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(+)-2-(1-Hydroxyl-4-oxocyclohexyl) ethyl caffeate suppresses solar UV-induced skin carcinogenesis by targeting PI3-K, ERK1/2 and p38

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Abstract

For decades, skin cancer incidence has increased, mainly due to oncogenic signaling pathways activated by solar ultraviolet (UV) irradiation (i.e., sun exposure). Solar UV induces multiple signaling pathways that are critical in the development of skin cancer, and therefore the development of compounds capable of targeting multiple molecules for chemoprevention of skin carcinogenesis is urgently needed. Herein, we examined the chemopreventive effects and the molecular mechanism of HOEC, [(+)-2-(1-hydroxyl-4-oxocyclohexyl) ethyl caffeate], isolated from *Incarvillea mairei* var. *grandiflora* (Wehrhahn) Grierson. HOEC strongly inhibited neoplastic transformation of JB6 C141 cells without toxicity. PI3-K, ERK1/2 and p38 kinase activities were suppressed by direct binding with HOEC *in vitro*. Our *in silico* docking data showed that HOEC binds at the ATP-binding site of each kinase. The inhibition of solar UV-induced PI3-K, ERK1/2 and p38 kinase activities resulted in suppression of their downstream signaling pathways and AP-1 and NF-κB transactivation in JB6 cells. Furthermore, topical application of HOEC reduced skin cancer incidence and tumor volume in SKH-1 hairless mice chronically exposed to solar UV. In summary, our results show that HOEC exerts inhibitory effects on multiple kinase targets and their downstream pathways activated by solar UV *in vitro* and *in vivo*. These findings suggest that HOEC is as a potent chemopreventive compound against skin carcinogenesis caused by solar UV exposure.

Keywords

2-(1-hydroxyl-4-oxocyclohexyl) ethyl caffeate; solar-UV; skin carcinogenesis; natural compound

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Conflict of Interest

The authors state no conflict of interest.

Introduction

Skin cancer is one of the most common types of cancers in the United States (1). Epidemiological studies on cancer incidence show that in the last decade, skin cancer in men has increased, which is in contrast to the decreases observed in three major cancers, prostate, lung and colon (2, 3). In any type of skin cancer, the major etiological factor is solar ultraviolet (solar UV, i.e., sunlight) irradiation (4, 5). Solar UV consists of UVA, comprising the largest portion (95%), which has a relatively weaker effect on causing skin cancer compared to UVB. UVB comprises a smaller portion (5%) of solar UV, but is considered to be a complete carcinogen (6). UVA and UVB cause DNA damage (7) and activate various signaling pathways for skin tumor promotion (8). Recent reports suggest that phosphatidylinositol 3-kinase (PI3-K) (9, 10) and mitogen-activated protein kinase (MAPK) family members, including extracellular signal-regulated kinases (ERKs) (11–15) and p38 (14–18), are major factors in skin carcinogenesis. This conclusion is based on research findings showing that these kinases activate oncogenic transcription factors, activator protein-1 (AP-1) and nuclear factor-kappaB (NF- κ B) (19, 20). Thus, an effective strategy might be to directly suppress the activities of these kinases or their common regulators (21–24) for chemoprevention of skin cancer.

Elucidating the anticancer effects of plant-derived compounds might be a successful approach for chemoprevention (24). HOEC, (+)-2-(1-hydroxyl-4-oxocyclohexyl) ethyl caffeate (Fig. 1), is found in *Incarvillea mairei* var. *grandiflora* (Wehrhahn) Grierson. Although the plants of the *Incarvillea* genus are widely grown for ornamental purposes, especially in China, *Incarvillea delavayi*, one of the *Incarvillea* species, recently received attention for its anti-nociceptive effects (25). In particular, HOEC isolated from *Incarvillea mairei*, is reported to exhibit anti-inflammatory effects on inflammation-induced mice (26). However, the potential anticancer effects and molecular targets of the plant extract or its lead compound have not yet been studied.

Herein, we report that HOEC directly targets and inhibits the kinase activities of PI3-K, ERK1/2, and p38 and down-regulates their downstream signaling pathways in solar UV-treated JB6 Cl41 mouse epidermal cells. The inhibition of these pathways leads to a reduction of AP-1 and NF- κ B transcriptional activities. In an *in vivo* model of solar UV-induced skin carcinogenesis, HOEC significantly reduced tumor volume and tumor number by inhibiting the PI3-K, ERK1/2 and p38 signaling pathways.

