JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Vanillin Suppresses Metastatic Potential of Human Cancer Cells through PI3K Inhibition and Decreases Angiogenesis in Vivo

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Vanillin, a food flavoring agent, has been shown to suppress cancer cell migration and metastasis in a mouse model, but its mechanism of action is unknown. In this report, we have examined the antimetastatic potential of vanillin and its structurally related compounds, vanillic acid, vanillyl alcohol, and apocynin on hepatocyte growth factor (HGF)-induced migration of human lung cancer cells by the Transwell assay. Vanillin and apocynin could inhibit cell migration, and both compounds selectively inhibited Akt phosphorylation of HGF signaling, without affecting phosphorylation of Met and Erk. Vanillin and apocynin could inhibit the enzymatic activity of phosphoinositide 3-kinase (PI3K), as revealed by an in vitro lipid kinase assay, suggesting that inhibition of PI3K activity was a mechanism underlying the inhibitory effect on cancer cell migration, and the presence of an aldehyde or ketone group in the vanillin structure was important for this inhibition. Vanillin and apocynin also inhibited angiogenesis, determined by the chick chorioallantoic membrane assay.

KEYWORDS: Vanillin; apocynin; PI3K; cell migration; signal transduction; antimetastatic; antiangiogenesis

INTRODUCTION

A major cause of death in cancer patients is "metastasis", where cancer cells in the primary tumor invade surrounding tissues and penetrate into blood and lymphatic vessels, allowing them to reach distant sites. Cancer invasion is a key event in metastasis and is considered to be a therapeutic target for cancer treatment and chemoprevention. Dysregulation of cell migration can lead to cancer invasion. Antimetastatic therapy requires long-term treatment using agents with little or no cytotoxic activity to retard the invasion of cancer cells.

Vanillin, the major component of natural vanilla flavor widely used in foods, beverages, drugs, and perfumes, has been given GRAS (generally regarded as safe) status by the Flavor and Extract Manufacturers Association (FEMA) and is recognized as suitable for food use by the Food and Drug Administration (FDA) (*1*). The oral LD_{50} of vanillin in rat has been reported as 1.58−2.8 g/kg (*1*), and the concentrations of vanillin used in food and beverage products cover a broad range of 0.3−33 mM (*2*). The chemopreventive properties of vanillin include scavenging reactive oxygen species and preventing oxidative damage of lipids and proteins (*2*), decreasing mutagenesis (*3*), inhibiting procarcinogen activation and enhancing detoxification of aflatoxin in rats (*4*), and decreasing the rate of chemical-induced carcinogenesis in rats (*5*). Recently, we reported that mice implanted with 4T1 mouse mammary adenocarcinoma cells showed decreased lung metastasis, after oral administration with vanillin [100 mg/(kg day) for 1 month], and in vitro studies showed that vanillin inhibited invasion and migration of the breast cancer cells (*6*).

In human, rat, and rabbit, vanillin is metabolized by the liver, predominantly by oxidation of the aldehyde group catalyzed by aldehyde oxidase to yield vanillic acid, and to a lesser extent by reduction of the aldehyde group, yielding vanillyl alcohol as a minor metabolite (*7*). Our previous studies showed that vanillin had an inhibitory effect on cancer cell invasion and migration, while vanillic acid did not show this inhibition, suggesting that the aldehyde group at the C-1 position of vanillin is important for the inhibitory effect (*6*).

Hepatocyte growth factor (HGF) is a cytokine produced by mesenchymal cells, which has paracrine action on epithelial cells in tissues. Fibroblast-derived HGF enhances invasion of several

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10.1021/jf803366f CCC: $40.75
Published on Web 03/10/2009

carcinoma cell types (*8*). Elevated HGF levels are observed in the pleural fluid of lung cancer patients, and they appear to be responsible for stimulatory effects of the fluid on in vitro invasion and migration of human lung cancer cells, suggesting that HGF may play an important role in metastasis of lung cancer (*9*). HGF binds to its receptor (Met) and regulates cell migration through activation of phosphoinositide 3-kinase (PI3K)/Akt and Ras/Erk signaling pathways, leading to Actin reorganization, cell polarity formation, and cytoskeleton contraction (*10*).

PI3Ks are a group of ubiquitously expressed lipid kinase enzymes which are important players in a major pathway of cell signaling. The constitutive activation of PI3K/Akt signaling is frequently found in cancer (*11*). Among the members of the PI3K superfamily, class I PI3Ks are responsible for cell migration regulated by growth factor receptors (*12*). The important role of PI3Ks in metastasis has been demonstrated by using the siRNA technique, where intravenous administration of siRNA specific to class I PI3K (p110 alpha) resulted in suppression of metastasis of colon cancer cells in a mouse model (*13*). One member of the PI3K superfamily is DNA-dependent protein kinase (DNA-PK), a key enzyme in DNA double strand break repair, which contains a catalytic subunit (DNA-PK_{cs}) sharing the structure of the catalytic core domain with PI3Ks (*14*). Since vanillin has been reported to be a novel inhibitor of DNA-PK_{cs} (*15*), we hypothesized that vanillin could also inhibit PI3K activity and suppress PI3K-dependent behavior of cancer cells, such as cell migration.

During cancer progression, a tumor can induce formation of new blood vessels from pre-existing capillaries (tumor angiogenesis) for oxygen and nutrient feeding and also providing an escape path, so metastasis can be suppressed by inhibition of angiogenesis. Angiogenesis takes place after activation of endothelial cells by angiogenic stimuli, so that the activated endothelial cells degrade the basement membrane and extracellular matrix, proliferate, and migrate into new areas, finally differentiating to form new vascular tube structures. Induction of angiogenesis by a variety of angiogenic stimuli, such as VEGF, HGF, basic fibroblast growth factor, and angiopoietins, is mediated by PI3K/Akt signaling (*11*). Most steps in angiogenesis are similar to cancer invasion, and many signaling pathways and processes used by invading cancer cells are also used by activated endothelial cells during angiogenesis: PI3K signaling is the key player in both (*11*). Thus, if vanillin can affect PI3K activity, it is interesting to see whether vanillin can also inhibit angiogenesis.

In the present work, we have investigated the effects of vanillin and its derivative compounds on HGF-induced cell migration and phosphorylation of Met, Akt, and Erk in A549 human lung cancer cells, as well as their inhibitory effects on PI3Ks enzymatic activity and in vivo angiogenesis. The compounds studied included vanillin, vanillic acid, vanillyl alcohol, and apocynin, which differ in the functional group attached to the C-1 position.

MATERIALS AND METHODS

Chemicals and Antibodies. Vanillin, vanillic acid, vanillyl alcohol, apocynin, ascorbic acid, dimethylsulfoxide (DMSO), agarose type I, and protease inhibitor cocktail for use with mammalian cells and tissue extracts were purchased from Sigma-Aldrich (St. Louis, MO). Wortmannin and PD98059 were obtained from Calbiochem (San Diego, CA). All the test compounds were dissolved in DMSO and kept as stock solutions at −20 °C. The final concentration of DMSO was kept below 0.2% (v/v) throughout the study. Cell culture medium, antibiotics, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Hepatocyte growth factor was purchased from R&D system (Minneapolis, MN). Primary antibody against Met (SC-12) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), primary antibodies against phospho-Met (Y1230/1234/1235), Akt, phospho-Akt (S473), Erk1/2, and phospho-Erk1/2 (T202/Y204) were obtained from Cell Signaling Technology (Beverly, MA). Rhodamine-conjugated phalloidin and 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Molecular Probes (Eugene, OR).

