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Growth Inhibition of Human Lung Cancer Cells via Down-regulation of Epidermal Growth Factor Receptor Signaling by Yuanhuadine, a Daphnane Diterpene from *Daphne genkwa*

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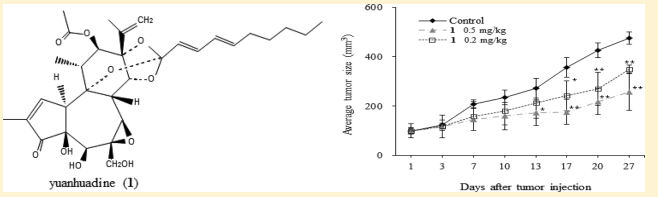
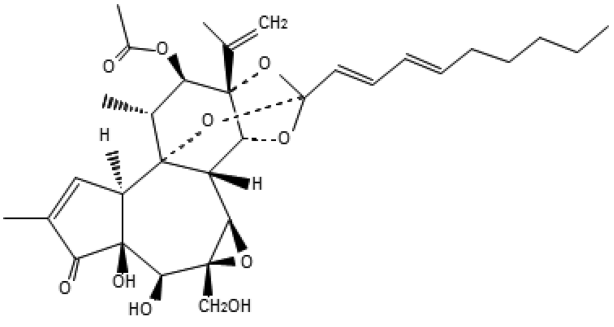
ABSTRACT: The growth inhibition and antitumor activities of yuanhuadine (**1**), a daphnane diterpenoid from the flowers of *Daphne genkwa*, were investigated in human lung cancer cells. Compound **1** exhibited a relatively selective growth inhibition against human lung cancer cells compared to other solid human cancer cell lines. The potent antiproliferative activity by **1** was associated with cell-cycle arrest and modulation of cell-signaling pathways. Cell-cycle arrest in the G0/G1 and G2/M phase was induced by **1** in A549 human non-small-cell lung cancer cells, and these events were correlated with the expression of checkpoint proteins including the up-regulation of p21 and down-regulation of cyclins, cyclin-dependent kinases 2 (CDK2) and 4 (CDK4), and c-Myc. Compound **1** also suppressed the expression of the Akt/mammalian target of rapamycin (mTOR) and its downstream effector molecules including p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1). Ligand-induced epidermal growth factor receptor (EGFR) and c-Met signaling were also inhibited by **1**. The oral administration of **1** (0.5 mg/kg body weight, daily) for 14 days significantly inhibited tumor growth in athymic xenograft nude mouse model bearing human lung A549 cells, without any overt toxicity. Synergistic antiproliferative effects of compound **1** were also found in combination with the EGFR inhibitor gefitinib. Cell-cycle arrest and suppression of Akt/mTOR and EGFR signaling pathways might be plausible mechanisms of actions for the antiproliferative and antitumor activity of **1** in human non-small-cell lung cancer cells.



Lung cancer is the most common form of this disease in the world and the leading cause of cancer-related death.[1] For lung cancer therapy, along with surgery and radiotherapy, chemotherapy is one of the most common treatments. During the past decade, the quality of life and overall survival have been improved significantly in advanced non-small-cell lung cancer (NSCLC) using platinum-based combinations with newer chemotherapeutic agents such as vinorelbine, gemcitabine, and the taxanes.[2,3] Nevertheless, the one-year survival rates are typically 35%, and the two-year survival rates approach only 15−20% in patients with advanced NSCLC.[4] Therefore, the development of novel approaches to prevent and treat lung cancer is an important mission.

The flowers of *Daphne genkwa* Sieb. et Zucc. (Thymelaeaceae) ("Genkwa Flos"), a medicinal plant distributed mainly in mainland China and Korea, have been used traditionally for its abortifacient, anticancer, antitussive, diuretic, and expectorant effects.[5] Phytochemical investigation of this species has revealed as constituents flavonoids, coumarins, and daphnane diterpenoids.[6−8] Daphnane diterpenoids are found only in the plant families Euphorbiaceae and Thymelaeaceae. Abortifacient, antileukemic, neurotropic, and piscicidal effects have been reported for such diterpenoids.[9−11] In an ongoing program to search for anticancer agents from natural products, several daphnane diterpenoids were isolated from *D. genkwa* including yuanhuadine and some new compounds.[12] Yuanhuadine (**1**) has been reported to have moderate neurotrophic activity and inhibitory activities on DNA topoisomerase I and on the growth of human cancer cells.[7,10,13,14] Our group has found that **1** exhibited the most potent antiproliferative activity among several daphnane diterpenoids isolated from *D. genkwa* against human lung cancer cell growth.[12] However, the anticancer potential and underlying mechanism of action of **1** remain to be further elucidated.



yuanhuadine (**1**)

An important target in cancer chemotherapy is the regulation of receptor tyrosine kinase (RTK) and its subsequent downstream signaling pathway, which are associated with the uncontrolled