Materials and Methods

Reagents

HOEC (2-(1-hydroxyl-4-oxocyclohexyl)ethyl caffeate) is a natural compound isolated from the whole plants of *Incarvillea mairei* and the compound used in this study was synthesized according to previous reports (27). The purity of HOEC was assessed by HPLC and found to be greater than 97%. Active p110 (PI3-K), active ERK1, active ERK2, active p38 α , inactive RSK2 and inactive ATF2 recombinant proteins for kinase assays were purchased from Millipore (Temecula, CA). Antibodies to detect phosphorylated tyrosines (p-Tyr, i.e., p-

p110), phosphorylated Akt (p-Akt S473), phosphorylated ERK1/2 (p-ERK1/2 Thr202/Tyr204), phosphorylated p38 (p-p38 Thr180/Tyr182), phosphorylated RSK2 (p-RSK2 Ser380), phosphorylated MSK1 (p-MSK1 Ser376), phosphorylated ATF2 (p-ATF2 Thr69/71), phosphorylated S6 ribosomal protein (p-S6 ribosomal protein Ser235/236), phosphorylated c-Fos (p-c-Fos Ser32), phosphorylated c-Jun (p-c-Jun Ser63), total p110, total ERKs, total RSK, total Akt, total ATF2, total MSK, total S6 ribosomal protein, total c-Fos, and total c-Jun were purchased from Cell Signaling Technology (Beverly, MA). The antibody against β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA). CNBr-sepharose 4B beads were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). The luciferase assay substrate and the Cell Titer 96 Aqueous One Solution Reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] kit for the cell proliferation assay were from Promega (Madison, WI).

Cell culture

The JB6 Cl41 murine epidermal cell line (a promotion-sensitive clone of the JB6 P+ cell line) was cultured in Eagle's Minimum Essential Medium (MEM) and HaCaT human keratinocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM)/high glucose containing penicillin (100 units/mL), streptomycin (100 μ g/mL), and 4% or 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA), respectively. Cells were maintained in a 5% CO₂, 37°C humidified incubator. 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.

In vitro kinase assay

The kinase assay was performed according to the instructions provided by Millipore. Briefly, for ERK1, ERK2, or p38 α activity analysis, the relevant active protein (100 ng) was incubated with HOEC (0, 10 or 20 μ mol/L) for 30 min at 30°C. Then each reaction mixture was mixed with isotope-unlabeled ATP and 10 μ Ci [γ -³²P] ATP with each compound in 10 μ L of reaction buffer containing 20 mmol/L HEPES (pH 7.4), 10 mmol/L MgCl₂, 10 mmol/L MnCl₂, and 1 mmol/L dithiothreitol (DTT). After incubation at 30°C for 30 min, the reaction was stopped by adding 5 μ L protein loading buffer and the mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For measuring PI3-K activity, an active p110 α protein (100 ng) was incubated with HOEC (0, 10 or 20 μ mol/L) for 30 min at 30°C. Then, 20 μ L of 0.5 mg/mL phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) were added, and the mixture was incubated for 5 min at room temperature. Reaction buffer (100 mmol/L HEPES, pH 7.6, 50 mmol/L MgCl₂, 250 μ mol/L ATP) containing 10 μ Ci [γ -³²P] ATP was added, and the reaction was incubated for an additional 30 min at 30°C. The reaction was stopped by the addition of 15 μ L of 4 N HCl and 130 μ L of chloroform:methanol (1:1) and mixed by vortexing. The lower chloroform phase (30 μ L) was spotted onto a 1% potassium oxalate-coated silica-gel plate (previously activated for 1 h at 110°C) and subjected to thin-layer chromatography and autoradiography to visualize the ³²P-labeled phosphatidylinositol 3-phosphate (PIP) product. Each experiment was repeated twice and the relative amounts of incorporated radioactivity were assessed by autoradiography.

***In vitro* and *ex vivo* pull-down assays**

Recombinant p110 α , ERK1, ERK2 or p38 α (200 ng) proteins or solar UV-treated JB6 Cl41 cell lysates (500 μ g) were incubated with HOEC-sepharose 4B (or sepharose 4B only as a control) beads (50 μ L 50% slurry) in reaction buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% NP-40, and 2 mg/mL bovine serum albumin). After incubation with gentle rocking overnight at 4°C, the beads were washed 3 times with buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, and 0.01% NP-40) and binding was visualized by Western blotting.