Cell Culture. Human lung adenocarcinoma cell line (A549) was purchased from the American type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 containing 25 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, 12.5 μg/mL amphotericin B, and 10% FBS in humidified atmosphere, 95% air, 5% CO_2 at 37 °C.

Cell Viability Assay. Cell viability was determined by the MTT method (*16*) with some modifications. Briefly, cell suspensions in culture medium were seeded in 96-well plates (100 μL/well) and incubated at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h, additional medium (100 μL) containing test sample was added to each well, followed by further incubation for 24 h. Then, the wells were replaced and incubated with fresh culture media containing MTT (0.5 mg/mL) for 2 h at 37 °C. Finally, the media were removed and DMSO was added to the wells (100 μL/well), and absorbance was measured at 550 nm in a microplate reader, subtracted with absorbance at 650 nm. The number of viable cells was determined from the absorbance. Assays were performed in quadruplet wells. Data were expressed as percent viability compared with control.

Cell Migration Assay. Cell migration was assayed in Transwell cell culture chambers with 8.0 μm pore size (Costar, Cambridge, MA), as previously described (*6*) with some modifications. Briefly, cell suspension in culture medium containing the test compound was incubated for 30 min, at 37 °C, and then seeded into the upper chamber at 5×10^4 cells (200 μL/well). Culture media (500 μL) containing the same concentration of the test compound and HGF (10 ng/mL) was added into the lower chamber. After incubation for 24 h in humidified atmosphere, 95% air, 5% CO_2 at 37 °C, the cells on the upper surface were removed by wiping with a cotton swab, and the filter was fixed with 25% methanol and stained with 0.5% (w/v) crystal violet solution. The filters containing the stained cells were removed from the Transwell chambers and individually transferred to separate wells in a 96-well plate. The crystal violet dye retained on the filters was extracted with a solution of 0.1 N HCl in methanol, and the absorbance was measured at 550 nm using a microplate reader. The number of migrated cells was determined from the absorbance of crystal violet dye extracted from the filters. Assays were performed in duplicate wells. Data were expressed as percent migration compared with control.

Fluorescence Microscopy. Filamentous Actin (F-Actin) and cell nuclei were stained with phalloidin and DAPI, respectively, and observed by using a fluorescence microscope. Cells were seeded on glass coverslips placed in 6-well plates and allowed to adhere for 4 h. Cells were pretreated with the test compounds for 30 min before incubation with HGF (10 ng/mL) for 20 h in humidified atmosphere, 95% air, 5% CO_2 at 37 °C. Cells were washed with phosphate buffer saline (PBS), fixed in a freshly prepared solution of 4% paraformaldehyde and 3% sucrose in PBS, for 30 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS, for 1 h at room temperature. Cells were stained with rhodamine-conjugated phalloidin and DAPI for 30 min, and washed with PBS. Then the coverslips were mounted with 75% glycerol in PBS on glass slides. Images were captured by using an inverted fluorescence microscope (Nikon Eclipse TE 2000-U, Japan).

Western Blot Analysis. A monolayer of cells were starved in serum-free medium containing 0.5% bovine serum albumin for 24 h, and the cells were pretreated with the test compounds for 30 min before HGF stimulation (10 ng/mL) for a further 30 min. Cells were washed with PBS, scraped and collected by centrifugation, and lysed by sonication in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM Na_3VO_4, protease inhibitor cocktail). The protein concentration of the cell lysate was determined using the Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA). Cell lysates (30−50 μg protein) were subjected to electrophoresis in 7.5% SDS-

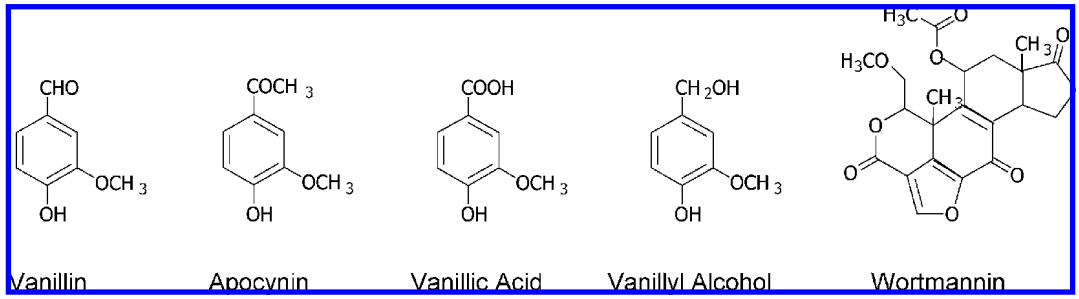

Figure 1. Chemical structure of vanillin derivatives and wortmannin—a specific PI3K inhibitor.

PAGE. Proteins were electrophoretically transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, U.K.), and the membrane was immunoblotted with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody (antirabbit IgG). Bands were visualized using ECL reagents (GE Healthcare). Band intensity on scanned films was quantified using Bio-Rad Quantity One software (Bio-Rad Laboratories) and expressed as relative intensity compared with control.

PI3K Assay. The inhibitory effect of test compounds on enzymatic activity of PI3K isoforms was determined by an in vitro lipid kinase assay (KinaseProfiler; Millipore, Dundee, U.K.), at ATP concentration equal to K_m for each isoform. Assays were performed in duplicate. Data were expressed as percent kinase inhibition compared with control.

DPPH Radical Scavenging Assay. The antioxidant activity of test compounds was determined from the capability to scavenge free radicals by using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as previously described (*17*) with some modification. Briefly, DPPH, a stable radical compound (Fluka-Chemika, Buchs, Switzerland), was dissolved in ethanol to a final concentration of 100 μM; this solution (195 μL) was mixed with 5 μL dilutions of test compound (in DMSO) in a 96-well plate and left at room temperature for 30 min. After that, the residual DPPH radicals were determined from absorbance measured at 515 nm in a microplate reader. Ascorbic acid (final concentration of 500 μM) was used as a reference of 100% radical scavenging, and solvent control was used as 0% radical scavenging. Assays were performed in triplicate wells. Data was calculated as percent radical scavenging. Concentrations that were required for 50% radical scavenging (SC_{50}) were extrapolated from the plot of % radical scavenging versus concentration.

Agarose Pellet Preparation. The test compound (dissolved in DMSO) was mixed with warm 2% agarose solution to a final concentration of 1.8% agarose solution; then the gel solution (10 μL) was allowed to solidify on 5 mm diameter Teflon rods.

