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Identification of 3'-Hydroxymelanetin and Liquiritigenin as Akt Protein Kinase Inhibitors

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Abstract The signal transduction system is one of the most important devices involved in maintaining life, and many protein kinases are included in the cellular signal transduction system. Finding a protein kinase inhibitor is very valuable, as it can be used to study cell biology and applied to pharmaceuticals. For the efficient and rapid screening of protein kinase inhibitors, two assay systems were combined; the nonradioactive protein kinase assay system that uses an FITC-labeled IRS-2 peptide and the cell-based paper disc assay system that uses *Streptomyces griseus* as the indicator strain. Among 330 kinds of herb extracts tested, the extract of *Dalbergia odorifera* exhibited the strongest inhibitory activity in the two assay systems and was selected for further isolation. Based on solvent extraction and many steps of chromatography, seven compounds were finally separated to homogeneity and their structures determined by ^{1}H and ^{13}C NMR spectroscopies. Four were to be flavonoids and identified as butin ($C_{15}H_{12}O_5$, Mw=272.07), 3'-hydroxymelanetin ($C_{16}H_{12}O_6$, Mw=300.06), liquiritigenin ($C_{15}H_{12}O_4$, Mw=256.07), and 2'-hydroxyformononetin ($C_{16}H_{12}O_5$, Mw=284.07). 3'-Hydroxymelanetin inhibited the phosphorylation of the GSK3 protein by Akt to 37% at a concentration of 10 μg/ml and showed the strongest cytotoxicity (ED_{50}<50 μg/ml) against the human cancer cell line HCT116. Under the same conditions, liquiritigenin also inhibited the phosphorylation of GSK3 by Akt to 26%, and its cytotoxicity against the HCT116 cell line was lower than 100 μg/ml.

Key words: Akt inhibitor, nonradioactive protein kinase assay, cell-based paper disc assay

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Protein phosphorylation is a major signal transduction system in eukaryotic and prokaryotic cells. Tyrosine protein kinases [2] and serine/threonine protein kinases [9] have been found in eukaryotic cells and are known to regulate cell division, cell differentiation, and homeostatsis. Akt (protein kinase B) is one of the serine/threonine kinases regulated by PI3K located on the cell membrane and controls the survival and death of the cell via phosphorylation of the target proteins [9, 16]. For this reason, regulation of the Akt-related signal transduction pathway is considered a good target for the development of pharmaceuticals to treat cancer, diabetes, and Alzheimer's disease [13, 15, 17, 19].

Most of the protein kinases found in prokaryotes are histidine and aspartic acid kinases [1]. These two-component kinases compose the major regulatory networks in a prokaryotic cell, which enable the cell to sense and respond to intra- or extracellular signals for survival. *Streptomyces* is the most evolved prokaryote and has unique life cycle characteristics. One spore can germinate, grow to form mycelia, and make spore chains at the tip of aerial mycelia with the concomitant biosynthesis of secondary metabolites [22]. Interestingly, these eukaryotic-type characteristics, including morphological and physiological differentiation, are not regulated by histidine/aspartic acid kinases, but rather by eukaryotic type serine/threonine kinases. Therefore, the treatment of serine/threonine/tyrosine kinase inhibitors inhibits the formation of aerial mycelia or sporulation without killing the cell in *Streptomyces griseus* [7]. As such, a cell-based assay system using *S. griseus* was designed for the screening of kinase inhibitors based on observing their inhibitory effects on cellular differentiation and sporulation on plates.

During subsequent studies of Akt kinases, the present authors developed a novel nonradioactive protein kinase assay system that uses a fluorescein isothiocyanate (FITC)-labeled peptide designed from the consensus motifs phosphorylated by Akt kinases [9]. In addition, it was found that the designed peptide could also be phosphorylated by some protein kinases of *S. griseus* [6]. This finding then prompted the use of the nonradioactive protein kinase assay system for the efficient screening of protein kinase inhibitors based on applying a crude cell extract of *Streptomyces* as the source for protein kinases.

Accordingly, this paper describes the combined application of the nonradioactive protein kinase assay system and a cell-based assay system for the screening of protein kinase inhibitors, including the separation, structural determination, and biological activities of inhibitors isolated from the medicinal plant *Dalbergia odorifera*.