ATP and HOEC competition assay

In order to determine whether HOEC inhibits a protein's kinase activity by interacting with the protein's ATP binding pocket, we performed an ATP and HOEC competition assay. ATP (0, 1, 10 or 100 μ mol/L) was mixed with active p110 α , ERK1, ERK2 or p38 α (200 ng) for 30 min. After incubation, HOEC-sepharose 4B (or sepharose 4B only as a control) beads (50 μ L 50% slurry) were added in reaction buffer and kept at 4°C overnight. The beads were washed and proteins (p110, ERK1, ERK2, or p38) were detected by Western blot analysis.

Solar UV irradiation system

The solar UV source comprised UVA-340 lamps purchased from Q-Lab Corporation (Cleveland, OH). The UVA-340 lamps 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 (28). The percentage of UVA and UVB emitted from UVA-340 lamps was measured by a UV radiometer and was recorded as 94.5 and 5.5%, respectively. Specifically, uncovered dishes of serum-starved cells were exposed to UV irradiation under UVA-340 lamps. The dose of solar UV radiation was 48 kJ/m² UVA/2.9 kJ/m² UVB, which was the same dose used in the animal study.

Western blot analysis

Cells were disrupted on ice for 40 min in cell 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 PMSF [phenylmethylsulfonyl fluoride]). After centrifugation at 12,000 rpm for 10 minutes, the supernatant fraction was harvested as the total cellular protein extract. The protein concentration was determined using the Bio-Rad protein assay reagent (Richmond, CA). Total cellular protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) 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 1 $\times$ TBS containing 0.05% Tween 20 (TBS-T) and incubated with antibodies against p-Tyr (p-p110), p110 α , p-Akt, Akt, p-ERK1/2, ERK1/2, p-RSK, RSK, p-p38, p38, p-ATF2, ATF2, p-S6 ribosomal protein (p-S6), S6 ribosomal protein (S6), p-MSK1, MSK1, p-c-Fos, c-Fos, p-c-Jun, c-Jun or β -actin. Blots were washed 3 times in 1X TBS-T buffer, followed by incubation with an appropriate HRP-linked IgG. The specific proteins in the blots were visualized using the enhanced chemiluminescence (ECL) detection reagent.

***In silico* target identification and molecular modeling**

To identify the potential binding proteins of HOEC, a shape similarity method (28) using the PHASE module of Schrödinger's molecular modeling software package was performed to search for biological targets of HOEC based on its structure. The atom-type parameter for volume was set at pharmacophore, which means the queries were used not only to consider shape similarity but also to align potential pharmacophore points between the queries and the targets. The target library was obtained from the Protein Data Bank (29) and our in-house library. To provide more structural orientations for possible alignment, we set the maximum number of conformers per molecule to be generated to 100, while retaining up to 10 conformers per rotatable bond. We filtered out conformers with similarity below 0.75. We then obtained the PDB ID associated with each aligned target molecule and used these PDB IDs to search the online Protein Data Bank to identify the protein. Based on the shape similarity results, ERK2, PI3-K and p38 α were identified as potential protein targets of HOEC. To study the HOEC interaction with PI2-K, ERK1/2 and p38 α , computational docking and modeling studies were performed. First, an X-ray diffraction structure of p110 with a resolution of 2.8 Å (PDB ID 3HHM, Chain A) (30), an X-ray diffraction structure of ERK1 with a resolution of 2.39 Å (PDB ID 2ZOQ) (31), an X-ray diffraction structure of ERK2 with a resolution of 2.4 Å (PDB ID 2OJJ) (32) and an X-ray diffraction structure of p38 with a resolution of 2.9 Å (PDB ID 3DS6) (33), were downloaded. Hydrogen atoms were added consistent with a pH of 7 and all water molecules removed. Finally, an ATP binding site-based receptor grid for docking with each respective kinase was generated. HOEC was prepared for docking by default parameters using the LigPrep program in Schrödinger. Then HOEC-protein docking was accomplished with the program Glide using default parameters under the extra precision (XP) mode allowing the acquisition of the best-docked representative structure.