Chick Chorioallantoic Membrane (CAM) Assay. In vivo antiangiogenic activity was assessed by the CAM assay as previously described (*18*). The antiangiogenic activity is determined from inhibition of minor blood vessel formation on CAM between day 3 and day 4 of chick embryo development. Test compound was applied as agarose pellets placed on the CAM of day 3 eggs. Briefly, fertilized chicken eggs (White leghorn, Freddy's Hühnerhof GmbH & Co. KG, Mainz, Germany) were incubated for 3 days at 37.8 °C and 75% relative humidity, in an automatically turning egg incubator (Ehret, Germany). On day 3, a hole was created at the base of the egg for removal of albumen (8−10 mL); then the hole was sealed with tape. A window was created in the upper surface of the egg by using an egg shell cutter to uncover the underlying CAM, and the window was sealed with transparent tape. The eggs were further incubated without turning at 100% humidity. After 2 h, the agarose pellets containing the test compound were placed on the area vasculosa of the CAM, and the eggs were returned to the incubator for a further 24 h incubation. At the end of the experiment, the pellets were removed and the CAMs were photographed using a digital camera attached to a stereomicroscope (Sony XC ST 50 monochrome CCD/Stereomicroscope Leica MZ 7-5, Germany). Areas of blood capillaries (angiogenesis) were analyzed and calculated from the CAM images with the customized image analysis software Leica QWin (Leica Microsystems, Bensheim, Germany), and at least 10 eggs for each sample were used for this calculation. The data were expressed as percent angiogenesis compared with the control.

Statistical Analysis. Data are expressed as mean ± SD and analyzed by the Student *t*-test to determine the significance of differences between groups. A p-value < 0.05 was considered to be significant.

RESULTS

Effect of Vanillin and Its Derivatives on HGF-Induced Cell Migration. Vanillin, apocynin, vanillic acid, and vanillyl alcohol differ in the functional group at the C-1 position of their structures (**Figure 1**). The effect of the test compounds on the migration of A549 cells was studied in Transwell chambers in the presence of test compounds for 24 h, with HGF (10 ng/mL) being used as a chemoattractant. Treatment with wortmannin (a specific PI3K inhibitor) inhibited cell migration, with 34% inhibition observed at 5 μM (**Figure 2**), indicating the role of PI3Ks in HGF-induced A549 cell migration.

In the range of noncytotoxic concentrations (cell viability of all the treatments remained >77%), vanillin and apocynin exhibited higher potency for inhibition of HGF-induced cell migration than vanillic acid and vanillyl alcohol. Among the test compounds which differ in the functional group at the C-1 position, 35−57% inhibition was observed for vanillin and 20−40% inhibition was observed for apocynin over the range of test concentrations used (1−4 mM), in a dose-dependent manner (**Figure 2**). However, vanillic acid and vanillyl alcohol treatment (1−4 mM) had a marginal effect and did not change in a dose-dependent manner, with the inhibitory effect of vanillic acid and vanillyl alcohol observed at the highest test concentration (4 mM) being 17% and 8%, respectively (**Figure 2**). Marked inhibition of cell migration by vanillin and apocynin could be observed at the lowest test concentration (1 mM), where the cell viability was 97% (**Figure 2**). Comparison between vanillin and apocynin at each concentration in terms of inhibition of cell migration showed no statistically significant difference between vanillin and apocynin at all concentrations studied. The results indicated that the carbonyl group at the C-1 position in vanillin and its derivatives has an important role in the inhibitory effect on cell migration, with the aldehyde or ketone group being more potent than the carboxylic group.

During cell migration, the cells become polarized in the direction of the gradient of the stimulating agent, and they extend membrane protrusions such as lamellipodia at the leading edge: these morphological changes are driven by cytoskeleton reorganization and local Actin polymerization. As shown in **Figure 3**, HGF stimulation (10 ng/mL) for 20 h induced morphological changes in A549 cells, as revealed by the polarized cell shape. Lamellipodia formation was observed in the HGF-stimulated cells after F-Actin staining, compared with the case for unstimulated cells, which did not migrate and were almost unpolarized (**Figure 3**). Pretreatment of cells with 4 mM vanillin

