in a sitting drops plate by mixing protein with precipitant solution comprised of 1.1 to 1.4 mol/L ammonium sulfate, 2% PEG 500 MME, and 0.1 mol/L HEPES-NaOH (pH 7.5). The ERK2 crystals were soaked in precipitant solution containing 2.5 mmol/L caffeic acid in 2.5% dimethyl sulfoxide (DMSO) for 1 to 2 days. The crystals were cryoprotected in precipitant solution (1.8 mol/L ammonium sulfate) with
the addition of 20% xylitol, and flash-cooled in liquid nitrogen. The ERK2 mutant, Q105A, was purified, crystallized, and soaked with caffeic acid in a manner similar to wild-type (WT) ERK2. ERK2 was also cocryystallized with 2 mmol/L AMP-PNP and 5 mmol/L MgCl₂ under similar crystallization conditions. Despite all attempts, electron density was clearly visible only for the adenosine moiety.

ERK2/coffeic acid cocystal structure determination

The high-resolution diffraction data to a resolution of 1.8 Å were collected at the Advanced Photon Source NE-CAT beamline 24ID-E using a 30- × 50-μm beam and the Quantum 315 CCD detector. X-ray diffraction data were integrated and scaled using the HKL2000 package (21). The structure was resolved by molecular replacement using a starting model of the refined ERK2/norathyriol structure (PDB code 3SAO; ref. 20). All calculations were performed using PHENIX (22). The coupling cycles of slow-cooling annealing, positional, restrained isotropic temperature factor, and Translation/Rotation/Screw (TLS) refinements were followed by visual inspection of the electron density maps, including omit maps, coupled with a manual model rebuilt using the graphics program COOT (23). The refined electron density clearly matched the amino acid sequence of ERK2 with the exception of the N- and C-terminus (residues 1–5 and 358–360) and a weak electron density was observed for residues 23 to 34 and 336 to 340. The values of the free R-factor were monitored during the course of the crystallographic refinement and the final value of free R-factors did not exceed the overall R-factor by more than 4%. The structures of the ERK2-mutant Q105A and ERK2 in complex with adenosine (AMP-PNP was used for cocryystallization) were refined using a similar protocol (data not shown). The coordinates and structure factors for ERK2 complexed with caffeic acid have been deposited in the Protein Data Bank under accession code 4N0S.

In vitro ERK1 and 2 kinase assays

The recombinant nonphosphorylated His-tagged RSK2 (1 μg) was used as a substrate in an in vitro kinase assay with 200 ng of active ERK1 or 2 (Millipore). Reactions were conducted at 30℃ for 60 minutes in kinase buffer (25 mmol/L Tris–HCl pH 7.5, 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na₃VO₄, 10 mmol/L MgCl₂) containing 100 μmol/L unlabeled ATP with or without 10 μCi of [γ-32P] ATP. Reactions were stopped by adding 5× protein loading buffer and proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

In vitro pull-down assay

Caffeic acid–conjugated Sepharose 4B or Sepharose 4B beads were prepared as reported (24). For the in vitro or ex vivo pull-down assay, active ERK1 or 2 (200 ng) or lysates from HaCaT cells (1 μg) were mixed with 100 μL of caffeic acid–conjugated Sepharose 4B or Sepharose 4B beads in 400 μL of reaction buffer (50 mmol/L Tris–HCl pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, and 0.01% NP-40, and 2 mg/mL BSA). After incubation with gentle rocking at 4℃ overnight, the beads were washed five times with washing buffer (50 mmol/L Tris–HCl pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, and 0.01% NP-40), and proteins bound to the beads were analyzed by Western blotting.

ATP and caffeic acid competition assay

For the ATP competition assay, active ERK2 (200 ng) was incubated with different concentrations of ATP (0, 10, or 100 μmol/L) in reaction buffer at 4℃ for 2 hours. Caffeic acid–conjugated Sepharose 4B or Sepharose 4B beads (control) were added followed by incubation at 4℃ for 2 hours. After washing five times with washing buffer, the proteins bound to the beads were analyzed by Western blotting.