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Table 1. Inhibitory Effects of Yuanhuadine (1) on the Proliferation of Human NSCLC Cells

	IC_{50} (μM)	
cell line	yuanhuadine (**1**)	ellipticine[a]
A549	12×10^{-3}	1.1
H292	$<10^{-6}$	1.0
SK-MES-1	$<10^{-6}$	0.2
H1993	4.7×10^{-3}	0.2
H1299	5.6	0.6
H358	9.1	0.8
H292-Gef	$<10^{-6}$[c]	NT[b]
H1993-Gef	4.1×10^{-3}[d]	NT

[a] Ellipticine was used as a positive control. [b] NT: not tested. [c] The IC_{50} values of gefitinib (Gef) in H292 and in H292-Gef cells were 0.02 and 0.41 μM, respectively. [d] The IC_{50} values of gefitinib (Gef) in H1993 and in H1993-Gef cells were 0.1 and 20.0 μM, respectively.

proliferation of cancer cells. The Akt/mammalian target of rapamycin (mTOR) kinase cascade is a major signaling pathway of RTK that also has been identified as an important target in cancer chemotherapy.[15,16] Akt regulates cell growth through its effects on the mTOR kinase pathways, as well as on the cell cycle and cell proliferation, through its direct action on the CDK inhibitors p21 and p27 and its indirect effect on the levels of cyclin D1 and p53.[15,17,18]

Epidermal growth factor receptor (EGFR) plays an essential role in normal cell growth and differentiation and is involved in tumor proliferation and survival. EGFR overexpression is a common feature in solid malignancies, including NSCLC, and is associated with poor clinical prognosis.[19] Thus, the EGFR pathway is also an important target in the treatment of advanced NSCLC. Indeed, gefitinib and erlotinib have been developed as therapeutic agents for NSCLC treatment and are small-molecule EGFR-targeting tyrosine kinase inhibitors (TKIs).[20,21]

c-Met is a RTK that is expressed during normal development but has been reported to be altered in a number of malignancies.[22,23] c-Met-dependent signaling in human cancers is activated via binding ligand hepatocyte growth factor (HGF), gene amplification, and mutation. Activation of c-Met signaling can lead to enhanced cell proliferation, motility, angiogenesis, production of reactive oxygen species, and transformation of cells.[24] Because c-Met is involved in a variety of physiological and pathopharmacological signalings, it is important to target this pathway in particular. Recently, small molecules such as SU11274, PHA665752, and PF-2341066 have been reported as potent and selective c-Met inhibitors in the development of anticancer drugs.[24−26] Therefore, the suppression of activated RTK-mediated signaling pathways of cancer cells with natural product-derived compounds might be a plausible approach to regulate the uncontrolled proliferation of cancer cells. In the present study, the antiproliferative potential of **1** and its potential mechanism of action against A549 human NSCLC cells were investigated in both in vitro and in an in vivo animal model.

RESULTS AND DISCUSSION

To determine the effects of yuanhuadine (**1**) on the growth of human lung cancer cells, its growth inhibitory potential was evaluated in a panel of human NSCLC cell lines and drug-resistant lung cancer cells. As summarized in Table 1, compound **1** exhibited a potent antiproliferative effect with IC_{50} values ranging from <1 pM to 9.1 μM. Specifically, A549, H292, H1993, and SK-MES-1 cells were sensitive compared to other NSCLC cells such as H1299 and H358 cells. This difference in potency may be the consequence of genetic background or of the biologically diverse characteristics in these cells. In general, however, compound **1** showed some selectivity for human lung cancer cells when compared to other solid cancer cell lines including colon (HCT-116; 10.2 μM, SW480; 11.8 μM), breast (T47D; 12.7 μM), stomach (SNU-638; >20 μM), liver (SK-HEP-1; 12.9 μM), and prostate (PC-3; 7.4 μM) cancer cells. In addition, **1** also exhibited potential antiproliferative activity in gefitinib-resistant lung cancer cells, and the activity was similar to the corresponding parent cancer cells. Therefore, it is likely that **1** might have the potential to overcome resistance induced by EGFR inhibitors in NSCLC cells. On the basis of its potent antiproliferative activity, further investigation on the mechanism of action of **1** in the regulation of cell proliferation was performed using the NSCLC A549 cell line, which is sensitive to **1** and is one of the most widely used human lung cancer cell lines. When compared to human normal lung epithelial cells (MRC-5), compound **1** inhibited preferentially the proliferation of the A549 cell line (Figure 1A). To determine whether or not the antiproliferative effect of **1** is reversible, cells were exposed to **1** for 24 h, washed, and cultured in drug-free fresh medium for an additional 24 h. Antiproliferative effects were similar in both groups, indicating that the growth inhibitory effect for the A549 cells by **1** is irreversible (Figure 1B). Additionally, cells exposed to **1** for 48 h were compared to assess the time-dependent effects of **1**. The cessation of treatment of **1** stopped any antiproliferative action against the cells, indicating that **1** has a growth inhibitory effect only when present in the cells. These results suggest also that the antiproliferative effect of **1** is irreversible. To further characterize the growth inhibitory action of **1**, A549 cells were treated with this compound for 24 h and were then grown in drug-free medium for 14 days. Colony formation was analyzed after staining with Giemsa. The number of colonies was reduced greatly by treatment with **1** (Figure 1C), indicating that yuanhuadine sustains its inhibitory effect on A549 cells in a significant manner.