Reporter gene activity of *AP-1* or *NF- κ B* transactivation

JB6 Cl41 cells stably transfected with an *AP-1* or *NF- κ B* luciferase reporter plasmid were seeded (1×10^4 viable cells/well) into a 24-well plate. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO₂ and were starved in serum-free medium for another 24 h. The cells were exposed to solar UV (48 kJ/m²) and then treated with HOEC (0, 10 or 20 μ mol/L). After 6 h, cells were harvested and disrupted with 100 μ L of lysis buffer (0.1 mmol/L potassium phosphate pH 7.8, 1% Triton X-100, 1 μ mol/L dithiothreitol (DTT), and 2 μ mol/L EDTA). Then firefly luciferase activities were measured by a luminometer (Luminoskan Ascent, Thermo Fisher Scientific, Lafayette, CO) using substrates provided in the reporter assay system (Promega).

Solar UV-induced mouse skin carcinogenesis study

Female SKH-1 hairless mice, 6 weeks old, were purchased from Charles River (Wilmington, MA) and maintained under conditions based on the guidelines established by Research Animal Resources and the University of Minnesota Institutional Animal Care and Use Committee (IACUC). Skin carcinogenesis in mice was induced using UVA-340 lamps. Mice were divided into 5 groups. In the control groups, the dorsal skin was topically treated with acetone as vehicle (n = 9) or HOEC (0.5 mg/mouse, n = 12) in 200 μ L acetone. In the

solar UV-treated group (n = 21), the dorsal skin was topically treated with 200 μ L acetone after exposure to solar UV. The mice in 1 group (0.5 mg/mouse, n = 20) received topical application of HOEC for a total of 30 weeks twice a week. The UV absorption spectra of HOEC indicated that HOEC absorbed UV in the solar UV range between 250 and 380 nm, which means that HOEC could act as a sun-blocking agent. Therefore, to eliminate the UV blocking effect of HOEC, we treated mice with HOEC for 12 weeks after solar UV exposure and for an additional 18 weeks without UV exposure.

The HOEC (0.5 mg/mouse) was dissolved in 200 μ L of acetone, which is sufficient to cover all of the dorsal skin area of each mouse, which measured approximately $2 \times 4.5 \text{ cm}^2$. The dose of solar UV was gradually increased by 10% each week for 6 weeks to acclimate the mice to the protocol and prevent sunburn and hyperplasia while maintaining the tumor-promoting effect of solar UV. At week 1, the solar UV dose was 30 kJ/m^2 UVA/1.8 kJ/m^2 UVB given twice/week. At week 6, the dose was 48 kJ/m^2 UVA/2.9 kJ/m^2 UVB and this dose was maintained for weeks 6 to 12 and then UV treatment was discontinued and tumor growth was monitored for an additional 18 weeks. A tumor was defined as an outgrowth of at least 1 mm in diameter that persisted for 2 weeks or more. Mouse weight and tumor number and volume were recorded every week until the end of the experiment (30 weeks). Tumors and skin were frozen for Western blot analysis. Tumor volume was calculated according to the following formula: tumor volume (mm^3) = length $\times$ width $\times$ height $\times$ 0.52.

Statistical analysis

All quantitative results are expressed as mean values $\pm$ S.D. Statistically significant differences were obtained using the Student's t test or by one-way ANOVA. A *p*-, value of < 0.05 was considered to be statistically significant.

Results

HOEC binds with p110, ERK1, ERK2, or p38 at their respective ATP binding site

To better understand how HOEC interacts with p110 α (Fig. 2Aa-b), ERK1 (Fig. 2Ba-b), ERK2 (Fig. 2Ba, c), or p38 α (Fig. 2Ca-b), we performed a computational docking study using the Glide docking program included in Schrödinger Suite 2012. In the docked models, HOEC binds well at the ATP-binding pockets of all four kinases (Fig. 2). Taken together with the *in vitro* and *ex vivo* pull-down assay results (supplemental Fig. S1A, B, C), our data confirmed that HOEC binds to the respective ATP binding site and inhibits the kinase activity of p110 α , ERK1, ERK2, or p38 α . The images were generated using the UCSF Chimera program (34).

PI3-K, ERK1, ERK2, and p38 α are major targets of HOEC

To rule out the possibility that the inhibitory effect of HOEC was due to toxicity, we conducted a cell toxicity assay and results indicated that HOEC had no cytotoxic effect on normal JB6 Cl41 murine epidermal cells or HaCaT human keratinocytes (data not shown). To identify direct target(s) of HOEC, we conducted several *in vitro* kinase assays. HOEC selectively inhibited PI3-K (10 $\mu\text{mol/L}$, 48%; 20 $\mu\text{mol/L}$, 97%; Supplemental Fig. S1A-a), ERK1 (10 $\mu\text{mol/L}$, 6%; 20 $\mu\text{mol/L}$, 16%; Supplemental Fig. S1A-b), ERK2 (10 $\mu\text{mol/L}$, 8%;