Western blot analysis

For Western blotting, cells (2 × 10⁶) were cultured in 10 cm dishes for 24 hours. The cells were cultured in DMEM without FBS for 24 hours to eliminate the influence of FBS on the activation of MAPKs. Cells were treated with caffeic acid (0–80 μmol/L) for 1 hour before being exposed to STIV (60 kJ TIA/m²) and 3.6 kJ (IVB/m²) and harvested 15 minutes later. Cells were disrupted on ice for 30 minutes in lysis buffer [20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)]. After centrifugation at 20,817 × g for 15 minutes, the supernatant fraction was harvested as the total cellular protein extract. The protein concentration was determined using the Bio-Rad protein assay reagent. Total cellular protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes in 20 mmol/L Tris-HCl (pH 8.0), containing 150 mmol/L glycine, and 20% (v/v) methanol. Membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (TBS-T) and incubated with primary antibodies against phosphorylated (p)-ERK1/2, p-p90RSK, p90RSK, p-Elk1, Elk1, p-c-Myc, c-Myc, or β-actin at 4℃ overnight. Blots were washed three times in TBS-T buffer, followed by incubation with the appropriate horseradish peroxidase–linked IgG. Specific proteins were visualized using an enhanced chemiluminescence detection reagent.

Xenograft mouse model

Female BALB/c (nu/nu) mice (6-week-old) were purchased from The Jackson Laboratory and maintained under "specific pathogen-free" conditions. All studies were performed following guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee (Minneapolis, MN). Mice were divided into 6 groups (n = 8): (i) mice injected with A431 sh-mock cells and treated with vehicle; (ii) mice injected with A431 sh-mock cells and treated with 10 mg/kg caffeic acid; (iii) mice injected with A431 sh-mock cells and treated with 100 mg/kg caffeic acid; (iv) mice injected with A431 sh-ERK2 cells...
and treated with vehicle; (v) mice injected with A431 sh-ERK2 cells and treated with 10 mg/kg caffeic acid; and (vi) mice injected with A431 sh-ERK2 cells and treated with 100 mg/kg caffeic acid. Mice were administered vehicle (10% DMSO and 20% polyethylene glycol 400 in PBS) or caffeic acid in vehicle i.p. every day for 3 consecutive weeks, beginning 3 days before injection of cells. A431 cells (3 x 10^6 in 50 μL PBS with 50 μL Matrigel) were injected s.c. into the right flank of each mouse. Tumor volume (length x width x depth x 0.52) was measured three times per week. Body weights were recorded once per week.

Mouse skin tumorigenesis study

Female SKH-1 hairless mice were purchased from Charles Rivers Laboratories and maintained according to guidelines established by the Research Animal Resources and Institutional Animal Care and Use Committee, University of Minnesota (Minneapolis, MN). SKH-1 mice (5–6-week-old; 25 g mean body weight) were divided into 6 age-matched groups: (i) vehicle-treated (n = 12); (ii) 20 μmol/L caffeic acid (n = 12); (iii) vehicle + SUV (n = 15); (iv) 10 μmol/L caffeic acid administered before SUV (n = 15); (v) 20 μmol/L caffeic acid administered before SUV (n = 15); (vi) 20 μmol/L caffeic acid administered after SUV (n = 15). The dose of SUV was progressively increased (10% each week) because of the ensuing hyperplasia that can occur with SUV irradiation of the skin. The vehicle was acetone and SUV irradiation was administered three times per week for 15 weeks with the dose at week 1 at 30 kJ/m² UVA and 1.8 kJ/m² UVB. At week 6, the dose of SUV reached 48 kJ/m² (UVA) and 2.9 kJ/m² (UVB) and this dose was maintained until 15 weeks. Mice were weighed and tumors measured by caliper once a week until week 29 or tumors reached a total volume of 1 cm³. At that point mice were euthanized and half of the dorsal skin was immediately fixed in 10% neural-buffered formalin and processed for hematoxylin and eosin (H&E) staining and IHC. The other half was frozen for Western blot analysis.