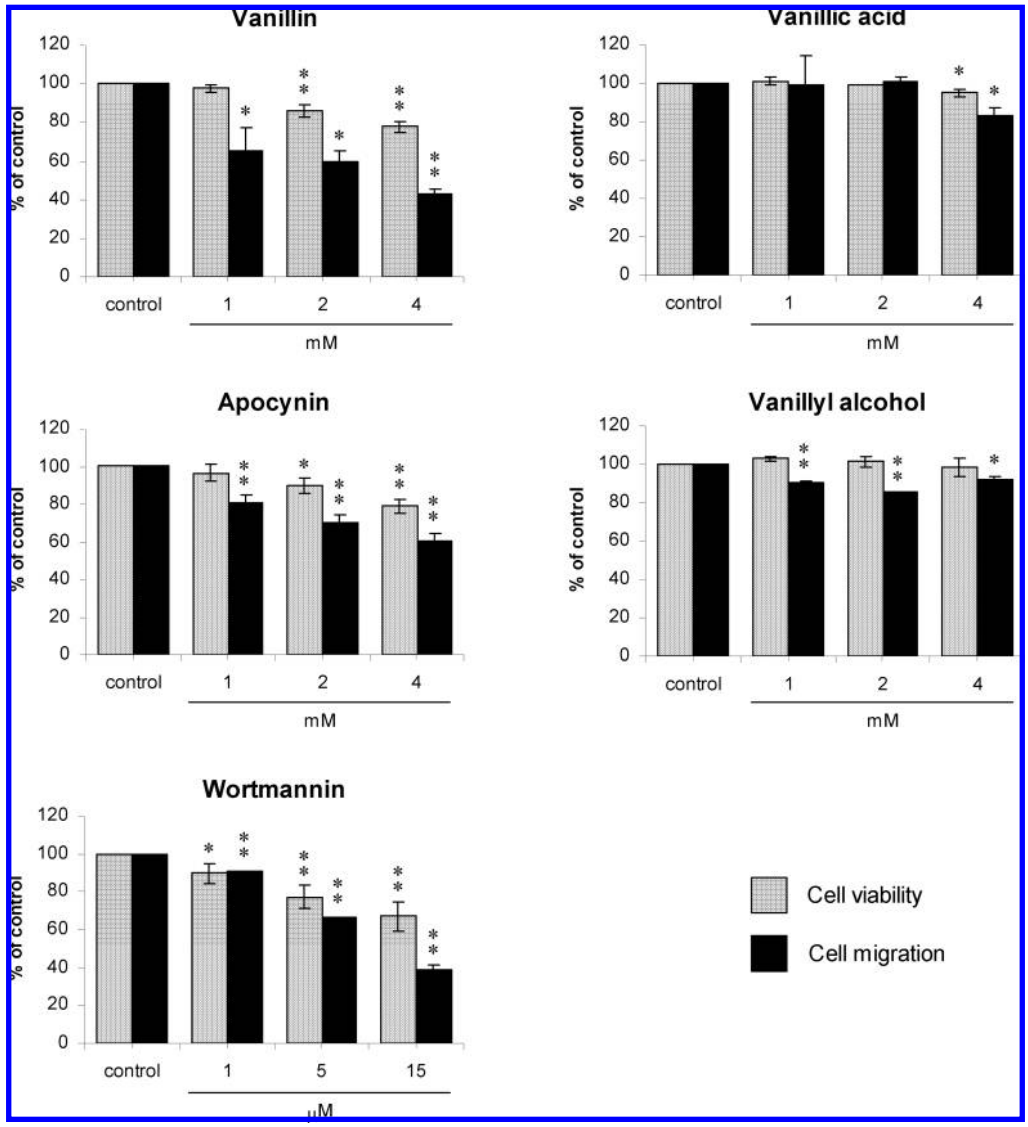

Figure 2. Inhibitory effect of vanillin and its derivatives on HGF-induced cancer cell migration. A549 cells were allowed to migrate in Transwell chambers in the presence of test compounds for 24 h, with HGF (10 ng/mL) being used as a chemoattractant. The number of migrated cells was determined by the colorimetric method, as described under Materials and Methods. Cell viability after a 24 h treatment with test compounds was determined by the MTT method. Data are expressed as mean ± SD from two independent experiments. Significant differences from control are shown by * ($p < 0.05$), ** ($p < 0.01$).

or apocynin before HGF stimulation decreased the number of polarized cells and inhibited morphological changes (**Figure 3**). Vanillin and apocynin treatment for 20 h did not impair the nuclear integrity of the cells, indicating that the compounds did not induce cell death under conditions where cell migration was inhibited, so the antimigration effect of vanillin and apocynin was independent of cytotoxic activity.

Effect of Vanillin and Its Derivatives on HGF-Met Signaling Pathways. We hypothesize that vanillin may possibly inhibit PI3K activity in the model of HGF-induced cell migration; therefore, we determined the effect of vanillin and its C-1 derivative compounds on phosphorylation of Akt as an indicator of PI3K activity inside the cells. A549 cells were treated with the test compounds at 4 mM before stimulation with HGF. Autophosphorylation of Met was not affected by any vanillin derivative tested (**Figure 4A**). PI3K/Akt and Ras/Erk pathways are downstream of HGF/Met activation, and strong inhibition of Akt phosphorylation was obtained by vanillin and apocynin treatments, while vanillic acid and vanillyl alcohol showed no inhibition. On the other hand, Erk phosphorylation was not affected by any test vanillin derivative compound (**Figure 4A**). In the range of concentrations used in the cell migration assay (1−4 mM), inhibition of Akt phosphorylation by vanillin and apocynin were observed at 41−74% and 73−92%, respectively (**Figure 4B**), corresponding to their inhibitory effects on cell migration. In contrast, vanillic acid and vanillyl alcohol, which showed minimal effect on migration inhibition, did not inhibit Akt phosphorylation. Comparison between vanillin and apocynin at each concentration for inhibition of Akt phosphorylation showed that there was no statistically significant difference between vanillin and apocynin at all concentrations. The results suggested that PI3Ks are targets of vanillin and apocynin and are responsible for the inhibition of HGF-induced cell migration by vanillin and apocynin.

Inhibitory Effect of Vanillin and Apocynin on the Lipid Kinase Activity of PI3K Isoforms. Since PI3K-induced Akt phosphorylation is mediated by phosphoinositide-dependent protein kinases (PDKs), to confirm that PI3Ks are targets of vanillin and apocynin, we determined the effects of vanillin and apocynin on the enzymatic activity of PI3Ks. However, class I PI3Ks have several isoforms of catalytic subunit (p110), so we determined the effects of the compounds on each catalytic

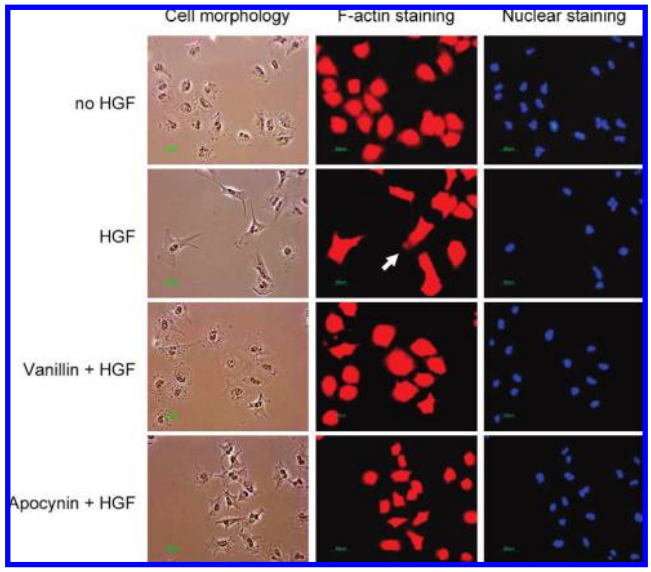

Figure 3. Effect of vanillin and apocynin on HGF-induced cell morphological changes and cytoskeleton reorganization. A549 cells were seeded on coverslips and allowed to adhere for 4 h. Cells were pretreated with 4 mM vanillin or apocynin for 30 min before HGF stimulation for 20 h. After fixation, filamentous Actin (F-Actin) was stained with rhodamine-conjugated phalloidin (red fluorescence), while nuclei were stained with DAPI (blue fluorescence). The white arrow indicates F-Actin-rich lamellipodia. Scale bar = 50 μm. Images were captured by using an inverted fluorescence microscope. Original magnification was $\times$400.

subunit isoform (p110 α, β, γ, and δ) by an in vitro lipid kinase assay. The results in **Table 1** revealed that vanillin and apocynin (at 4 mM) could inhibit the lipid kinase activity of all isoforms tested, with 23−36% inhibition for vanillin and 32−44% inhibition for apocynin, indicating that vanillin and apocynin are novel inhibitors of class I PI3Ks but showed no isoform-specific inhibition. Comparison between vanillin and apocynin for the inhibition of class IA PI3Ks (α, β, and δ isoforms) showed that there was no statistically significant difference between vanillin and apocynin inhibition of each PI3K isoform. However, significant difference in inhibition between vanillin and apocynin were observed for class IB PI3K γ (p = 0.022).

Antioxidant Activity of Vanillin and Its Derivatives. Because vanillin possesses antioxidant activity, we further investigated whether the antioxidant activities of vanillin and its derivatives correlate with their inhibitory effects on cell migration or PI3K activity. The antioxidant activities of the test compounds were determined by capability to scavenge DPPH radicals, using ascorbic acid as a reference antioxidant compound. All the test compounds showed the capability to scavenge DPPH radicals; vanillyl alcohol, the most reduced form of tested vanillin derivatives, exhibited the highest antioxidant activity with an SC_{50} of 0.137 mM, compared to 0.027 mM of ascorbic acid, while SC_{50} values of vanillin, apocynin, and vanillic acid were observed in the range of 16.2−42.0 mM (**Table 2**). There appears to be no correlation between the antioxidant activity of the tested vanillin derivatives and their inhibitory effects on cell migration.