Cell proliferation is generally controlled by the progression of three distinctive phases (G0/G1, S, and G2/M) of the cell cycle, and cell-cycle arrest is considered one of most common causes of the inhibition of cell proliferation. To determine whether **1** affects cell-cycle distribution, A549 cells were treated with **1** for 24 or 48 h, and the distribution of cell-cycle progression was analyzed by flow cytometry. When treated with 20 nM **1** for 24 h, the G0/G1 phase was effectively increased from 49.6% to 68.2% and the G2/M phase was also increased from 18.9% to 24.5%, whereas the S phase was decreased significantly from 31.6% to 7.3% (Figure 2A). Cell-cycle arrest in the G0/G1 and G2/M phase was apparent also at 48 h, but the degree of cell-cycle arrest was much lower compared to the data at 24 h. Time- or dose-dependent modulation of cell-cycle progression was not observed in **1**-treated cells, indicating cell-cycle modulation might contribute partially to the growth inhibitory activity of this diterpenoid. In addition, when compound **1** was applied to the relatively high concentration of 100 nM for 24 or 48 h, the sub-G1 peak, indicative of apoptotic cell death, was not detected. This indicated that **1** did not evoke obvious apoptotic cell death when compared to conventional cytotoxic anticancer agents. These data suggest that **1** induces cell-cycle arrest at the G0/G1 and G2/M phases without inducing apoptotic cell death.

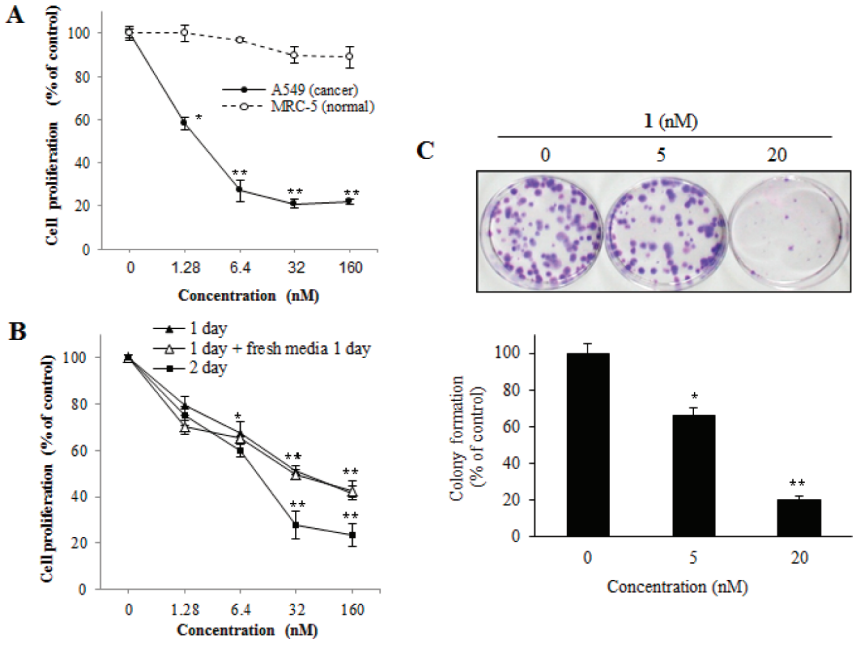

Figure 1. Antiproliferative effects of yuanhuadine (**1**) on A549 human lung cancer cells. (A) Human lung cancer cells (A549) or human normal lung epithelial cells (MRC-5) were plated at a density of 5×10^4 cells/mL in 96-well plates and incubated with the indicated concentrations of **1** for three days. The values of % cell proliferation were calculated by the mean absorbance of **1**-treated cells/absorbance of vehicle-treated control cells. Data are represented as means ± SE ($n = 3$) ($^*p < 0.05$, $^{**}p < 0.01$ indicates statistically significant differences from the control group). (B) Irreversible inhibitory effect of **1** on A549 cell proliferation. Cells were treated with various concentrations of **1** for 24 and 48 h. For 1 day + fresh medium 1 day, cells were treated with **1** for 24 h, and then the cells were washed with PBS and cultured in a drug-free medium for 24 h. Cell viability was measured by the SRB assay. Values indicate means ± SE in triplicate tests. (C) Inhibitory effect of **1** on colony formation. Cells were plated in 35 mm culture dishes (100 cells/dish) and treated with the indicated concentrations of **1** for 24 h. Cells were then washed, and incubations were continued for two weeks in drug-free medium. After staining, colonies were counted and the number of colonies was depicted as shown in the graph.

