**Research Article**

**A Derivative of Chrysin Suppresses Two-Stage Skin Carcinogenesis by Inhibiting Mitogen- and Stress-Activated Kinase 1**

Haidan Liu1,3,4,5, Joonsung Hwang1, Wei Li3,6, Tae Woong Choi1, Kangdong Liu1,7, Zunnan Huang3,8, Jae-Hyuk Jang9, N.R. Thimmegowda1, Ki Won Lee3, In-Ja Ryoo7, Jong-Seog Ahn9, Ann M. Bode3, Xinmin Zhou4, Yifeng Yang4, Raymond L. Erikson9, Bo-Yeon Kim1,2, and Zigang Dong1,3

**Abstract**

Mitogen- and stress-activated kinase 1 (MSK1) is a nuclear serine/threonine protein kinase that acts downstream of both extracellular signal-regulated kinases and p38 mitogen-activated protein kinase in response to stress or mitogenic extracellular stimuli. Increasing evidence has shown that MSK1 is closely associated with malignant transformation and cancer development. MSK1 should be an effective target for cancer chemoprevention and chemotherapy. However, very few MSK1 inhibitors, especially natural compounds, have been reported. We used virtual screening of a natural products database and the active conformation of the C-terminal kinase domain of MSK1 (PDB id 3KN) as the receptor structure to identify chrysin and its derivative, compound 69407, as inhibitors of MSK1. Compared with chrysin, compound 69407 more strongly inhibited proliferation and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation of JB6 P+ cells with lower cytotoxicity. Western blot data demonstrated that compound 69407 suppressed phosphorylation of the MSK1 downstream effector histone H3 in intact cells. Knocking down the expression of MSK1 effectively reduced the sensitivity of JB6 P+ cells to compound 69407. Moreover, topical treatment with compound 69407 before TPA application significantly reduced papilloma development in terms of number and size in a two-stage mouse skin carcinogenesis model. The reduction in papilloma development was accompanied by the inhibition of histone H3 phosphorylation at Ser10 in tumors extracted from mouse skin. The results indicated that compound 69407 exerts inhibitory effects on skin tumorigenesis by directly binding with MSK1 and attenuates the MSK1/histone H3 signaling pathway, which makes it an ideal chemopreventive agent against skin cancer. *Cancer Prev Res;* 7(1): 74–85. ©2013 AACR.

**Introduction**

Mitogen- and stress-activated kinase 1 (MSK1) is a nuclear serine/threonine protein kinase that acts downstream of both extracellular signal-regulated kinases (ERK) and p38 mitogen-activated protein kinase (MAPK) in response to growth factors, cellular stress stimuli such as UV or radioactive irradiation, and proinflammatory cytokines/factors. The well-established function of MSK1 involves the regulation of gene expression by phosphorylation of transcription factors, including CAMP-responsive element-binding protein (CREB; ref. 1), ATF1 (1), and the p65 subunit of NF-κB (2). MSK1 has also been reported to phosphorylate histone H3 at Ser10 and Ser28 (3). MSK1 is activated in response to tumor promoters, such as epidermal growth factor (EGF; ref. 4), 12-O-tetradecanoylphorbol-13-acetate (TPA; ref. 5), or UVB (6). It plays a positive role in the control of proliferation of HaCaT keratinocytes (7) and is involved in TPA- or EGF-induced neoplastic transformation of JB6 CI41 cells (4). Stable knockdown of MSK1 caused loss of the malignant phenotype of Ras-transformed mouse fibroblasts, as shown by the absence of...
anchorage-independent growth in soft agar (8). Recently, in vivo evidence showed that MSK1/2 knockout mice developed significantly fewer skin tumors compared with wild-type mice (9). MSK1/2 signaling represents a novel tumor-promoting axis in skin carcinogenesis.

Skin tumor formation occurs in three stages: initiation, promotion, and progression (10). Chemical carcinogenesis in mouse skin has been used for several decades and remains a powerful model for understanding multistage carcinogenesis in humans. The most common chemical carcinogenesis regimen is a two-stage induction that includes an initiating application of 7,12-dimethylbenz (a)anthracene (DMBA), which induces an irreversible and specific mutation in mouse skin. Initiation with DMBA is followed by multiple, regular applications of the phorbol ester, TPA. Alterations in signal transduction pathways, including the aberrant activation of ERKs, were found to contribute to genesis and progression of mouse skin cancer (11). MSK1 is an important downstream effector of the stimulated ERKs pathway and plays a role in the process of carcinogenesis in mouse skin (9). Therefore, inhibiting MSK1 activity might be an effective strategy for skin cancer chemoprevention.