MATERIALS AND METHODS

Chemicals and Cells

The synthetic FITC-labeled peptide was synthesized by Peptron Co., Korea, [γ-^{32}P]ATP (5,000 Ci/mol) purchased from Amersham International, and all the chemicals obtained from Sigma Chemical Co., U.S.A. The *S. griseus* IFO13350 [11] was received from S. Horinouchi at the University of Tokyo, Japan. The human cancer cell line HCT116 was purchased from KCLB, Korea. The chemicals for the Western blot analysis were from Upstate Biotechnology, U.S.A.

Preparation of Plant Extracts

Three-hundred-and-thirty kinds of dried plants supplied by Ildong Pharmaceutical Co. Ltd., Korea, were immersed in methanol (10 volume of dry weight) for 3 days and filtered through Whatman filter paper (No. 3) twice. The filtrates were then concentrated to dryness using a rotary vacuum evaporator and dissolved in a small amount of methanol to a final concentration of 100 mg/ml. When the extract was applied to the assay systems, the solvent was removed using the rotary vacuum evaporator, and dimethylsulfoxide (DMSO) added to give a final concentration of 10 mg/ml or 100 mg/ml.

Preparation of Kinase Enzyme

The *S. griseus* IFO13350 was cultured in 100 ml of a YMPD broth [12] containing 0.2% yeast extract, 0.2% meat extract, 0.4% bactopeptone, 0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, and 1.0% glucose at pH 7.2 in a 500-ml baffled-flask at 28°C for 2.5 days on a reciprocal shaker at 250 rpm. The cultured cells obtained after centrifugation at 5,000 ×*g* for 10 min were washed with 50 ml of a lysis buffer (50 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, pH 7.5), and then resuspended in 10 ml of the lysis buffer. Next, the cells were treated with lysozyme (2 mg/ml lysozyme) at 4°C for 1.5 h, and disrupted by sonication for 1 min five times. The cell debris was removed by centrifugation at 20,000 ×*g* for 30 min, and the resulting supernatant used as the kinase source for phosphorylation. PMSF, a serine-protease inhibitor, was added to the crude cell extract to a 1 mM final concentration to prevent digestion by protease. The protein concentrations were measured using the method of Bradford [3].

Nonradioactive Protein Kinase Assay

The nonradioactive protein kinase assay was performed using a fluorescein-conjugated Akt1 substrate peptide (IRS-2; FITC-VR*R*S*R*TDSL_A), synthesized by Peptron Co., Korea. The fluorescein-labeled oligopeptide (1 μg) was incubated with 10 μl of the crude cell lysate and 2 μl of a herb extract in 20 μl of a protein kinase reaction buffer (20 mM HEPES [pH 7.2], 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.02 mM ATP) at 30°C for 30 min. The reactions were stopped by heating at 95°C for 10 min. The phosphorylated peptide was then separated on a 0.8% agarose gel at 100 V for 15 min. Since the phosphorylated products gained one more negative charge, they migrated to the anode. After electrophoresis, the gel was photographed on a transilluminator [6].

Cell-Based Paper Disc Assay

To check the *in vivo* influence of the herb extracts and protein kinase inhibitors on *S. griseus* IFO13350, spores (~10^5 cells) of *S. griseus* IFO13350 were spread on a solid YMPD agar medium, and then paper discs containing different concentrations of the herb extracts and protein kinase inhibitors were placed on the plates after 5 h of incubation at 28°C. The aerial mycelium formation, sporulation, and pigment production were examined after 3 to 7 days of growth at 28°C.

Isolation of Protein Kinase Inhibitors from *Dalbergia odorifera*

Solvent Extraction. The *Dalbergia odorifera* (dry weight; 1.5 kg) purchased from EKD Inc., Korea, was immersed in 50 l of methanol for 3 days and filtered through Whatman filter paper. The filtrate was then concentrated in a vacuum to dryness and redissovled in 685 ml of 95% methanol in distilled water. The crude extract was further extracted with 4 l of n-hexane, 8 l of dichloromethane, and 6 l of ethylacetate in order of increasing polarity. The insoluble part remaining after extraction with ethyl acetate was dissolved in distilled water, and then in methanol for storage.