Cyclins and cyclin-dependent kinases (CDKs) are cell-cycle regulators and trigger their functions through the formation of cyclin−CDK complexes, and the functions are also regulated by CDK inhibitors such as p16, p21, and p27.[27] In particular, the transition from the G1 to the S phase is regulated by the cyclin E−CDK2 and cyclin D1−CDK4 complexes and by inactivation of the pRb tumor suppressor and CDK inhibitors. To examine further whether cell-cycle arrest is associated with the expression of cell-cycle regulatory proteins, cells were treated with various concentrations of **1** for 24 or 48 h, and then Western blot analysis was performed. As shown in Figure 2B, CDK4, which participates in G1 cell-cycle progression, was down-regulated in cells treated with **1**. Down-regulation of CDK2, cyclin E, cyclin A, and c-myc and phosphorylation of Rb expression, along with up-regulation of the CDK inhibitor p21 expression, were shown to be involved in cell-cycle arrest at the G0/G1 phase produced by **1**. In addition, the induction of G2/M phase cell-cycle arrest by **1** was also partially associated with the suppression of cyclin B1 and cell division cycle 2 (CDC2) expression. These findings indicate that **1** induces G0/G1 and G2/M phase cell-cycle arrest by modulating the cell-cycle regulators in A549 cells.

A further study was conducted to correlate the antiproliferative effect of **1** with the regulation of the cellular signal transduction pathway. Accumulating evidence has suggested that the up-regulation of the Akt/mTOR signaling pathway is central to the growth and survival of NSCLC cells.[28] Indeed, A549 cells also possess a constitutively active Akt/mTOR signaling pathway. Akt is a serine/threonine kinase that promotes cell survival and triggers a network that positively regulates the G0/G1 cell-cycle progression through regulation of substrates of Akt such as GSK3β, the forkhead family transcription factors, p21, and c-Myc.[18] As shown in Figure 3A, the treatment of **1** (5 to 40 nM) demonstrated the suppression of the activation of Akt and ERK1/2. Furthermore, **1** caused a significant dose-dependent decrease in not only the phosphorylation of Akt upstream kinase phosphoinositide-dependent kinase-1 (PDK-1) but also its downstream effectors, namely, glycogen synthase kinase-3β (GSK3β), and signal transducer and activator of transcription 3 (STAT3). Therefore, the inhibition of Akt/GSK-3β or Akt/c-Myc signaling by **1** resulted in the suppression of cyclin/CDK4 expression, leading to the dephosphorylation of the Rb protein. These events might thus evoke the arrest of G0/G1 phase cell-cycle progression. In addition, the down-regulation of PDK-1 might enhance the negative regulation of the overall Akt signaling pathway by **1**.

mTOR is another downstream effector of the Akt signaling pathway and a central modulator of cell proliferation in lung cancer cells.[29] Akt sequentially activates mTOR by phosphorylation of mTOR at Ser2448. mTOR has also been known to be involved in the translational initiation of many survival proteins via activating p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E(eIF4E)-binding protein 1 (4EBP1).[30] Therefore, the targeting of mTOR signaling is considered a promising approach for lung cancer chemotherapy. As shown in Figure 3A, compound **1** suppressed the phosphorylation of mTOR and the downstream effectors p70S6K and 4E-BP1. These data suggest that **1** inhibits the proliferation of A549 cells through a blockade of the constitutively activated Akt/mTOR signaling pathway.

Many previous studies have reported that the Akt/mTOR pathway is activated by growth factor receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and c-Met.[22,31]