Antiangiogenic Activity of Vanillin and Apocynin. Since cell migration is a crucial step in angiogenesis, and PI3K/Akt signaling is an important regulator of angiogenesis in vivo, we therefore examined the effect of vanillin and apocynin on in vivo angiogenesis by using the CAM assay. After exposure to vanillin and apocynin for 24 h, significant inhibition of the minor blood vessels formation of day 4 eggs in the areas under the agarose pellets was observed at dosage ranges of 100−500 nmol/(pellet egg) (**Figure 5A**), with 20−28% for vanillin and 32−37% inhibition for apocynin (**Figure 5B**).

DISCUSSION

Our data demonstrate for the first time that vanillin and apocynin inhibit class I PI3Ks, by either the in vitro lipid kinase assay or Western blot analysis of Akt phosphorylation as an indicator for cellular activity of PI3Ks. Data from cell migration assay and Western blot analysis suggest that carbonyl groups of vanillin and apocynin are critical for the inhibitory effect on PI3K activity. With vanillic acid, the negative charge of the carboxylate group at physiological pH might influence the binding or reactivity of the compound to the target sites on proteins. With vanillyl alcohol, the inhibitory effect on cell migration was marginally observed and was not dose-dependent. Since this compound could not inhibit cellular PI3K activity, as shown by a lack of inhibition of HGF-induced Akt phosphorylation, the alcohol group of this compound appears to be unable to bind or to react with the target sites, compared with the aldehyde or ketone group. The observed inhibitory effect of vanillyl alcohol may instead result from nonspecific interactions with cellular components. It has been proposed that some chemopreventive properties of vanillin are due to its antioxidant activity and its ability to scavenge free radicals (*2*). However, our data showed that the radical scavenging activity of vanillin is not related to its inhibitory effect on cell migration or PI3K/Akt signaling. It seems that the radical scavenging activity of vanillin does not participate in PI3K inhibition.

Comparison of vanillin and apocynin for inhibition of cell migration, Akt phosphorylation, and lipid kinase activity of class IA PI3Ks (α, β, and δ isoforms) showed that there was no statistically significant difference between the two compounds. Although apocynin was more potent than vanillin for inhibition of lipid kinase activity of the class IB PI3K γ isoform, however, PI3K γ is activated by chemokines through G-protein coupled receptors and shows restricted expression in the hematopoeitic system (*19*). Therefore, PI3K γ does not participate in HGF-induced cell migration and Akt phosphorylation in A549 cells. The inhibitory effects of vanillin and apocynin in our model should thus result from inhibition of class IA PI3Ks. The PI3K isoforms show some differences in amino acid sequence in the ATP-binding sites, and PI3K γ is the most distinct from other isoforms (*19*). We suggest that the mechanisms of vanillin and apocynin for inhibiting PI3K lipid kinase activity are conserved among PI3K isoforms, but the difference between the potencies of vanillin and apocynin might be due to differences in the capability of the compounds to interact with amino acids at the target sites.

The mechanisms of well-known pharmacological inhibitors of PI3Ks such as wortmannin and LY294002 are quite different. Wortmannin binds to the ATP-binding site of PI3Ks, and it irreversibly inhibits the enzyme by forming a covalent linkage between the furan C-20 of wortmannin and a critical lysine residue (Lys-802) which is essential for the phosphate transfer reaction (*20*). The C-20 of wortmannin is attacked by the ε-amino group of Lys-802, followed by furan ring opening and formation of an enamine which is in equilibrium with a Schiff base form. LY294002 is a competitive inhibitor of PI3Ks, which binds to the ATP-binding site, occupying the space filled by ATP and mimicking interactions between ATP and the enzyme (*21*). Vanillin can also form Schiff bases with proteins (*22*), but the mode and mechanism of inhibition by vanillin and apocynin need to be further studied.