Here, we used virtual screening of a natural products database (NPD) to identify MSK1 inhibitors. We identified compound 69407, a natural compound derivative of chrysin, as a novel MSK1 inhibitor. Our results indicated that compound 69407 is more potent and less toxic than chrysin, as a novel MSK1 inhibitor. Our results indicated that compound 69407 is more potent and less toxic than chrysin. Further studies revealed that compound 69407 exerted a significant antipromotion effect. Further studies revealed that compound 69407 seemed to exert its inhibitory effects on TPA-induced skin tumor promotion through direct inhibition of MSK1/histone H3 signaling. These data suggest that compound 69407 is a potential compound for chemoprevention of skin cancer.

Materials and Methods

General Materials and Methods are included in Supplementary Materials and Methods.

Anchorage-independent cell growth assay

TPA-induced neoplastic transformation was investigated in JB6 P+ cells. JB6 cells (8 × 10^3/mL) were exposed to TPA (10 ng/mL) and compound 69407 (0, 2.5, 5, 10, or 20 μmol/L) in 1 mL of 0.33% basal medium Eagle agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO₂ incubator for 10 or 14 days and colonies were counted under a microscope. Cell transformation is presented as colony number per 8,000 seeded cells in soft agar as described by Colburn and colleagues (12).

In vitro kinase assay

MSK1 and MSK2 in vitro kinase assays were performed as described previously (4) with some modification. Different concentrations of compound 69407 were incubated with active recombinant MSK1 or MSK2 at 30°C for 10 minutes. Then, 1 μg purified CREB or histone H3 was added and reactions were carried out in 1× kinase buffer (25 mmol/L Tris/HCl, pH 7.5, 5 mmol/L β-glycerophosphate, 0.1 mmol/L Na₃VO₄, 10 mmol/L MgCl₂, and 2 mmol/L dithiothreitol) containing 50 μmol/L unlabeled ATP with or without 10 μCi of [γ-³²P] ATP at 30°C for 30 minutes. Reactions were stopped by adding 6× SDS sample buffer. Phosphorylation of CREB was visualized by autoradiography and phosphorylation of the histone H3 protein at Ser10 or Ser28 was detected by Western blotting with specific antibodies. The RSK2 in vitro kinase assay (13) and Aurora B in vitro kinase assay (14) were performed as previously described, respectively.

Tumor induction and treatment

FVB/N mice (6 weeks of age, male) were purchased from Jackson Laboratories. Animals were maintained under "specific pathogen-free" conditions and all animal studies were conducted according to guidelines approved by the KRIIB-IACUC (Korea Research Institute of Bioscience & Biotechnology-Institutional Animal Care and Use Committee). Groups of six mice each were used for the two-stage skin carcinogenesis studies. Mouse skin tumors were induced by the initiation-promotion regimen as previously described (15). The backs of mice were shaved before initiation. All mice received a topical application of a single 200 nmol dose of DMBA in 0.2 mL of acetone to the shaved dorsal skin. Two weeks after initiation, mice were treated topically twice weekly with vehicle (acetone) only or 200 nmol compound 69407 in 0.2 mL acetone followed 30 minutes later by promotion with 17 nmol TPA in 0.25 mL acetone. Mice were weighed, photographed, and tumors measured once a week beginning when first measurable tumors (1 mm³) were observed (week 9). Tumor volume was calculated using the following formula: tumor volume = 4π/3(l/2)(w/2)(h/2), where l is the length, w is the width, and h is the height (15). At the end of the experiment, mice were sacrificed by cervical dislocation and the tumors and skin were removed and snap frozen in liquid nitrogen and lysates were prepared for Western blot analysis.