20 $\mu\text{mol/L}$, 54%; Supplemental Fig. S1A-c), and p38 α (10 $\mu\text{mol/L}$, 53%; 20 $\mu\text{mol/L}$, 68%; Supplemental Fig. S1A-d) kinase activities in a concentration-dependent manner, but had no effect on EGFR or JNKs kinase activity (data not shown). To confirm and identify additional kinase targets of HOEC, we used a commercial *in vitro* kinase profiling system (Kinase Profiler, Millipore). The Kinase Profiler results showed that all other kinases tested were not affected by HOEC (Supplemental Table S1). Then to determine whether HOEC interacts directly with p110 α , ERK1, ERK2, or p38 α , we performed *in vitro* (Supplemental Fig. S1B-a) and *ex vivo* (Supplemental Fig. S1B-b) pull-down binding assays. Recombinant p110 α , ERK1, ERK2, and p38 α strongly interacted with HOEC-conjugated sepharose 4B beads (Supplemental Fig. S1B-a). In addition, these proteins in solar UV-treated JB6 Cl41 cell lysates were pulled down with HOEC-sepharose 4B beads (Supplemental Fig. S1B-b). To determine whether the binding of HOEC to p110 α , ERK or p38 α occurs in an ATP-competitive manner, we performed an ATP (0, 1, 10, or 100 $\mu\text{mol/L}$) competitive kinase pull down assay. Results showed that the binding of HOEC to p110 α , ERK1, ERK2 or p38 α decreased in an ATP-competitive manner (Supplemental Fig. S1C). These results suggest that PI3-K, ERK1, ERK2, and p38 α are major targets of HOEC.

HOEC down-regulates solar UV-induced phosphorylation of PI3-K, ERKs, and/or p38 downstream signaling pathways

Our data showed that HOEC binds to the ATP binding site of p110 α , ERK1, ERK2, or p38 α (Fig. 2) and suppresses their respective activity (Supplemental Fig. S1A-a-d). Therefore, the inhibitory effects of HOEC likely suppress the respective downstream signaling pathways. The PI3-K/Akt and MAPKs pathways are highly activated in UV-induced skin cancers (10, 11, 16). HOEC decreased solar UV-induced phosphorylation of PI3-K downstream proteins, including Akt (10 $\mu\text{mol/L}$, 38%; 20 $\mu\text{mol/L}$, 62%) and S6 ribosomal protein (S6; 10 $\mu\text{mol/L}$, 5%; 20 $\mu\text{mol/L}$, 62%) compared to the same kinases in solar UV-induced, untreated JB6 Cl41 cells (Fig. 3A). ERK1/2 and p38 are MAPK family members and HOEC affects these MAPK signaling pathways. RSK2 and MSK1 are ERKs downstream signaling proteins and ATF2, c-Jun and c-Fos act downstream of p38. Results indicated that HOEC (20 $\mu\text{mol/L}$) suppressed solar UV-induced phosphorylation of RSK2 by 85%, MSK1 by 64% (Fig. 3B), c-Fos by 29%, and c-Jun by 64% (Fig. 3C). These findings suggest that HOEC suppresses solar UV-induced activation of PI3-K/Akt and MAPKs signaling pathways mediated by their well-known upstream kinases, PI3-K, ERK1, ERK2, or p38.

HOEC suppresses the transcriptional activity of AP-1 and NF- κ B

After stimulation with UV, the transcriptional activities of activator protein-1 (AP-1) and nuclear factor-kappa B (NF- κ B) are induced through the PI3-K/Akt or MAPKs signaling pathways (8, 35). To examine the effect of HOEC on the transcriptional activity of AP-1 and NF- κ B, we exposed JB6 Cl41 cells stably transfected with the *AP-1* or *NF- κ B* luciferase reporter plasmid to solar UV (48 kJ/m² UVA/2.9 kJ/m² UVB) and then treated cells with HOEC for another 6 h. HOEC (20 $\mu\text{mol/L}$) suppressed solar UV-induced transactivation of AP-1 by 47% and NF- κ B by 80% (Fig. 4).