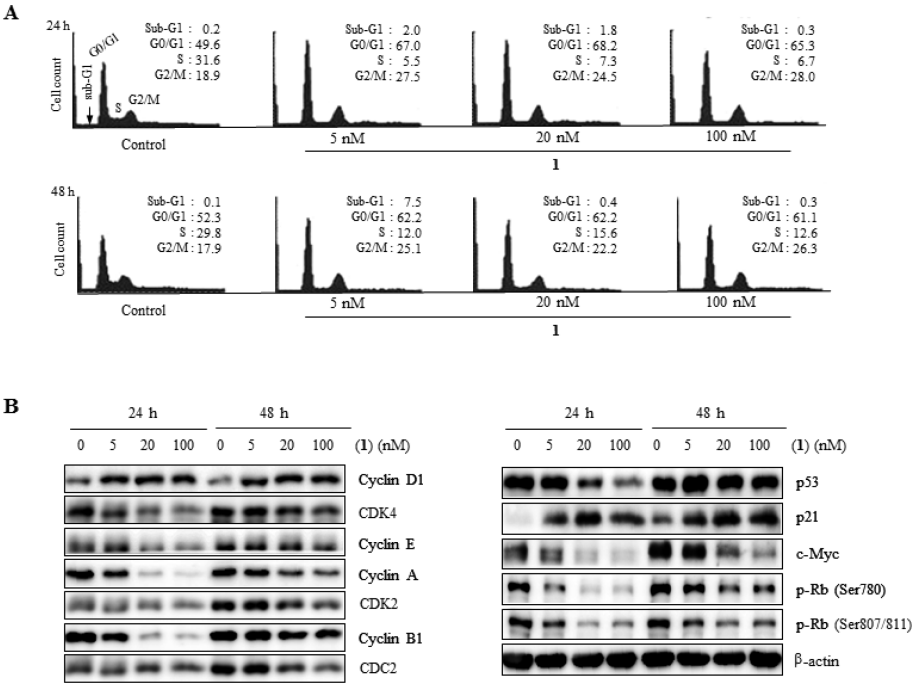

Figure 2. Analysis of cell-cycle distribution by yuanhuadine (**1**) in A549 cells. (A) Cells were treated with **1** for 24 or 48 h. The cell-cycle distribution was analyzed by flow cytometry. (B) Effects of **1** on the expression of biomarkers of cell-cycle progression and cell proliferation. Cells were treated with **1** for 24 and 48 h, and the protein expressions were analyzed by Western blot analysis. Data are representative of three independent experiments.

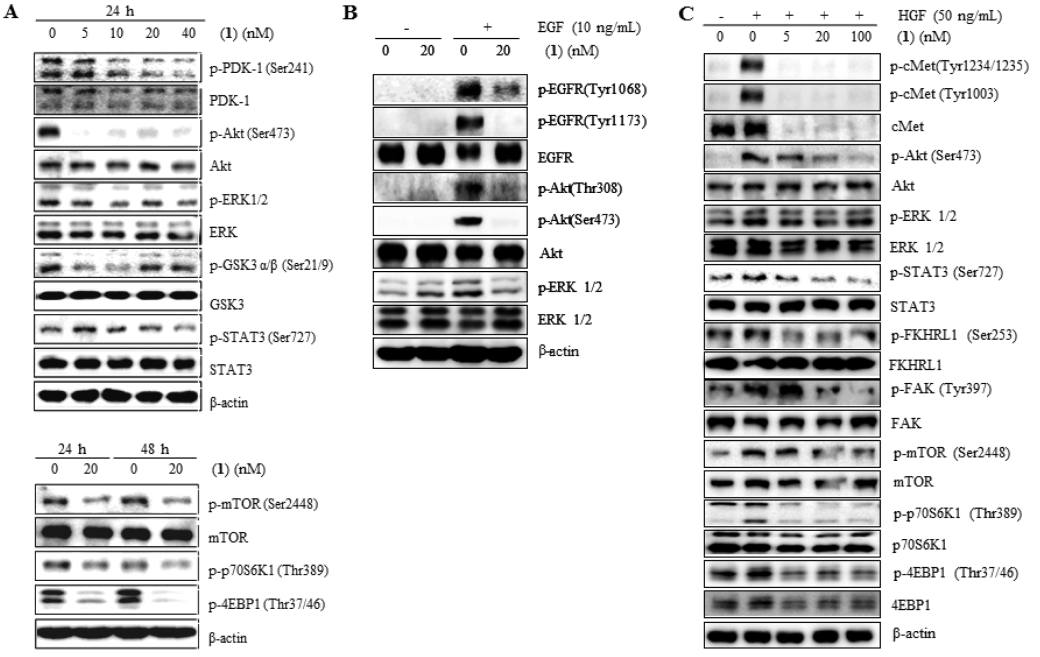

Figure 3. Suppression of cell-signaling pathways by yuanhuadine (**1**) in A549 cells. (A) Inhibition of the Akt/mTOR signaling pathways. Cells were treated with **1** for 24 or 48 h, and protein expression was analyzed by Western blot analysis. (B) Suppression of EGFR and ERK in EGF-stimulated A549 cells. Cells were serum starved for 24 h and then treated with 20 nM **1** for 2 h, followed by EGF (10 ng/mL) stimulation for 10 min. The expression of EGFR, Akt, and ERK was determined by Western blots. (C) Inhibition of c-Met-mediated signaling by **1**. Cells were stimulated with HGF (50 ng/mL, 10 min) in the absence or presence of **1** (5, 20, and 100 nM). c-Met-mediated downstream signaling was analyzed by Western blots.