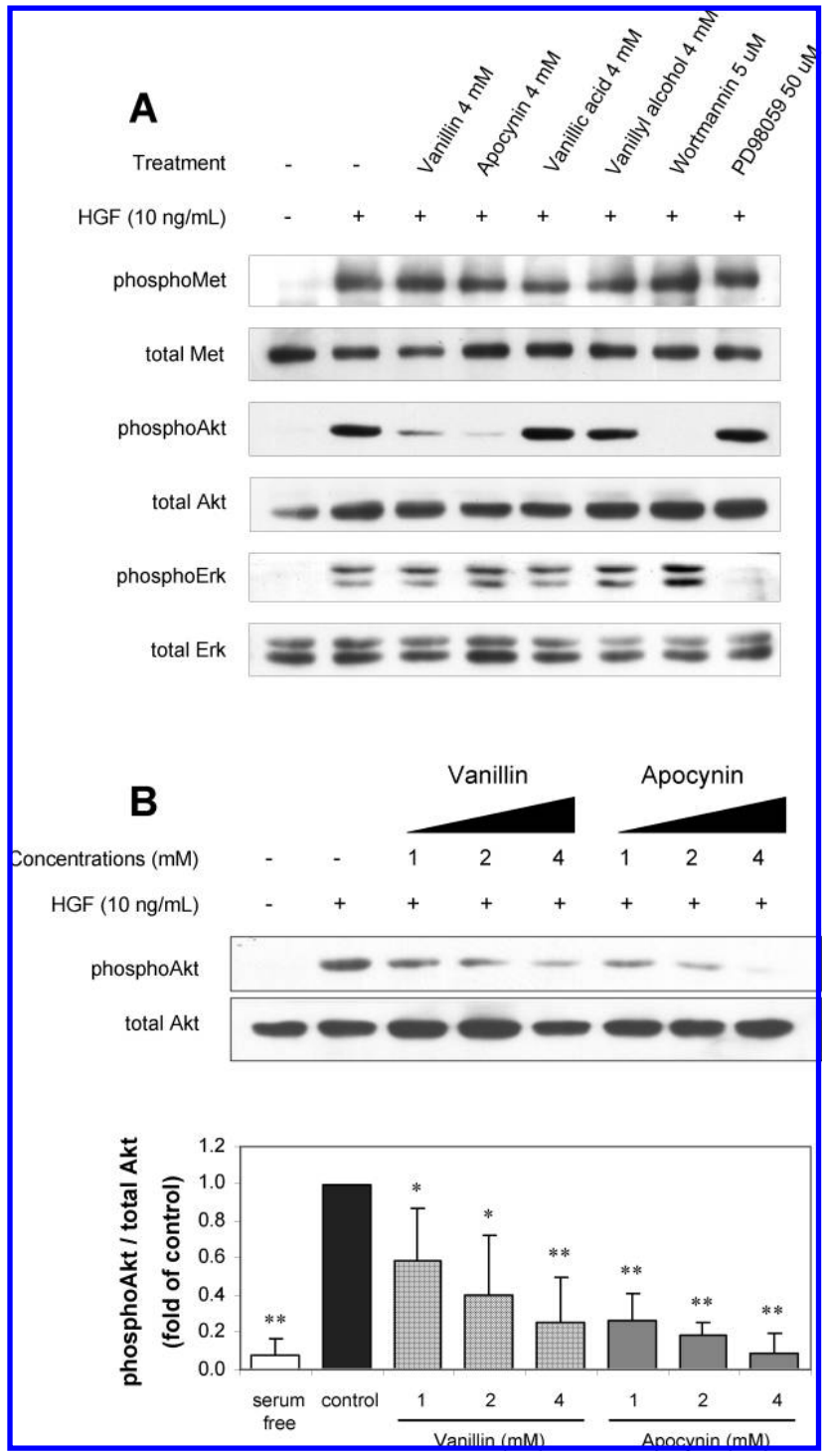

Figure 4. (**A**) Effect of vanillin and its derivatives on the HGF signaling pathway. A549 cells were starved in serum-free medium for 24 h and then treated with test compounds for 30 min before stimulation with HGF (10 ng/mL) for a further 30 min. Phosphorylation of Met, Akt, and Erk were detected by the Western blot technique, as described under Materials and Methods. Data are representative of three independent experiments. (**B**) Dose-dependent inhibition of Akt phosphorylation by vanillin and apocynin. Band intensity was quantified, and the relative intensity was expressed as fold of control. Data are expressed as mean ± SD from three independent experiments. Significant differences from control are shown by * ($p < 0.05$), ** ($p < 0.01$).

Vanillin and apocynin at concentrations of 4 mM showed an inhibitory effect on the in vitro lipid kinase activity of all class I PI3K catalytic subunit isoforms causing 23−44% inhibition; however, at the same concentration, a greater effect on cellular activity was observed in terms of 74−92% inhibition of Akt phosphorylation. Cellular PI3K activity is influenced by many factors which are absent from the cell-free solution of the in vitro assay, such as activation and feedback inhibition of the enzymes, protein−protein interactions, and cross-talk signaling. All of the above contribute to tight regulation of the rates of enzymatic reaction in cells to prevent excessive signaling. Another explanation is that vanillin and apocynin might affect other enzymes which enhance inhibition of PI3K downstream signaling, such as inhibition of PDKs or activation of lipid phosphatase enzyme (PTEN).

Growth factor-induced PI3K activation promotes cell migration and enhances cell survival and cell proliferation through two major effectors, Rho family small GTPases such as Rac

Table 1. Inhibitory Effect of Vanillin and Apocynin on the Lipid Kinase Activity of PI3K Isoforms[a]

PI3K isoforms	% kinase inhibition	
	vanillin (4 mM)	apocynin (4 mM)
class IA—PI3K-α (mouse)	36 ± 2**	44 ± 1**
class IA—PI3K-β (human)	28 ± 4**	38 ± 1**
class IA—PI3K-δ (human)	23 ± 11**	32 ± 4**
class IB—PI3K-γ (human)	27 ± 3**	40 ± 1**

[a] Lipid kinase activities of recombinant PI3Ks with different catalytic subunit isoforms of class I PI3Ks (p110 α, β, γ, and δ) were determined by an in vitro kinase assay (KinaseProfiler; Millipore, Dundee, U.K.), at an ATP concentration equal to K_m for each isoform. Test compounds were assayed at a concentration of 4 mM. Data are expressed as mean ± SD of duplicate wells. Significant differences from control are shown by ** ($p < 0.01$). Original species of the recombinant PI3Ks are indicated in parentheses.

Table 2. Antioxidant Activity of Vanillin Derivatives[a]

compound	SC_{50} (mM)
vanillin	42.0 ± 5.0
apocynin	39.5 ± 0.5
vanillic acid	16.2 ± 1.3
vanillyl alcohol	0.137 ± 0.040
ascorbic acid	0.027 ± 0.004

[a] Antioxidant activities of test compounds were determined from the capability to scavenge DPPH radicals. Concentrations required for 50% radical scavenging (SC_{50}) were extrapolated from the plot of % radical scavenging versus concentration. Data are expressed as mean ± SD from three independent experiments.

and Cdc42 that control cytoskeleton reorganization, and Akt which regulates antiapoptotic signaling and cell-cycle machinery (*14*, *23*). VEGF-induced cell migration and cytoskeleton reorganization are impaired in the bovine lung microvascular endothelial cells harboring activation-deficient Akt, whereas the cells harboring constitutively active Akt exhbit increased basal cell migration and cytoskeleton reorganization even in the absence of VEGF stimulation (*24*). In A549 cells, it has been shown that TGF-β1-induced cell migration is abolished by inhibition of Akt activity, either by Akt inhibitor treatment or transfection of Akt dominant-negative allele (*25*). In this model, Akt activates the NFκB signaling pathway, resulting in up-regulation of β1 integrin expression which contributes to cell migration. It has also been proposed that activated Akt controls optimal cell spreading and stimulates cellular contraction through phosphorylation of Pak1 (*26*). So the inhibition of Akt phosphorylation by vanillin and apocynin might be involved in the antimigration effect of these compounds, in addition to PI3K inhibition.

Since Akt also plays a role in cell survival and cell proliferation, inhibition of Akt phosphorylation may lead to inhibition of cell growth. In our previous report on the antimetastatic effect of vanillin, we showed that oral administration of vanillin (100 mg/(kg day) for 1 month) significantly suppressed lung metastasis of the 4T1 cells but could not suppress the primary tumor growth at the implantation site (*6*). These suggested that the inhibitory effect of vanillin on cell invasion and migration, rather than the cell growth arrest effect, was responsible for the antimetastatic effect of vanillin in vivo.

The in vivo antiangiogenic activity of vanillin and apocynin is likely to be, at least in part, due to its inhibitory effect on PI3K activity toward endothelial cells, because PI3K/Akt signaling plays an important role in angiogenesis in vivo. Studies using fertilized hen eggs showed increasing angiogenesis in chick chorioallantoic membrane (CAM) after induction of constitutively active PI3K and Akt, while overexpression of PI3K negative regulator (PTEN) or dominant-negative PI3K resulted in inhibition of angiogenesis (*27*). Recently, the siRNA technique was used to confirm the role of PI3K in endothelial cell migration and angiogenesis, by which down-regulation of the PI3K subunit (p110 α) in human umbilical vein endothelial cells by siRNA transfection resulted in decreasing migration capacity of the transfected cells (*28*). The decreasing migration of endothelial tip cells was also observed in genetically engineered mice in which PI3K α was downregulated, by replacement of the endogenous p110 α gene with a kinase-dead allele (*28*). In the case of apocynin, it has been shown to inhibit cell migration and in vitro tube formation of human microvascular endothelial cells at concentrations of 2−10 mM and to