HOEC attenuates solar UV-induced skin carcinogenesis in SKH-1 hairless mice

To determine the chemopreventive effects of HOEC *in vivo*, we utilized a solar UV-induced skin carcinogenesis mouse model. We treated mice with solar UV and then topically applied vehicle or HOEC to the dorsal surface of SKH-1 hairless mice. Volume (Fig. 5A) and number (Fig. 5B) of tumors in mice treated with HOEC (0.5 mg/mouse) after solar UV exposure were significantly inhibited by HOEC ($p < 0.05$). At week 30, all mice were sacrificed and mouse skin and tumor tissues were collected for Western blot analysis. Phosphorylation of p110, Akt, S6K, RSK, MSK, p38, ATF2, c-Jun, and c-Fos was induced by solar UV and HOEC strongly suppressed the phosphorylation (Fig. 5C and Supplemental Fig. S2). The phosphorylation level of HOEC-targeted kinases was quantified by densitometry (Fig. 5C). RSK2, MSK1, S6K40, and c-Jun are well-known substrates of ERK1/2, p110, and p38, respectively. The phosphorylation levels of RSK2, MSK1, S6K40, and c-Jun were significantly induced by solar UV treatment and strongly inhibited by HOEC (40%, 50%, 77% and 77% inhibition, respectively) in skin tissues (Fig. 5C). Additionally, phosphorylated Akt, ATF2, and c-Fos levels were also decreased by HOEC treatment (Supplemental Fig. S2). Moreover, HOEC substantially decreased PCNA levels in skin tissues. However, HOEC did not affect caspase 7 or PARP cleavage. PCNA and the cleavage status of caspase 7 or PARP are representative markers of cell growth or cell death, respectively. Note that HOEC was topically applied after UV exposure to exclude the possibility of any sun-block effect of the HOEC compound (Supplemental Fig. S3). To eliminate the possibility that residual HOEC could remain on the skin between UV exposures, we examined the amount of HOEC remaining in the dorsal skin tissues (500 mg) from mice sacrificed at 0, 24, and 48 h after HOEC treatment. Mass spectrometry results indicated that skin tissues harvested at 24 or 48 h after HOEC treatment showed decreased HOEC levels of 50 or 90%, respectively, compared to skin treated with HOEC and harvested immediately (data not shown). This result supports the idea that residual HOEC does not block penetration of subsequent UV irradiation because most (90%) of the HOEC had disappeared within 48 h. Overall, our data show that HOEC binds its target kinases and inhibits their respective activities, blocking signal transduction to their downstream molecules resulting in decreased cell growth but not necessarily cell death. These results suggest that HOEC is a robust multi-targeted chemopreventive agent against solar UV-induced skin carcinogenesis that acts through mechanisms other than, but not entirely excluding, a sunblock effect.

Discussion

Cancer chemoprevention with natural compounds is a promising strategy because of the ability of these compounds to inhibit multiple signaling pathways activated by carcinogens (24) and tumor promoters. Solar UV, comprising both UVA and UVB, is a major factor in the increased incidence of skin cancers, including non-melanoma skin cancer and melanoma (36, 37). Moreover, multiple signaling pathways are activated by UV exposure and lead to the acquisition of gene alterations in skin cells (38, 39). Therefore, identifying chemopreventive compounds with multiple molecular targets against solar UV-induced skin carcinogenesis is crucial because of the continued increasing incidence of this cancer. Several groups have reported the skin photoprotective effects of various phytochemicals (11,

22, 40). We used the natural compound, HOEC, which is a caffeic ester from *Incarvillea mairei* var. *grandiflora* (Wehrhahn) Grierson (27, 41). HOEC was revealed to act as an anti-inflammatory agent targeting 5-LOX, which confirmed a key role of 5-LOX in the pathogenesis of inflammation, especially rheumatoid arthritis (26). HOEC may be a pro-drug of caffeic acid because of the chemical relevance and bio-functional similarity. However, the mechanism of the anti-cancer effects and direct targets of HOEC have not yet been elucidated.

In the present study, we found that HOEC directly binds with PI3-K, ERK1/2, and p38 and inhibits their kinases activities. PI3-K, ERK1/2, and p38 are known to play critical roles in several cancers, including skin cancer (9, 12, 16, 39). The PI3-K/Akt signaling pathways are activated by UV irradiation and can induce apoptosis in human keratinocytes (42). ERK1/2 signaling pathways are also stimulated by UV irradiation and UVB irradiation of keratinocytes results in the activation of ERK1/2 and p38 MAPK pathways (8, 14, 16, 39). Animal study results also revealed that treatment of mouse skin with HOEC inhibits solar UV-induced skin carcinogenesis by attenuating PI3-K, ERK1/2, and p38 activation leading to decreased downstream signaling. The effects of HOEC on UV-induced oxidative stress or UV-induced inflammation have not yet been elucidated. However, HOEC is a novel compound that suppresses solar-UV induced skin carcinogenesis by directly binding and inhibiting the activity of multiple kinases, resulting in the attenuation of UV-induced inflammation and skin carcinogenesis (14). Thus, we suggest that HOEC is a novel compound that functions as a multi-kinase inhibitor of solar UV-induced skin cancer.