Figure 1. Caffeic acid reduces human skin cancer cell colony formation. A, chemical structure of caffeic acid. B, caffeic acid inhibits A431, SK-MEL-5, or SK-MEL-28 colony formation dose-dependently. Representative photographs are shown and data, means ± SD of values from three independent experiments; the asterisk (*) indicates a significant (P < 0.05) decrease in colony formation in cells treated with caffeic acid compared with the DMSO-treated group.
Statistical analysis

All quantitative data are expressed as means ± SD or SE as indicated. The Student t test or a one-way ANOVA was used for statistical analysis. A P value of <0.05 was used as the criterion for statistical significance.

Results

Caffeic acid inhibits human skin cancer cell colony formation and EGF-induced neoplastic transformation of HaCaT cells

In the present study, we examined the effect of caffeic acid (Fig. 1A) on human skin cancer cell colony formation and EGF-induced transformation of HaCaT cells. Treatment of A431 skin cancer cells or SK-MEL-5 and SK-MEL-28 melanoma cells with caffeic acid significantly inhibited colony formation in soft agar in a dose-dependent manner (Fig. 1B). HaCaT cells were also treated with caffeic acid and was not cytotoxic (Supplementary Fig. S1A). However, caffeic acid dramatically inhibited EGF-promoted transformation dose-dependently (Supplementary Fig. S1B). Colony formation in soft agar is an ex vivo indicator and a key characteristic of the transformed cell phenotype (25) and these results indicate that caffeic acid can inhibit colony formation of human skin cancer cells and neoplastic transformation of HaCaT cells induced by EGF.

ERKs are identified by in silico screening as plausible targets of caffeic acid

To identify potential targets of caffeic acid, we conducted in silico screening by using a small molecule shape similarity...
approach. Caffeic acid was screened against all the crystal-lized ligands available from the Protein Data Bank and our in-house natural compound library. Screening results showed that caffeic acid has a shape and pharmacophore similarity of 0.76 with ERK00071, which is a known ERK2 inhibitor. Thus, ERK2 was suggested as potential protein target of caffeic acid.

**Caffeic acid inhibits ERK1/2 in vitro kinase activity and suppresses SUV-induced activation of ERKs signaling**

We performed an *in vitro* kinase assay using recombinant active ERK1 or 2 in the presence of various concentrations of caffeic acid. CAY10561, a well-known inhibitor of ERK2, was used as a positive control. The phosphorylation of RSK2, a well-known ERKs substrate, was inhibited by caffeic acid in a concentration-dependent manner (Fig. 2A). We also performed an *in vitro* binding assay using caffeic acid–conjugated Sepharose 4B beads. No obvious band was observed when the recombinant active ERK 1 or 2 was incubated with control Sepharose 4B beads, whereas a strong band was seen when ERK1 or 2 was incubated with caffeic acid–conjugated Sepharose 4B beads (Fig. 2B). An ATP-competitive pull-down assay demonstrated that caffeic acid binds to ERK2 in an ATP-competitive manner (Fig. 2C). Altogether, the results clearly indicate that caffeic acid binds to recombinant ERK 1 or 2 to inhibit their respective activity.

To confirm the effect of caffeic acid on ERKs signaling, we used SUV to induce the activation of various ERKs substrates using primary mouse epidermal keratinocytes or HaCaT cells. The results show that caffeic acid inhibited SUV-induced phosphorylation of ERKs and also reduced phosphorylation of their downstream target proteins, including RSK2, c-Myc, and Elk1 (Fig. 2D and Supplementary Fig. S2; ref. 26).

**Cocrystal structure of caffeic acid and ERK2**

To identify the binding mode of ERKs and caffeic acid, we resolved the crystal structure of ERK2 bound with caffeic acid at 1.8 Å resolution. The details of data collection and structure refinement for the ERK2/caffeic acid complex are presented in Supplementary Table S1. Caffeic acid is bound at the ATP-binding site (Fig. 3A–C). The catechol moiety of caffeic acid occupies a position at the adenine ring of AMP-PNP that is visible in electron density as adenosine. Two hydroxyl groups of caffeic acid form hydrogen bonds with amino acid residues of ERK2 located in the hinge region, including the carboxyl group of Q105 and the main chain of D106 and M108. To confirm the importance of the Q105 residue in binding with caffeic acid, we performed an *in vitro* pull-down–binding assay using caffeic acid–conjugated Sepharose 4B beads. Compared with WT ERK2, a purified recombinant ERK2 mutant, Q105A, showed decreased binding with caffeic acid–conjugated Sepharose 4B beads.
We attempted to obtain the Q105A-mutant crystal structure bound with caffeic acid. However, no electron density of caffeic acid was observed in the refined structure of the Q105A mutant despite identical soaking experiments. This result suggests that the Q105A mutant was not able to strongly bind with caffeic acid in the crystallized form.