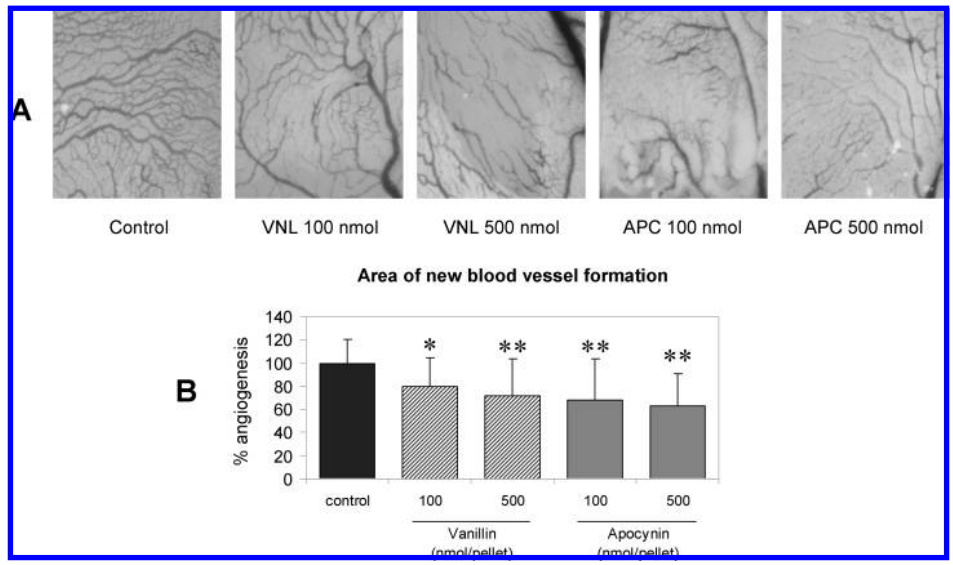

Figure 5. Inhibitory effect of vanillin and apocynin on angiogenesis in vivo determined by the chick chorioallantoic membrane (CAM) assay, as described under Materials and Methods. Agarose pellets containing the test compounds were placed on the CAM of day 3 hen eggs and further incubated for 24 h. (**A**) Representative photographs of the CAMs on day 4 after removal of the agarose pellets. (**B**) Area of minor blood vessels in images of the CAMs was calculated by image analysis software. Data are expressed as mean ± SD ($n = 10$), and significant differences from control are shown by * ($p < 0.05$), ** ($p < 0.01$).

suppress in vivo angiogenesis assessed by tissue engineering chambers implanted in rat femurs (*29*).

Although apocynin has long been known as an inhibitor of NADPH oxidase activation, however, its mechanism of action is unclear. Apocynin prevents translocation of NADPH oxidase components from cytosol to the plasma membrane, where it is assembled with other components (*30*). This process is also regulated by PI3Ks (*31*); therefore, the PI3Ks inhibition by apocynin might be another possible mechanism for the inhibiting activation of NADPH oxidase.

As noted earlier, vanillin is metabolized in the body to vanillic acid and to a lesser extent vanillyl alcohol (*7*), so in terms of physiological significance, other possible effects of the latter two compounds may also need to be considered. Vanillic acid has been shown to exhibit immunostimulatory effects in enhancing IFN-γ secretion and stimulating proliferation of human peripheral blood mononuclear cells (*32*). Mononuclear leukocytes such as macrophages and NK cells, when activated, are able to kill cancer cells. Thus, the action of vanillin in vivo might occur via a direct manner by suppressing cancer cell migration and metastasis, and reducing angiogenesis and/or by an indirect manner through metabolites such as vanillic acid, which enhances the immune response to destroy cancer cells. Whichever is the case, it is noteworthy that our previous studies showed that vanillin not only inhibited cell invasion and migration in mouse breast cancer cells in vitro to a similar extent to that observed in the present studies, but oral administration of vanillin at a high but nontoxic dose also showed in vivo inhibition of metastasis in the treated mice (*6*). The inhibition of migration of human A549 cells in vitro is therefore of potential interest physiologically.

In conclusion, we have provided evidence that vanillin and apocynin are novel inhibitors of class I PI3K enzymes. In addition, the suppression of HGF-induced cancer cell migration and the reduction of angiogenesis in vivo by vanillin or apocynin appear, at least in part, to be due to inhibition of PI3K activity. These findings might explain the mechanism underlying the previously reported antimetastatic effect of vanillin. Since dysregulation of the PI3K/Akt pathway is involved in many pathological conditions, such as cancer and inflammatory diseases (*33*), vanillin and apocynin might be of benefit for the treatment of these diseases, which require long-term treatment with nontoxic drugs. Wortmannin is of limited use in vivo due to its toxicity (oral LD_{50} in rats is about 1 mg/(kg day)) (*14*). Vanillin and apocynin have been shown to be less toxic, with the oral LD_{50} of vanillin in rats reported as 1.58−2.8 g/kg (*1*) and the oral LD_{50} value of apocynin in mice being reported as 9 g/kg (*34*). Taken together, vanillin and apocynin might be useful as lead compounds for development of less toxic therapeutic agents for PI3K inhibition.

ACKNOWLEDGMENT

We thank Pantipa Subhasitanont, Lukana Ngiwsara, Khajeelak Chiablaem, and Sitsom Wijitburaphat for their assistance.

LITERATURE CITED

(1) Opdyke, D. L. J. Fragrance raw materials monographs. Vanillin. *Food Cosmet. Toxicol.* **1977**, *15*, 633–638.

(2) Kamat, J. P.; Ghosh, A.; Devasagayam, T. P. A. Vanillin as an antioxidant in rat liver mitochondria: inhibition of protein oxidation and lipid peroxidation induced by photosensitization. *Mol. Cell. Biochem.* **2000**, *209*, 47–53.

(3) Shaughnessy, D. T.; Setzer, R. W.; DeMarini, D. M. The antimutagenic effect of vanillin and cinnamaldehyde on spontaneous mutation in Salmonella TA104 is due to a reduction in mutations at GC but not AT sites. *Mutat. Res.* **2001**, *480*−*481*, 55–69.

(4) Aboobaker, V. S.; Balgi, A. D.; Bhattacharya, R. K. In vivo effect of dietary factors on the molecular action of aflatoxin B1: role of non-nutrient phenolic compounds on the catalytic activity of liver fractions. *In Vivo* **1994**, *8*, 1095–1098.

(5) Tsuda, H.; Uehara, N.; Iwahori, Y.; Asamoto, M.; Iigo, M.; Nagao, M.; Matsumoto, K.; Ito, M.; Hirono, I. Chemopreventive effects of β-carotene, α-tocopherol and five naturally occurring antioxidants on initiation of hepatocarcinogenesis by 2-amino-3-methylimidazo(4,5-*f*)quinoline in the rat. *Jpn. J. Cancer Res.* **1994**, *85*, 1214–1219.

(6) Lirdprapamongkol, K.; Sakurai, H.; Kawasaki, N.; Choo, M. K.; Saitoh, Y.; Aozuka, Y.; Singhirunnusorn, P.; Ruchirawat, S.; Svasti, J.; Saiki, I. Vanillin suppresses in vitro invasion and in vivo metastasis of mouse breast cancer cells. *Eur. J. Pharm. Sci.* **2005**, *25*, 57–65.

(7) Panoutsopoulos, G. I.; Beedham, C. Enzymatic oxidation of vanillin, isovanillin and protocatechuic aldehyde with freshy prepared guinea pig liver slices. *Cell. Physiol. Biochem.* **2005**, *15*, 89–98.

(8) Nakamura, T.; Matsumoto, K.; Kiritoshi, A.; Tano, Y.; Nakamura, T. Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: in vitro analysis of tumor-stromal interactions. *Cancer Res.* **1997**, *57*, 3305–3313.