Epidermal growth factor (EGF) causes phosphorylation of EGFR and leads to the activation of a number of downstream cytoplasmic signaling molecules.[32] Therefore, it was determined further whether **1** affects EGF-activated signal transduction in A549 cells. As shown in Figure 3B, in serum-starved A549 cells, the phosphorylated EGFR level was negligible, but the stimulation of cells with EGF (10 ng/mL) for 10 min effectively induced the phosphorylation of EGFR. The EGF-stimulated activation of EGFR was

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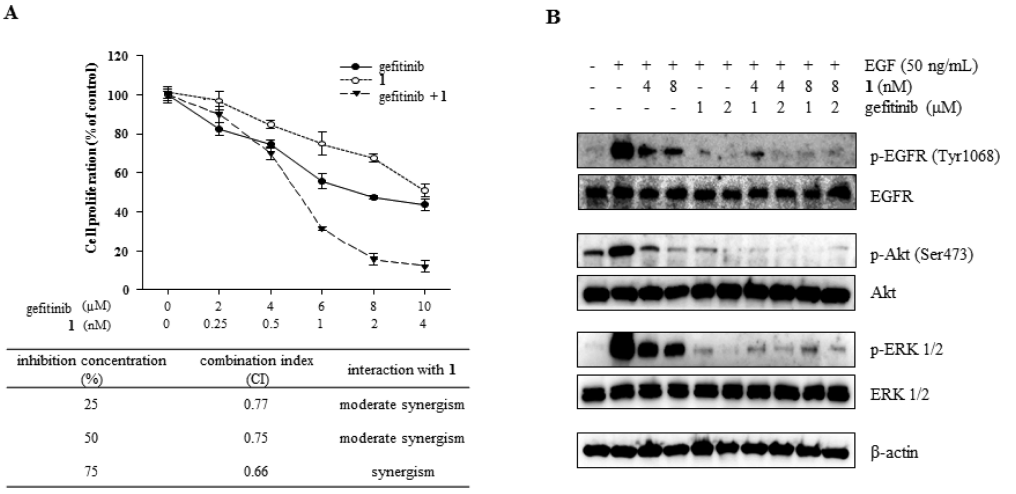

inhibition concentration (%)	combination index (CI)	interaction with 1
25	0.77	moderate synergism
50	0.75	moderate synergism
75	0.66	synergism

Figure 4. Growth inhibitory effect of yuanhuadine (**1**) in combination with gefitinib in A549 cells. (A) Cells were treated with gefitinib, **1**, or in combination for 72 h. Cell viability was measured using the SRB assay. Cell proliferation (% of control) was determined by comparison with vehicle-treated control cells. (B) Suppression of EGFR and its downstream effectors Akt and ERK expressions in combination with **1** and gefitinib. Cells were serum starved for 24 h and then treated with **1** for 2 h, followed by EGF (50 ng/mL) stimulation for 10 min. The protein expressions were determined by Western blots. Data are representative of three independent experiments.

alleviated significantly by pretreatment with 20 nM **1** without changing the relative expression of EGFR. The activation of Akt and ERK1/2, downstream effectors of EGFR, by EGF was also inhibited by **1**. These results suggest that the **1**-mediated antiproliferative activity of cell growth might be associated with the inhibition of EGFR signaling pathway.

c-Met is also classified as the receptor tyrosine kinase family.[22] However, the downstream signaling cascade and the generation of biological responses of c-Met are quite distinctive from those of EGFR. The overexpression or activation of c-Met kinase in NSCLC has been reported, and, in consequence, several recent studies have suggested that suppression of the c-Met signaling pathway in cancer cells may serve as a potential therapeutic target.[24,25]

To investigate the inhibitory effects of **1** on the hepatocyte growth factor/c-Met pathway, A549 cells were pretreated with **1** for 2 h and then stimulated with HGF (50 ng/mL) for 10 min. As shown in Figure 3C, **1** abrogated the HGF-induced autophosphorylation of c-Met at the activation loop site phosphoepitope [pY1230/1234/1235]. Similarly, tyrosine phosphorylation at the phosphoepitope [pY1003] was also inhibited by **1**. In addition, HGF-induced phosphorylation of Akt, ERK1/2, forkhead transcription factor like 1 (FKHRL1), focal adhesion kinase (FAK), STAT3, mTOR, p70S6K, and 4EBP1 were also inhibited by **1**. These results suggest that **1** modulates the activation of HGF/c-Met signaling and its downstream molecules such as Akt/mTOR and FAK in A549 cells.

Several studies have reported the beneficial effects of combined treatment of an EGFR inhibitor gefitinib with c-Met or mTOR inhibitors in preclinical models.[33,34] In the present study, it was found that **1** effectively inhibited the growth of gefitinib-resistant cells and also suppressed the Akt/mTOR, EGFR, and c-Met signaling pathways. Therefore, the combination effect of **1** with gefitinib was examined on the A549 cell growth. A combination of **1** with gefitinib exhibited a synergistic inhibitory effect on the growth of A549 cells (Figure 4A). To characterize further the downstream EGFR signaling that might correlate with the observed synergistic growth inhibition, the expression of several key regulators involved in the EGFR signaling pathway was examined. As shown in Figure 4B, the combination of **1** and gefitinib effectively enhanced the suppression of the expression of EGF-induced phosphorylation of Akt. Collectively, these data indicate that **1** potentiates the antiproliferative activity of the EGFR inhibitor gefitinib in combination in A549 cells.