Results

Compound 69407, a derivative of the natural compound chrysin, inhibits MSK1 kinase activity in vitro

Compound 69407 (Fig. 1A) was identified by the Virtual Screening Workflow docking screen of NPD using the active conformation of the C-terminal kinase domain of MSK1 (PDB id 3KN; ref. 16) as the receptor structure. To confirm the virtual screening result, the effect of compound 69407 on the kinase activity of MSK1 was first investigated using an in vitro kinase assay with GST-CREB, active MSK1, [γ-³²P] ATP, and different doses of compound 69407. The autoradiography results demonstrated that compound 69407 dose dependently inhibited the phosphorylation of...
CREB, indicating that the compound dose dependently suppressed MSK1 activity in vitro (Fig. 1B). Because MSK1 is a kinase of histone H3 at both Ser10 and Ser28 (17), the effect of compound 69407 on MSK1 activity was also assessed by histone H3 in vitro kinase assays. The results indicated that compound 69407 dose dependently inhibited the phosphorylation levels of histone H3 at Ser10 (Fig. 1C) and Ser28 (Fig. 1D). These results further confirmed that compound 69407 inhibits MSK1 activity. The effect of compound 69407 on kinases closely related to MSK1, such as MSK2 and RSK2, was assessed by in vitro kinase assays. The results indicated that compound 69407 did not inhibit MSK2 or RSK2, as assessed by in vitro kinase assays. The effect of compound 69407 on kinases closely related to MSK1, such as MSK2 and RSK2, was assessed by in vitro kinase assays. The results indicated that compound 69407 did not inhibit MSK2 or RSK2, as assessed by in vitro kinase assays.

Compound 69407 directly binds MSK1 kinase in vitro and ex vivo

To verify direct binding of compound 69407 with MSK1, we conducted a pull-down assay with compound 69407-conjugated beads and commercially available active MSK1. The results indicated that compound 69407 bound with MSK1 (Fig. 2A). Moreover, we confirmed in cell lysates that compound 69407 bound with endogenous MSK1 (Fig. 2B). Notably, ATP did not compete with compound 69407 for binding to MSK1. In an ATP competition assay, the binding of compound 69407 to MSK1 did not change as the concentration of ATP increased (Fig. 2C). These results suggest that compound 69407 inhibits MSK1 activity through ATP-dependent mechanisms.
independent binding. To predict the binding motif between compound 69407 and MSK1, we performed flexible-ligand flexible-protein docking using the Induced Fit Docking (IFD) Module (Schrödinger Suite 2011 Induced Fit Docking protocol; Glide v.5.7; Prime v.3.0). IFD has an advantage over regular Glide docking (18) because of its ability to capture the ligand-induced conformational changes in receptor active sites (19). We examined both the ATP-binding site and the allosteric site of the dual kinase domains of MSK1 using the crystal structures of PDB id 1VZO (20) and 3KN5 (16), respectively, as the receptor model structures. The docking results between compound 69407 and N-terminal domain of MSK1 were not good (data not shown). However, the binding affinity between compound 69407 and the C-terminal domain as determined from IFD docking results was predicted to be better in the allosteric site (Fig. 2D), with a score of −11.73 kcal/mol, than the binding in the ATP-binding pocket, with a score of −10.08 kcal/mol. Compound 69407 formed five hydrogen bonds with MSK1 (Fig. 2E). Three of the hydrogen bonds, including one-bifurcated, involve the backbone atoms of two residues Ala531 and Asp565, and the other two forms with the side chain atoms of Asp565 and Lys455, respectively. In addition, 11 residues around the allosteric site, including Val487, Ile469, Thr465, Leu496, Leu484, Leu472, Phe566, Ile481, Val482, Ile563, and Ala531, formed hydrophobic interactions with the carbons of compound 69407 (Fig. 2E). Notably, these residues are mainly
distributed into two hydrophobic clusters, one including Val487, Ile469, Thr465, Leu496, Leu484 and the other including Phe566, Ile481, Val482, Ile563, and Ala531, to form two hydrophobic pockets for accommodating the benzene ring at each side of compound 69407. Therefore, those residues should play an important role in the binding of compound 69407 with MSK1. The computational results corresponded well with the experimental results, suggesting that compound 69407 was an ATP noncompetitive inhibitor of MSK1 and located inside the allosteric-binding site rather than the ATP pocket of MSK1.