Knockdown of ERK2 decreases the anticancer effects of caffeic acid

We determined whether knocking down ERK2 expression could influence the sensitivity of human skin cancer cells or HaCaT cells to caffeic acid. We first determined the efficiency of shRNA knockdown, as well as the effect of shRNA transfection on anchorage-independent growth. The expression of ERK2 in each cell line was obviously decreased after shRNA transfection (Fig. 4A and Supplementary Fig. S3A). Colony number dramatically decreased after ERK2 shRNA transfection compared with the sh-mock group (Fig. 4B and Supplementary Fig. S3B). Next, cells transfected with ERK2 shRNA or sh-mock were treated with caffeic acid or vehicle and subjected to a soft agar assay. The results showed that caffeic acid (40 μmol/L) inhibited colony formation of each cell type transfected with sh-mock by more than 80%. In contrast, the same dose of caffeic acid inhibited colony formation of each cell type transfected with ERK2 shRNA by no more than 50% (Fig. 4C and Supplementary Fig. S3C). These results suggested that ERK2 plays an important role in the sensitivity of human skin cancer cells or HaCaT cells to the antiproliferative effects of caffeic acid.

Using a xenograft model, we found that administration of vehicle or caffeic acid (10 or 100 mg/kg) had no effect on body weight (Fig. 5A). More importantly, we observed that

![Figure 4](https://example.com/f4.png)
knocking down ERK2 expression decreased xenograft tumor volume (Fig. 5B and C), but also decreased the sensitivity of xenograft skin cancer growth to caffeic acid (Fig. 5B and D). These results indicated that when ERK2 levels were decreased, caffeic acid lost the ability to exert its preventive effects against skin cancer development.

**Caffeic acid suppresses SUV-induced skin carcinogenesis in SKH-1 hairless mice in vivo**

To further study the antitumorigenic activity of caffeic acid in vivo, we evaluated the effect of caffeic acid in an SUV-induced mouse skin tumorigenesis model. We used SUV to mimic sunlight because although UVB is a major etiologic factor for the development of skin cancer, UVA is the most abundant component of SUV irradiation (5). The results demonstrated that topical application of caffeic acid to dorsal skin–inhibited skin cancer development in mice that were exposed to SUV compared with mice treated with vehicle only and exposed to SUV (Fig. 6A). Caffeic acid (10 or 20 μmol/L) significantly inhibited the average number (P < 0.05, Fig. 6B) or volume (P < 0.05, Fig. 6C) of tumors per mouse. Notably, application of caffeic acid either before or after UV resulted in similar observations; a significant reduction in tumor formation. Skin and tumor samples were processed for H&E and IHC staining. After treatment with SUV, epidermal thickness in the vehicle/SUV group was increased by edema and epithelial cell proliferation, whereas caffeic acid–treated groups showed a smaller increase in epidermal thickness and less inflammation (Fig. 6D). IHC data showed that Ki-67, which is a well-known cellular marker for proliferation, was dramatically increased in the vehicle/SUV group compared with the vehicle group. However, Ki-67 expression was decreased in the SUV–caffeic acid–treated groups, compared with the vehicle/SUV group (Fig. 6D). The IHC staining for phosphorylation of c-Myc, which is downstream of ERKs, showed a significantly reduced level of phosphorylation in mice treated with caffeic acid (Fig. 6D). In addition, Western blotting analysis of mouse skin showed that the phosphorylation of p90RSK, c-Myc, and Elk1 induced by SUV was dramatically suppressed in the caffeic acid–treated groups (Fig. 6E). Overall, these results indicate that caffeic acid might serve as an effective chemopreventive agent against SUV-mediated skin cancer acting by suppressing the activity of ERKs.