The in vivo efficacy of **1** was evaluated in a nude mouse tumor xenograft model implanted with A549 human lung cancer cells. A549 cells (3×10^6 cells/mouse) were injected sc into the right flank region of female nu/nu mice. When the tumor size reached ca. 100 mm^3, **1** was administered orally once a day for 14 days. Tumor volume in the control group was about 500 mm^3 on day 27 after treatment was started. However, yuanhuadine (0.2 or 0.5 mg/kg) significantly inhibited tumor growth, and the inhibitory effect in the **1**-treated group (0.5 mg/kg) was approximately 50% compared with the vehicle-treated control group (Figure 5A). No overt toxicity or body weight change was apparent in the treatment of **1** compared to the control group (Figure 5B).

In summary, the present study has demonstrated the potent growth inhibitory activity of the diterpenoid yuanhuadine (**1**) in both cell culture and an in vivo tumor xenograft model against human non-small-cell lung cancer cells. A plausible mechanism of action for the antiproliferative activity of **1** has been proposed for the first time and involves cell-cycle arrest and suppression of the Akt/mTOR, EGFR, and cMet signaling pathways. Yuanhuadine (**1**) may be suggested as a promising new chemotherapeutic candidate for the management of non-small-cell lung cancer.

EXPERIMENTAL SECTION

General Experimental Procedures. Fetal bovine serum (FBS), an antibiotics−antimycotic solution, and trypsin−EDTA were purchased from Invitrogen (Grand Island, NY). Bovine serum albumin, sulforhodamine B (SRB), trichloroacetic acid, propidium iodide, mouse monoclonal anti-β-actin antibody, and ellipticine were obtained from Sigma-Aldrich (St. Louis, MO). Gefitinib was provided by AstraZeneca (Wilmington, DE). Mouse monoclonal anti-phospho-ERK (Tyr 204), anti-p53, rabbit polyclonal anti-cyclin A, anti-cyclin B1, anti-CDK2,

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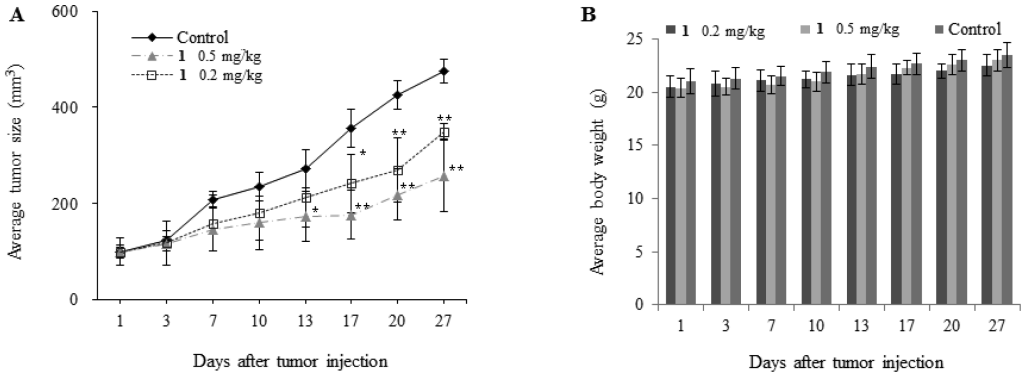

Figure 5. Inhibition of tumor growth by yuanhuadine (**1**) in an A549 xenograft model. (A) A549 cells (3×10^6 cells) were injected subcutaneously into the right flank of nude mice. When tumor volumes reached ca. 100 mm^3, treatment was initiated. Compound **1** (0.2 or 0.5 mg/kg) was administered orally daily for 14 days. Tumor volumes were measured with a caliper every 2−3 days ($^*p < 0.05$, $^{**}p < 0.01$ indicate statistically significant differences from the control group). (B) Body weight changes of the mice were monitored during the experiments.

anti-CDK4, anti-CDC2, anti-p21, anti-p27, anti-ERK1 (p44), anti-ERK 1/2, anti-STAT3, anti-EGFR, and anti-cMet were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-cyclin D1, anti-cyclin E, and anti-GSK-3β antibodies were purchased from BD Biosciences (San Diego, CA). Rabbit polyclonal anti-phospho-Akt (Ser 473 and Thr 308), anti-Akt, anti-phospho-GSK-3α/β (Ser 21/9), anti-phospho-mTOR (Ser 2448), anti-mTOR, anti-phospho-cMet (Tyr 1003 and Tyr 1234/1235), anti-phospho-p70S6K (Thr 389), anti-p70S6K, anti-phospho-4EBP1 (Thr 37/46), anti-4EBP1, anti-EGFR (Tyr 1068, Tyr 1173), anti-phospho-PDK1 (Ser 241), anti-PDK1, and anti-phospho-STAT3 (Ser 727) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-FAK was purchased from Biosource (San Diego, CA). Yuanhuadine (**1**; purity >98.5%) was isolated and characterized from a $CHCl_3$-soluble extract of the flowers of *Daphne genkwa*, as described previously.[12]