**Compound 69407 inhibits growth of JB6 P+ cells by inducing cell-cycle G2–M phase accumulation**

The cytotoxicity of compound 69407 was evaluated using the WST-1 assay. Concentrations of compound 69407 up to 80 μmol/L and exposure for 48 hours had a minimal effect on the viability of JB6 cells (Fig. 3A). To examine whether this compound could affect proliferation, cells were treated with different concentrations of the compound dissolved in dimethyl sulfoxide (DMSO) as vehicle for 24, 48, 72, or 96 hours. Results indicated that compound 69407 strongly inhibited growth in a dose-dependent manner (Fig. 3B). Furthermore, cell-cycle distribution analysis of asynchronously growing JB6 P+ cells treated with various doses of compound 69407 for 48 hours, the fluorescence-activated cell sorting (FACS) result indicated that G2–M arrest occurred, especially at relatively high doses (10 or 20 μmol/L) of compound 69407 (Fig. 3C, *P* < 0.05). The G2–M arrest caused by compound 69407 was not due to the inhibition of Aurora B activity because compound 69407 has no effect on Aurora B kinase activity (Supplementary Fig. S2A and S2B). We observed that treatment with compound 69407 produced a slight blockade of S phase although this was not statistically significant. These results suggested that compound 69407 inhibits cell growth, at least in part, by inducing cell-cycle G2–M arrest.

**Compound 69407 suppresses TPA-induced anchorage-independent growth of JB6 P+ cells**

To examine the effect of compound 69407 on TPA-induced neoplastic transformation of JB6 P+ cells, soft agar
assays were performed. With TPA stimulation, JB6 P+ cells readily form colonies in soft agar, exhibiting an anchorage-independent growth that is a distinctive characteristic of neoplastic transformation (21). Treatment with compound 69407 significantly inhibited TPA-promoted neoplastic transformation in a dose-dependent manner (Fig. 4A). The compound at 5, 10, or 20 μmol/L caused a significant decrease to 49.3%, 24.9%, or 15.9% of the TPA only control (Fig. 4A). The inhibition of colony formation by this compound was not caused by cytotoxicity because the effective concentration range for inhibiting cell transformation did not affect JB6 P+ cell viability (Fig. 3A). To determine whether compound 69407 is comparable with chrysin in its ability to suppress cell proliferation and transformation, we examined the effects of both compounds on proliferation and TPA-induced neoplastic transformation of JB6 P+ cells. Results showed that although either compound effectively inhibited cell proliferation and transformation, compound 69407 was much more effective (Fig. 4B and C). These data indicated that compound 69407 is more potent than chrysin at suppressing proliferation and the anchorage-independent growth capability of JB6 P+ cells.

**Knockdown of MSK1 decreases the sensitivity of JB6 P+ cells to compound 69407**

Because compound 69407 was shown to specifically target MSK1, we then examined whether knockdown of MSK1 expression could influence the sensitivity of JB6 P+ cells to compound 69407. The efficiency of short hairpin RNA (shRNA) knockdown was confirmed by an obvious decrease of MSK1 protein after shRNA transfection (Fig. 5A). Results of cell viability assay indicated that JB6 P+ cells expressing sh-MSK1 were resistant to the inhibitory effect of compound 69407 on growth compared with cells

**Figure 4.** Compound 69407 inhibits TPA-induced anchorage-independent growth of JB6 P+ cells. A, compound 69407 suppresses TPA-induced anchorage-independent growth of JB6 P+ cells. Soft agar assay was performed as described in Materials and Methods. The cultures were incubated for 14 days and then colonies were counted. B, compound 69407 inhibits cell growth more strongly than chrysin. JB6 P+ cells were treated with compound 69407 or chrysin at 10 μmol/L in 5% FBS/MEM and growth was measured at the indicated times using the WST-1 assay. MEM, minimum essential medium. C, compared with chrysin, compound 69407 more strongly inhibits TPA-induced transformation of JB6 P+ cells. Soft agar assay was performed as described in Materials and methods. The cultures were incubated for 14 days and then colonies were counted. Data, mean ± SE of two independent experiments performed in triplicate and significant differences were determined by a t test. The asterisk (*, P < 0.001) indicates a significant difference between cells treated with DMSO plus TPA and cells treated with compound 69407 plus TPA (A); or in cells treated with compound 69407 and cells treated with chrysin plus TPA (C). The asterisks (**, P < 0.01) indicate a significant difference in cells treated with compound 69407 and cells treated with chrysin (B).
expressing sh-Mock (Fig. 5B). In addition, JB6 P+ cells stably transfected with sh-Mock or sh-MSK1 were treated with vehicle or compound 69407 and subjected to a soft agar assay to examine colony formation in response to TPA stimulation. Results showed that compound 69407 (10 µmol/L) inhibited TPA-induced anchorage-independent growth of JB6 P+ cells transfected with sh-Mock by about 75%. In contrast, the inhibition was only about 50% in JB6 P+ cells transfected with sh-MSK1 (Fig. 5C). These results suggested that MSK1 plays an important role in the sensitivity of JB6 P+ cells to the anticancer effects of compound 69407. Notably, sh-MSK cells formed fewer colonies than sh-Mock cells.