**Discussion**

Many research groups have observed that coffee consumption is linked to a reduced risk of several cancers, including liver, colorectal, mouth and throat cancer, and breast cancer in premenopausal women (1, 11, 27–31). Caffeic acid is one of the main metabolites produced by the hydrolyzation of chlorogenic acid, a major phenolic phytochemical found in various foods, especially coffee. Previous studies have shown that caffeic acid inhibits skin tumor promotion induced by 12-O-tetradecanoylphorbol-13-acetate (PMA) in human keratinocytes (28). Our findings provide new evidence that caffeic acid inhibits skin tumorigenesis after UV exposure by inhibiting the activity of ERKs.
13-acetate in mouse skin (31). Also caffeic acid has been implicated as a protective agent against UVB-induced skin damage (32). Although accumulating evidence suggests that caffeic acid has the potential to inhibit skin cancer development, the actual effects and the molecular mechanisms remain unclear. In the present study, we showed a chemopreventive effect of caffeic acid against SUV-induced skin cancer development and identified ERK1 and 2 as its possible protein targets.

UV radiation in sunlight is the most prominent and ubiquitous physical carcinogen in our natural environment. Studies have shown that sunlight induced signal transduction is an important step for development of both nonmelanoma skin cancer and melanoma (33). Cells respond to signals produced from UV exposure by activating signaling cascades, including the MAPK pathway. MAPKs regulate multiple critical cellular functions, including proliferation, growth, and senescence (34).
Studies in various skin cell lines demonstrated that EGF receptors (35), MAPks (36), and PI3K (37) are specific signaling molecules in UVB-induced skin carcinogenesis. Some studies showed that when HaCaT cells were irradiated by UVB (0.2 kl m-2), ERK1 and 2 were phosphorylated after 15 minutes and remained activated for 6 hours (38). In our previous study, phosphorylation of ERKs and RSK2 was increased by exposure to UVB (4 kl m-2) in HaCaT cells (39), suggesting that ERKs might be a useful anticancer target.

In the present work, we showed that caffeic acid effectively inhibited colony formation of human skin cancer cells and EGF-induced neoplastic transformation of HaCaT cells (Fig. 1 and Supplementary Fig. S1). Long-term topical treatment of mouse dorsal skin with caffeic acid either before or after SUV significantly reduced skin tumor formation (Fig. 6). The Ki-67 protein is a known cellular marker for proliferation (40) and our IHC results showed that expression of Ki-67 was dramatically decreased after treatment with caffeic acid.

We provided systemic evidence, indicating that caffeic acid directly targets ERKs. According to our ERK2/caffeic acid cocystal structure (Fig. 3), caffeic acid strongly binds to the ATP-binding cleft through the formation of hydrogen bonds between hydroxyl groups and amino acids Q105, D106, and M108 located at the hinge loop. This is the best evidence available showing that caffeic acid can target ERKs at the molecular level. We further showed that caffeic acid suppressed SUV-induced ERKs phosphorylation and downstream signaling in primary mouse epidermal keratinocytes and HaCaT cells (Fig. 2D and Supplementary Fig. S2). Caffeic acid was highly effective in decreasing SUV-induced skin carcinogenesis, in vivo, whether applied before or after exposure to SUV (Fig. 6A and B). Western blot analysis of mouse skin tissue clearly confirmed that the SUV-induced phosphorylation of several of ERKs' downstream targets, including RSK2, Elk1, and c-Myc, was suppressed by caffeic acid (Fig. 6D). In addition, knocking down ERK2 expression significantly reduced expression of Ki-67 and suppressed the activity of their downstream substrates, RSK2, Elk1, and c-Myc. Therefore, caffeic acid might be a good chemopreventive agent that is highly effective against SUV-induced skin cancer.

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

Authors' Contributions
Conception and design: G. Yang, A.M. Bode, Z. Dong, Z. Dong
Development of methodology: G. Yang, K. Yao, Z. Dong
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Yang, Y. Fu, M. Malakhova, I. Kurinov, F. Zhu, H. Li, A.M. Bode, Z. Dong
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Study supervision: Z. Dong

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