Cell Culture. The human non-small-cell lung cancer cell lines, A549, SK-MES-1, and H358, were provided from the Korean Cell Line Bank (Seoul, Korea), and H292 and H1993 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotic−antimycotic solution (100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 250 ng/mL amphothericin B). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Proliferation Assay. Cells (5×10^4 cells/mL) were treated with various concentrations of yuanhuadine (**1**) for 3 days. After treatment, cells were fixed with 10% TCA solution, and cell viability was determined with a sulforhodamine B (SRB) assay.[35] The results are expressed as percentages, relative to solvent-treated control incubations, and IC_{50} values were calculated using nonlinear regression analysis (percent survival versus concentration).

Colony Formation Assay. A549 cells were plated in a 35 mm culture dish at a density of 100 cells/dish. Twenty-four hours later, fresh medium containing compound **1** was added to culture dishes. After treatment for 24 h, the cells were washed with PBS and allowed to grow in drug-free medium for 14 days. Colonies were fixed with methanol, stained with Giemsa (Fisher Scientific, Itasca, IL), enumerated, and expressed as a percentage, relative to DMSO-treated controls.[36]

Analysis of Cell Cycle Distribution. The analysis of cell-cycle dynamics was performed by flow cytometry as described previously.[36] Briefly, A549 cells were plated at a density of 1×10^5 cells per 100 mm culture dish and incubated for 24 h. Fresh medium containing test samples was added to the culture dishes. After 24 or 48 h, adherent and floating cells were harvested, washed twice with PBS, fixed with 100% methanol, and incubated with a staining solution containing 0.2% NP-40, propidium iodide (50 μg/mL), and RNase A (50 μg/mL) in phosphate-citrate buffer (pH 7.2) for 30 min at room temperature. Cellular DNA content was analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). At least 20 000 cells were used for each analysis, and the results are displayed as histograms. The distribution in each phase of the cell cycle was determined using the ModFit LT 2.0 program.

Western Blot Analysis. Cells were incubated with various concentrations of **1**. The proteins from cell lysates were resolved by 6−15% SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). Membranes were blocked with blocking buffer (5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature. After washing three times with PBST, membranes were incubated with primary antibodies diluted in 3% nonfat dry milk in PBST (1:200−1:2000) overnight at 4 °C. Membranes were washed three times with PBST and incubated with corresponding secondary antibodies diluted in 3% nonfat dry milk in PBST (1:1000−1:5000) for 2 h at room temperature. Membranes were washed three times with PBST and detected using ECL reagent (Lab Frontier, Suwon, Korea). Blots were imaged by LAS 3000 (Fuji Film Corp., Tokyo, Japan).

Analysis of Combination Effect. Cells (1×10^4 cells/well) were plated in 96-well plates with various concentrations of **1** and gefitinib. After 72 h incubation, the growth inhibition was measured by the SRB assay. The combination effect of **1** plus gefitinib was analyzed by the calculation of the combination index (CI), which was calculated using the following equation: $CI = D_1/(D_x)_1 + D_2/(D_x)_2$. Thus, D_1 and D_2 are the concentrations of **1** and gefitinib in the mixture that achieve the expected effect, and $(D_x)_1$ and $(D_x)_2$ are the concentrations that achieve the same effect when the compounds are used alone. In this study, the effective level was chosen at 50% inhibition. The calculated CI was then compared to the reference values reported by Chou.[37]

In Vivo Tumor Xenograft Study. Female nude mice (5 weeks old, BALB/c-nu (nu/nu)) were purchased from Central Laboratory Animal, Inc. (Seoul, Korea) and maintained in pathogen-free conditions. All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Ewha Womans University, as approved by the Korean Association of Laboratory Animal Care (permission number: EWHA2007-2-14). A549 cells were injected subcutaneously into the flanks of the mice (3×10^6 cells in 200 μL of medium), and tumors were allowed to grow. When the tumor volume reached approximately 100 mm^3, treatment was initiated. The mice were randomized into vehicle control and treatment groups of five animals per each. Compound **1** (0.2 or 0.5 mg/kg body weight) dissolved in a volume of 100 μL of solution (ethanol−Tween 80−H_2O, 1:1:98) was administered orally once a day for 14 days. The control group was treated with an equal volume of vehicle. Tumor volume was

monitored for 27 days three times per week using calipers, and tumor volume was estimated according to the following formula: tumor volume (mm^3) = 3.14 × L × W × H/6, where L is the length, W is the width, and H is the height.

Statistical Analysis. Data are presented as means ± SE for the indicated number of independently performed experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with the Dunnett's t-test.

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