**Compound 69407 inhibits MSK1-mediated association of phosphorylated histone H3 with the c-fos promoter in response to TPA in JB6 P+ cells**

MSK1 is a well-known histone H3 kinase responsible for TPA-induced phosphorylation of histone H3 at Ser10 and Ser28 (5, 17). To investigate further whether compound 69407 suppresses proliferation and TPA-induced transformation by inhibiting MSK1 activity in cells, the phosphorylation status of histone H3 at Ser10 and Ser28 was assessed. In addition, because H89 is a well-characterized MSK1 inhibitor, we compared the inhibitory activity of compound 69407 with that of H89 in intact cells. Immunoblot results showed that compound 69407 dose dependently inhibited TPA-stimulated H3 phosphorylation at both Ser10 and Ser28 in JB6 P+ cells (Fig. 6A and B). This effect was not due to the inhibition of the MSK1 upstream ERKs kinase or p38 kinase activation because the phosphorylation of ERKs in response to TPA or the phosphorylation of p38 in response to UV radiation, a much stronger activator than TPA for p38 activation (1, 22), was unaffected by treatment of the cells with compound 69407 (Supplementary Fig. S3A and S3B). In contrast with the blockade of TPA-induced histone H3 phosphorylation at Ser10 and Ser28, compound 69407 had no effect on CREB phosphorylation at Ser133 elicited by TPA (Fig. 6A and B). As an MSK1 inhibitor, H89 had no effect on the phosphorylation of ERKs (Supplementary Fig. S3A), p38 (Supplementary Fig. S3B), or MSK1 at Ser376 (Fig. 6A and B), but slightly inhibited phosphorylation of CREB at Ser133 (Fig. 6A and B), which agrees with a previous report (23). However, both compound 69407 and H89 inhibited TPA-induced phosphorylation of MSK1 at Thr581 (Fig. 6A and B), a site critical for MSK1 activation (24), suggesting that compound 69407 and H89 suppress MSK1 activity by inhibiting the phosphorylation of this site. The UVC-induced phosphorylation of other MSK1 downstream effector molecules, such as the
p65 subunit of NF-kB at Ser276 (25) and CREB at Ser133 (1), was not affected by compound 69407 treatment (Supplementary Fig. S3C). These data indicate that at the concentration used, compound 69407 does not have a nonspecific inhibitory effect upon the activation of upstream ERKs or p38 MAPK. Studies have provided evidence to suggest that the phosphorylation of histone H3 at Ser10 or Ser28 following TPA stimulation plays an important role in mediating transcriptional activation of immediate-early genes such as c-fos and c-jun, which correlates with MSK1 activity (3, 23, 26). Phosphorylation events on Ser10 or Ser28 apparently do not occur together on the same promoter (26), and histone H3 at Ser10 has been shown by chromatin immunoprecipitation (ChIP) assay to associate with the promoter region of the IE c-fos gene upon TPA stimulation (27). We thus examined by ChIP assay whether MSK1-mediated association of phosphorylated histone H3 (Ser10) with the c-fos promoter is affected by compound 69407 treatment. The amount of c-fos promoter DNA present in immunoprecipitated chromatin fractions was then determined by PCR. Immunoprecipitation of equivalent amounts of chromatin from TPA-stimulated cells with the phospho-H3 (Ser10) antibody showed an increase in the association of phosphorylation H3 with the c-fos promoter compared with that of unstimulated cells. Treatment with compound 69407 dose dependently reduced the enrichment of phosphorylation H3 at the c-fos gene promoter in response to TPA stimulation (Fig. 6C). These findings