The pursuit of molecular protein targets of natural compounds in skin cancer or other cancers is challenging. HOEC is a novel and unique compound because it targets at least three important proteins that play significant roles in solar UV-induced skin cancer, which makes it very potent in suppressing solar UV-induced skin carcinogenesis. Compared to taxifolin (43) or norathyriol (11), which each effectively suppressed solar UV-induced skin carcinogenesis in mice, HOEC has stronger effects on its target kinases' activities and downstream transcription activities. For example, the effect of HOEC against PI-3K ($IC_{50} = 10.3 \mu\text{mol/L}$), is 7.6 fold greater than that of taxifolin ($IC_{50} = 78.4 \mu\text{mol/L}$). The inhibition of ERK2 and p38 by HOEC ($IC_{50} = 20.9$ and $12.9 \mu\text{mol/L}$, respectively) is 1.4 and 2.3 fold greater than the inhibition of EGFR by taxifolin ($IC_{50} = 29.0 \mu\text{mol/L}$). Moreover, the inhibition of AP-1 and NF- κ B transcription activity by HOEC was 1.4 and 2.8 fold greater than the effect of norathyriol (11).

UV-induced activation of these kinases leads to increased AP-1 and NF- κ B transcription activities (43, 44). With UV irradiation, AP-1 is stimulated in human keratinocytes and promotes tumor development and UVB-induced photocarcinogenesis is reduced by repression of AP-1 and NF- κ B activation (45, 46). Because AP-1 and NF- κ B activate many oncogenes in carcinogenesis, the inhibitory effect of HOEC on AP-1 and NF- κ B transactivation activity might account for its ability to suppress EGF-induced anchorage-independent growth of JB6 Cl41 P+ murine epidermal cells and HaCaT human keratinocytes.

This result is also supported by our *in silico* identification and computational docking model. The theory of shape-similarity screening is derived from the idea that molecules possessing similar shape and electrostatic capabilities might exhibit analogous biological activity. According to our *in silico* computer screening results, HOEC is similar in shape to well-known kinase inhibitors of PI3-K, ERK2, and p38. The specific similarity score of HOEC to each kinase is 0.83 with (Z)-5-((5-(4-fluoro-2-hydroxyphenyl)furan-2-yl)methylene)thiazolidine-2,4-dione (47), which is a known ERK2 inhibitor; 0.83 with 1-(4-bromophenyl)-2-(5-(furan-2-yl)-4H-1,2,4-triazol-3-ylthio)ethanone (48), which is a known PI3-K alpha inhibitor; and 0.81 with 1-(5-tetra-butyl-2-methyl-2H-pyrazol-3-yl)-3-(4-chloro-phenyl)-urea (BMU) (49), which is a known p38 inhibitor, respectively. Based on these results, ERK1/2, PI3-K and p38 are potential protein targets of HOEC.

Most studies focusing on UV-induced signaling have involved mainly UVB irradiation. However, in this study, we used solar UV treatment, which comprises both UVA and UVB, making it similar to our natural environment. An effective strategy to suppress solar UV-induced skin carcinogenesis might include the use of a multi-targeted inhibitor because solar UV activates multiple signaling pathways. We and others have found that determining the most important target among many signals activated by UV is highly challenging and to attempt to suppress only one signaling molecule is probably not feasible (24). Notably, the chemopreventive effects of HOEC are not due to its absorption of UV (as described in Materials and Methods), but because of a direct inhibition of key signaling pathways.