(9) Uchiyama, A.; Morisaki, T.; Beppu, K.; Kojima, M.; Matsunari, Y.; Nakatsuka, A.; Mizumoto, K.; Matsumoto, K.; Nakamura, T.; Tanaka, M. Hepatocyte growth factor and invasion-stimulatory activity are induced in pleural fluid by surgery in lung cancer patients. *Br. J. Cancer* **1999**, *81*, 721–726.

(10) Birchmeier, C.; Birchmeier, W.; Gherardi, E.; Vande Woude, G. F. Met, metastasis, motility and more. *Nat. Rev. Cancer* **2003**, *4*, 915–925.

(11) Brader, S.; Eccles, S. A. Phosphoinositide 3-kinase signalling pathways in tumor progression, invasion and angiogenesis. *Tumori* **2004**, *90*, 2–8.

(12) Hawkins, P. T.; Anderson, K. E.; Davidson, K.; Stephens, L. R. Signalling through class I PI3Ks in mammalian cells. *Biochem. Soc. Trans.* **2006**, *34*, 647–662.

(13) Rychahou, P. G.; Jackson, L. N.; Silva, S. R.; Rajaraman, S.; Evers, B. M. Targeted molecular therapy of the PI3K pathway: therapeutic significance of PI3K subunit targeting in colorectal carcinoma. *Ann. Surg.* **2006**, *243*, 833–844.

(14) Wymann, M. P.; Zvelebil, M.; Laffargue, M. Phosphoinositide 3-kinase signaling—which way to target. *Trends Pharmacol. Sci.* **2003**, *24*, 366–376.

(15) Durant, S.; Karran, P. Vanillins—a novel family of DNA-PK inhibitors. *Nucleic Acids Res.* **2003**, *31*, 5501–5512.

(16) Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. Feasibility of Drug Screening with Panels of Human Tumor Cell Lines Using a Microculture Tetrazolium Assay. *Cancer Res.* **1988**, *48*, 589–601.

(17) Gerhauser, C.; Klimo, K.; Heiss, E.; Neumann, I.; Gamal-Eldeen, A.; Knauft, J.; Liu, G.-Y.; Sitthimonchai, S.; Frank, N. Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutat. Res.* **2003**, *523*−*524*, 163–172.

(18) Peifer, C.; Dannhardt, G. A novel quantitative chick embryo assay as an angiogenesis model using digital image analysis. *Anticancer Res.* **2004**, *24*, 1545–1552.

(19) Rückle, T.; Schwarz, M. K.; Rommel, C. PI3K γ inhibition: towards an 'aspirin of the 21st century'? *Nat. Rev. Drug Discovery* **2006**, *5*, 903–918.

(20) Wymann, M. P.; Bulgarelli-Leva, G.; Zvelebil, M. J.; Pirola, L.; Vanhaesebroeck, B.; Waterfield, M. D.; Panayotou, G. Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* **1996**, *16*, 1722–1733.

(21) Walker, E. H.; Pacold, M. E.; Perisic, O.; Stephens, L.; Hawkins, P. T.; Wymann, M. P.; Williams, R. L. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol. Cell* **2000**, *6*, 909–919.

(22) Zaugg, R. H.; Walder, J. A.; Klotz, I. M. Schiff base adducts of hemoglobin. *J. Biol. Chem*. **1977**, *252*, 8542–8548.

(23) Vivanco, I.; Sawyers, C. L. The phosphatidylinositol 3-kinase-Akt pathway in human cancer. *Nat. Rev. Cancer* **2002**, *2*, 489–501.

(24) Morales-Ruiz, M.; Fulton, D.; Sowa, G.; Languino, L. R.; Fujio, Y.; Walsh, K.; Sessa, W. C. Vascular endothelial growth factor-stimulated Actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ. Res*. **2000**, *86*, 892–896.

(25) Fong, Y. C.; Hsu, S. F.; Wu, C. L.; Li, T. M.; Kao, S. T.; Tsai, F. J.; Chen, W. C.; Liu, S. C.; Wu, C. M.; Tang, C. H. Transforming growth factor-β1 increases cell migration and β1 integrin up-regulation in human lung cancer cells. Lung Cancer 2008, 10.1016/j.jungcan.2008.07.010.

(26) Zhou, G. L.; Zhuo, Y.; King, C. C.; Fryer, B. H.; Bokoch, G. M.; Field, J. Akt phosphorylation of serine 21 on Pak1 modulates Nck binding and cell migration. *Mol. Cell. Biol*. **2003**, *23*, 8058–8069.

(27) Jiang, B. H.; Zheng, J. Z.; Aoki, M.; Vogt, P. K. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc. Natl. Acad. Sci. U.S.A*. **2000**, *97*, 1749–1753.

(28) Grauper, M.; Guillermet-Guibert, J.; Foukas, L. C.; Phng, L. K.; Cain, R. J.; Salpekar, A.; Pearce, W.; Meek, S.; Millan, J.; Cutillas, P. R.; Smith, A. J. H.; Ridley, A. J.; Ruhrberg, C.; Gerhardt, H.; Vanhaesebroeck, B. Angiogenesis selectively requires the p110α isoform of PI3K to control endothelial cell migration. *Nature* **2008**, *453*, 662–666.

(29) Jiang, F.; Zhang, G.; Hashimoto, I.; Kumar, B. S.; Bortolotto, S.; Morrison, W. A.; Dusting, G. J. Neovascularization in an arteriovenous loop-containing tissue engineering chamber: role of NADPH oxidase. *J. Cell. Mol. Med*. **2008**, *12*, 2062–2072.

(30) Stolk, J.; Hiltermann, T. J. N.; Dijkman, J. H.; Verhoeven, A. J. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am. J. Respir. Cell Mol. Biol*. **1994**, *11*, 95–102.

(31) Gao, X. P.; Zhu, X.; Fu, J.; Liu, Q.; Frey, R. S.; Malik, A. B. Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J. Biol. Chem*. **2007**, *282*, 6116–6125.

(32) Chiang, L. C.; Ng, L. T.; Chiang, W.; Lin, C. C. Immunomodulatory activities of flavonoids, monoterpenoids, triterpenoids, iridoid glycosides and phenolic compounds of Plantago species. *Planta Med*. **2003**, *69*, 600–604.

(33) Stein, R. C.; Waterfield, M. D. PI3-kinase inhibition: a target for drug development. *Mol. Med. Today* **2000**, *6*, 347–358.

(34) Van den Worm, E.; Beukelman, C. J.; Van den Berg, A. J. J.; Kroes, B. H.; Labadie, R. P.; Van Dijk, H. Effect of methoxylation of apocynin and anologs on the inhibition of reactive oxygen species production by stimulated human neutrophils. *Eur. J. Pharmacol*. **2001**, *433*, 225–230.

Received for review July 16, 2008. Revised manuscript received January 2, 2009. Accepted February 17, 2009. This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0090/2547) to K.L. and a research grant from the Chulabhorn Research Institute. J.S. is a Senior Research Scholar of the Thailand Research Fund. Part of this work was also supported by the Institute of Pharmacy, Johannes Gutenberg-University, Germany.

JF803366F