Figure 6. Compound 69407 inhibits MSK1-mediated association of phosphorylated histone H3 with the c-fos promoter in response to TPA in JB6 P+ cells. A, JB6 P+ cells were serum starved for 36 hours followed by treatment for 1 hour with DMSO, compound 69407, or H89 at the indicated concentrations. Cells were then exposed to TPA (0 or 10 ng/mL) for 10 minutes and harvested. Individual levels of phosphorylated and total proteins were visualized by Western blot analysis with specific antibodies. β-Actin was used as a loading control. B, band density of (A) was measured using the NIH Image J program (Version 1.41). The band intensities of phosphorylated proteins were normalized against their respective total proteins and phospho-CREB was normalized against β-actin. Data, mean ± SD of two separate experiments and significant differences were determined by a t test. *, P < 0.05, compared with their respective DMSO plus TPA-treated control. B, JB6 P+ cells were treated using the same protocol as described in A and subjected to ChIP assays with a phospho-H3 (Ser10)-specific antibody. The precipitated DNA fragments were subjected to PCR analysis to test for the presence of sequences corresponding to the c-fos promoter. Input material (10%) is shown for comparison. Data, mean ± SD of two separate experiments and significant differences were determined by a t test. *, P < 0.05, compared with the DMSO-treated group. **, P < 0.01, compared with the DMSO plus TPA-treated group.
demonstrate that TPA-stimulated phosphorylation of histone H3 (Ser10) at the c-fos promoter is dependent on the integrity of the MSK1 signaling pathway in JB6 P+ cells. The results imply that compound 69407 acts on histone H3, a downstream substrate of MSK1, to attenuate the proliferation and transformation of JB6 P+ cells.

**Compound 69407 attenuates TPA-promoted skin carcinogenesis in mice**

On the basis of the data showing that compound 69407 inhibits TPA-induced transformation of JB6 P+ cells (Fig. 4A), an experiment was conducted to determine whether topical treatment with compound 69407 could inhibit tumorigenesis in vivo. Two-stage skin carcinogenesis experiments with DMBA/TPA were carried out in FVB/N mice to evaluate the effect of compound 69407 on skin tumor development and growth. Groups of FVB/N mice 6 weeks of age were initiated with 200 nmol of DMBA. Two weeks later, mice were treated topically with vehicle (acetone) or 200 nmol compound 69407 in acetone followed 30 minutes later by topical application of 17 nmol of TPA in acetone. The selection of 200 nmol compound 69407 for treatment was based on our prior studies, in which we administered compound 69407 at respective doses of 100, 200, or 500 nmol. At a dose of 100 nmol, the compound only exhibited a weak effect. The doses of 200 and 500 nmol were not much different in their effectiveness (Supplementary Fig. S4A–S4C). All treatments were applied twice weekly for the duration of the experiment. Tumor multiplicity (average number of papillomas per mouse) was measured weekly for each group. Results indicated that compound 69407 exerted an antitumor-promoting effect (Fig. 7A and B). The group receiving topical application of 200 nmol of compound 69407 30 minutes before application of TPA had a significant reduction in papilloma development compared with the DMBA/TPA-only–treated control group (\( P < 0.05 \), Fig. 7B). In addition, mice treated with only TPA developed significantly more and larger skin tumors (Fig. 7C).

![Figure 7. Compound 69407 attenuates TPA-promoted skin carcinogenesis in mice. The shaved dorsal surface of each mouse was initiated by topical application of DMBA (200 nmol) in acetone. After 2 weeks, mice were topically treated with compound 69407 (200 nmol) in acetone followed 30 minutes later with TPA (17 nmol) in acetone. A, representative photographs of papilloma development in TPA (left) or TPA combined with compound 69407 (right) mice. B, average number of papillomas developed per mouse in response to TPA or TPA combined with compound 69407. C, average number of papillomas in mice treated with compound 69407 and TPA or TPA only. D, average volume of papillomas in mice treated with compound 69407 and TPA or TPA only. E, treatment with TPA combined with compound 69407 attenuates histone H3 phosphorylation at Ser10. Tumors were extracted from mouse dorsal skin at week 18 after DMBA treatment and lysates were subjected to SDS–PAGE and levels of phosphorylated and total histone H3 were analyzed by Western blot analysis. Densitometer analysis of each band was performed using the NIH ImageJ software program (Version 1.41). The asterisk (*) indicates a significant (\( P < 0.05 \)) difference.](http://cancerpreventionresearch.aacrjournals.org)
expression level, it is an ideal candidate for cancer research. Western blot results confirmed that tumor lysates from mice treated with compound 69407 and TPA exhibited significantly lower phosphorylation levels of histone H3 (Ser10) than mice treated with TPA alone (P < 0.05; Fig. 7E). These results suggest that topically applied compound 69407 led to inhibition of TPA-induced MSK1 downstream signaling, particularly through the histone H3 protein pathway, which inhibits the formation of skin tumors.