In summary, HOEC attenuated solar UV-induced PI3-K, ERK1/2 and p38 signaling pathways in JB6 Cl41 cells and decreased skin tumor formation in SKH-1 hairless mice exposed to solar UV. The chemopreventive effect of HOEC on skin carcinogenesis *in vitro* and *in vivo* is due to direct binding and inhibition of PI3-K, ERK1/2 and p38 activation, resulting in suppression of AP-1 and NF- κ B transactivation. Treatment with HOEC resulted in decreased cell growth but did not necessarily cause cell death in preventing skin carcinogenesis. Based on our results, we conclude that the identification of HOEC as a chemopreventive agent against skin cancer might contribute to the further development of novel multi-targeted agents to prevent solar UV-induced skin carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Chemical structure of HOEC

Fig. 2. Confirmation of the predicted interaction between HOEC and p110 α , ERK1, ERK2, or p38 α using a computer docking model

(A-a) HOEC can bind at the ATP binding pocket of p110 α . (A-b) Enlarged view of HOEC binding with p110 α at the ATP binding pocket and formation of hydrogen bonds. (B-a) HOEC can bind at the ATP binding pocket of ERK1 or 2. Enlarged view of HOEC binding with ERK1 (B-b) or ERK2 (B-c) at the ATP binding pocket and formation of hydrogen bonds. (C-a) HOEC can bind at the ATP binding pocket of p38 α . (C-b). Enlarged view of HOEC binding with p38 α at the ATP binding pocket and formation of hydrogen bonds. For A-C, hydrogen bonding is shown as cyan-colored lines.

Fig. 3. HOEC down-regulates solar-UV induced phosphorylation of PI3-K, ERKs, and/or p38 downstream signaling pathways

(A) HOEC inhibits solar UV-induced phosphorylation of Akt and S6 ribosomal protein. (B) HOEC inhibits solar UV-induced phosphorylation of RSK2 and MSK1. (C) HOEC suppresses solar UV-induced phosphorylation of ATF2, c-Fos, and c-Jun. JB6 Cl41 cells were serum-deprived with MEM containing 0.01% FBS and treated or not treated with solar UV (48 kJ/m² UVA/2.9 kJ/m² UVB) and HOEC (0, 10, or 20 μmol/L). Treated cells were disrupted 30 min after solar UV as described in Materials and Methods. The levels of phosphorylated and total proteins as indicated were determined by Western blot analysis. Representative blots from 3 independent experiments are shown. The relative abundance of each band is normalized to its own total protein or β-actin and quantified. The value is shown above each respective lane in each blot. The untreated control levels were set at 1 and all lanes are compared to the control.

Fig. 4. HOEC suppresses the transcriptional activity of AP-1 or NF- κ B

JB6 Cl41 cells stably transfected with an *AP-1* A) or *NF- κ B* B) *luciferase* reporter plasmid were cultured and serum-deprived with MEM containing 0.01% FBS. Serum-deprived cells were treated with solar UV (48 kJ/m² UVA/2.9 kJ/m² UVB) followed by treatment with HOEC (0, 10, or 20 $\mu\text{mol/L}$) for 6 h. Luciferase activity was measured as described in Materials and Methods. HOEC inhibits (A) AP-1 or (B) NF- κ B transcriptional activity in JB6 Cl41 cells. Data are shown as means $\pm$ S.D. of values from triplicate samples from 3 independent experiments. The asterisk (*) indicates a significant difference ($p < 0.05$)

between vehicle-treated and solar UV-induced transcriptional activity and the asterisks (**) indicate a significant difference ($p < 0.05$) between untreated and HOEC-treated cells.

Fig. 5. HOEC suppresses solar UV-induced skin carcinogenesis in SKH-1 hairless mice
 Mice were divided into 4 groups and treated as described in Materials and Methods. Groups are as follows: 1) vehicle and no solar UV; 2) HOEC (0.5 mg/mouse) and no solar UV; 3) solar UV + vehicle; 4) solar UV + HOEC (0.5 mg/mouse). HOEC was applied topically in acetone on the dorsal surface of each mouse after exposure to solar UV (48 kJ/m² UVA/2.9 kJ/m² UVB). Tumor volume (A) and average number (B) of tumors per mouse are shown. Data are represented as means $\pm$ S.D. The asterisk (*) indicates a significant difference between the vehicle-treated and HOEC-treated groups exposed to solar UV ($p < 0.05$). (C)

The levels of phosphorylated and total targeted proteins in mouse skin were analyzed by Western blot. Quantitative analysis of representative results from Western blot assay was performed and is shown as means $\pm$ S.D. (n = 3). The control (vehicle-treated and no solar UV group) levels were set at 100%. The asterisk (*) indicates a significant difference ($p < 0.05$) between the vehicle-treated and HOEC-treated groups exposed to solar UV. The asterisks (**) indicate a significant difference ($p < 0.05$) between untreated and HOEC-treated groups within the solar UV exposed groups.