Discussion

The Ras/Raf/MEK/ERKs pathway is critical for neoplastic cell transformation and two-stage mouse skin carcinogenesis (11). MSK1 is an important downstream effector of the stimulated ERKs pathway and plays a role in the process of tumorigenesis in mouse skin (9). We found that TPA was very effective in promoting the transformation of JB6 P+ cells (Fig. 4A), corresponding with the elevation of ERKs (Supplementary Fig. S3A) and MSK1 (Fig. 6A and B) activities and, in particular, phosphorylation of histone H3 at Ser10 and Ser28 (Fig. 6C). The increased MSK1 activation reportedly results in the phosphorylation of several immediate-early genes, including c-fos, c-myc, and c-jun (3, 23, 26, 27). Several lines of evidence have indicated that the aberrant expression of c-Fos and c-Jun of the AP-1 transcription factor causes neoplastic transformation and malignant progression. In addition, transactivation of c-jun and c-fos was significantly diminished in histone H3 Ser10-mutant–overexpressing JB6 Cl41 cells compared with histone H3 wild-type–overexpressing cells (29). Moreover, ChIP assay results indicated that TPA significantly increases MSK1-mediated association of phosphorylated histone H3 with the immediate-early gene c-fos promoter in JB6 P+ cells (Fig. 6C). These results indicated that induction of c-jun and c-fos gene expression is one of the critical mechanisms for neoplastic cell transformation elicited by MSK1-mediated phosphorylation of histone H3 at Ser10. Considering that MSK1 is a link between the signaling cascade and the primary response at the gene expression level, it is an ideal candidate for cancer chemotherapy and has immense potential in the treatment of cancers.

Besides its role in malignant transformation and cancer development, MSK1-mediated H3 phosphorylation plays a role in synaptic plasticity (30) and long-term potentiation (31–33), as well as phase resetting of the circadian clock (34) in postmitotic neurons. In the dentate gyrus of the hippocampus, MSK1-mediated H3 phosphorylation is relevant to the physiology of stress-related memory formation (35), whereas in the striatum, it is linked to the long-term effects of drugs of abuse and physiologic reward-controlled learning (36). A recent study found a deficit of MSK1 expression in the striatum of R6/2 transgenic mouse model of Huntington disease, which accounts for the absence of phosphorylation of histone H3 and is involved in transcriptional deregulation and striatal degeneration (37).

MSK1 is reportedly an efficient kinase for histone H3 in response to TPA, and its activity toward H3 is uniquely sensitive to H89. It strongly inhibits TPA-stimulated histone H3 phosphorylation at Ser10 but has relatively little effect on TPA-stimulated CREB phosphorylation at Ser133 in C3H 10T1/2 cells (23). These earlier findings suggested that MSK1 is a far more efficient kinase toward histone H3 than toward CREB in response to TPA. Similarly, our results showed that H89 has a relatively weak effect on TPA-stimulated phosphorylation of CREB at Ser133, whereas the same dose of H89 completely blocked TPA-induced phosphorylation of histone H3 at both Ser10 and Ser28 in JB6 P+ cells (Fig. 6A and B). In addition, compound 69407 at the same dose nearly completely blocked TPA-induced phosphorylation of histone H3 at both Ser10 and Ser28 in JB6 P+ cells (Fig. 6A and B). Moreover, our ChIP assay result indicated that compound 69407 dose dependently inhibits the MSK1-mediated association of phosphorylated histone H3 with the immediate-early gene c-fos promoter in response to TPA stimulation in JB6 P+ cells (Fig. 6C). The results suggested that the major physiologic target of compound 69407 and H89 might be MSK1/histone H3 rather than MSK1/CREB in response to TPA in JB6 P+ cells. The MSK1 kinase activity toward histone H3 is inhibited by compound 69407, suggesting that inhibition of carcinogenesis in intact cells occurs because compound 69407 acts directly on MSK1, blocking its ability to phosphorylate histone H3.

We found that compound 69407 strongly inhibits TPA-induced H3 phosphorylation at both Ser10 and Ser28 but has no effect on TPA-elicited CREB phosphorylation at Ser133 (Fig. 6A and B). However, as a positive control of MSK1 inhibition, H89 dramatically blocked TPA-elicited H3 phosphorylation and also had a small effect on TPA-stimulated phosphorylation of CREB at Ser133 (Fig. 6A and B), which is in agreement with an earlier report (23). In addition, phosphorylation of the p65 subunit of NF-κB at Ser276 or CREB at Ser133 elicited by UVC irradiation was not affected by compound 69407. However, both could be inhibited by H89 treatment (Supplementary Fig. S3C).

These data seem contradictory to the role of compound 69407 as a MSK1 inhibitor. We hypothesized that this
phenomenon might be due to different mechanisms by which compound 69407 and H89 inhibited MSK1 activity. Compound 69407 inhibits MSK1 activity in an ATP-non-competitive manner (Fig. 2C–E), whereas H89 is an ATP-competitive inhibitor (38). This difference in binding to MSK1 might cause dissimilar conformational changes in the MSK1 protein and might lead to a difference in the affinity MSK1’s interaction with a physiologic substrate and differentially contribute to phosphate transfer. Consequently, their inhibitory effects on downstream substrate phosphorylation are likely different. A recent study, which quantitatively evaluated the specific docking domains involved in mediating interactions between ERK2 and protein substrates and defined the contributions of these interactions to phosphate transfer by using surface plasmon resonance methodology (39), might provide some information that can further support the hypothesis.

Chemoprevention, the use of drugs or natural substances to inhibit carcinogenesis, is an important and rapidly evolving aspect of cancer research that is providing a practical approach to identify potentially useful inhibitors of cancer development. Chrysin (5,7-dihydroxyflavone), a natural flavonoid widely distributed in many plant extracts, honey, and propolis, has been reported to be a potential cancer-preventive agent (40, 41). Interest is increasing in the pharmacologic activity of chrysin and its derivatives (42–44). Herein, we report the chemopreventive potential of a derivative of chrysin, compound 69407. The results from in vitro and in vivo studies support compound 69407 as a novel compound with activity against cell proliferation and TPA-induced neoplastic transformation. Moreover, topical application of compound 69407 before TPA application inhibits tumorigenesis in a two-stage mouse skin model. In addition, compound 69407 displays much lower cytotoxicity compared with H89. More than 90% of JB6 P+ cells died when treated with H89 at 40 μmol/L for only 24 hours (data not shown). However, compound 69407, at concentrations up to 80 μmol/L and exposure times up to 48 hours, had little effect on the viability of cells (Fig. 3A).

The mechanism of action involved in the anticarcinogenesis effects of compound 69407 was also investigated. The results indicated that compound 69407 exerts inhibitory effects on skin tumorigenesis by directly binding with MSK1 and inhibiting MSK1/histone H3 signaling. In conclusion, compound 69407 could be an ideal chemopreventive agent against skin cancer.

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

**Authors’ Contributions**

**Conception and design:** H. Liu, J. Hwang, W. Li, Z. Huang, N.R. Thimme-gowda, K. W. Lee, Z. Dong  

**Development of methodology:** J. Hwang, N.R. Thimme-gowda, K.W. Lee  

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** H. Liu, J. Hwang, W. Li, X. Zhou, Y. Yang, Z. Dong  

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J. Hwang, T.W. Choi, K. Liu, Z. Huang, A.M. Bode, Z. Dong  

**Writing, review, and/or revision of the manuscript:** H. Liu, J. Hwang, Z. Huang, N.R. Thimme-gowda, A.M. Bode, R.L. Erikson, B.-Y. Kim, Z. Dong  

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J. Hwang, T.W. Choi, J.-H. Jang, I.-J. Ryoo, Z. Dong  

**Study supervision:** J. Hwang, K.W. Lee, J.-S. Ahn, R.L. Erikson, B.-Y. Kim, Z. Dong

**Grant Support**
This work was supported by the National Research Foundation of Korea grant funded by the Korea government (MSIP, No. 2010-0029233); Leap Research Program (No. 2010-0029233); World Class Institute Program (WCI 2009-002) funded by the Ministry of Science, ICT and Future planning, Korea; The Hormel Foundation and NIH grants CA120386, R37 CA08164, CA1666011, CA172457, and ES016548; and National Natural Science Foundation of China (No. 31170676).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 11, 2013; revised August 6, 2013; accepted August 26, 2013; published OnlineFirst October 29, 2013.

**References**


A Derivative of Chrysin Suppresses Two-Stage Skin Carcinogenesis by Inhibiting Mitogen- and Stress-Activated Kinase 1

Haidan Liu, Joonsung Hwang, Wei Li, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-13-0133

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2013/10/29/1940-6207.CAPR-13-0133.DC1

Cited articles
This article cites 44 articles, 27 of which you can access for free at:
http://cancerpreventionresearch.aacrjournals.org/content/7/1/74.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerpreventionresearch.aacrjournals.org/content/7/1/74.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.