Silica Gel Column Chromatography. The dichloromethane-extract was concentrated in a vacuum and dissolved in a small aliquot of methanol, and then applied to a silica gel column packed with Silica gel 60 (Merck KGaA, Germany, 100 mm×850 mm). The column

was washed with dichloromethane and eluted with a gradient of 100% dichloromethane to 50% dichloromethane in methanol at a flow rate of 20 ml/min. Thirty 700 ml fractions (S1–S30) were collected, and the active fraction used for the further purification steps.

Sephadex LH20 column chromatography. The active fraction from the Silica gel column chromatography was concentrated, dissolved in a small aliquot of methanol, and then applied to a Sephadex LH20 column (Amersham Pharmacia Biotech, Sweden). The column was eluted with 100% methanol at a flow rate of 4 ml/min. Twenty ml fractions were collected, and the active fraction pooled and used for the further purification steps.

HPLC. The active fraction from the Sephadex LH20 column chromatography was further purified by preparative HPLC, which was carried out using a Waters equipped with a Waters 600 binary pump, Waters 600 controller, and Waters 486 tunable absorbance detector. The separation was performed on a YMC-pack ODS-A column (20× 150 mm, YMC Co.) at an ambient temperature with a sample injector volume of 1 ml and wavelength of 254 nm. The column was washed with 40% methanol in distilled water and eluted with a gradient of 40% to 50% methanol in water at a flow rate of 18 ml/min for 30 min. Each fraction with peaks was collected. To optimize the conditions for separation, analytical HPLC was also performed with the same HPLC system equipped with an analytical column (YMC-pack ODS-A column, 4.6×150 mm) using a sample injector volume of 10 μl and gradient of 40% to 60% methanol in water at a flow rate of 1 ml/min.

In Vitro Phosphorylation Protocol

The effect of the protein kinase inhibitors isolated from *Dalbergia odorifera* on the *in vitro* phosphorylation of the cellular proteins of *S. griseus* IFO13350 was examined [7]. The crude cell-free extract (7 μg) of *S. griseus* IFO13350 was preincubated with 1 μg of the isolated compounds in a reaction mixture containing 10 mM Tris-Cl [pH 7.4], 4 mM $MgCl_2 \cdot 6H_2O$, 3 mM β-mercaptoethanol, 2 mM $MnCl_2$, and 1 mM EDTA in a total volume of 10 μl for 5 min at room temperature. Next, 5 μCi of [γ-^{32}P]ATP was added to the reaction mixture, and the reaction terminated after another 10 min of incubation at 30°C by the addition of a 4× SDS sample buffer containing 250 mM Tris-HCl [pH 6.8], 40% glycerol, 8% SDS, 20% 2-mercaptoethanol, and 0.1% bromophenol blue. The samples were boiled for 2 min and the phosphorylated proteins separated by 0.1% SDS-10% polyacrylamide gel electrophoresis [7]. After the gel had been stained, destained, and dried, autoradiography was performed at −80°C using a Du Pont Cronex intensifying screen.

In Vitro Phosphorylation by Akt

The Akt/PKB protein was incubated with a GSK protein, a substrate protein of Akt, and the phosphorylating activity of GSK3 caused by Akt detected using a Western blot analysis (Upstate Biotechnology, U.S.A.). Briefly, 1 μl of the Akt/PKB protein kinase (10 unit/μl), 3 μl of a 10× kinase buffer (200 mM HEPES [pH 7.4], 143 mM $MgCl_2$, 10 mM EDTA), 3 μl of 20 mM DTT, 17.5 μl of distilled water, and 1.5 μl of the samples in DMSO were premixed. After standing for 10 min, 3 μl of 2 mM ATP and 1 μl of the GSK fusion protein (1 mg/ml) were mixed and incubated for 30 min at 37°C. The reactions were stopped by heating at 70°C for 5 min. After adjusting the reaction volume to 100 μl with distilled water, the mixture was moved to a dot-blot well, and then transferred onto PVDF membranes in a vacuum. The membranes were treated with boiling water for 5 min, incubated in a blocking buffer (5% dried milk in PBS and 0.05% Tween-20), probed with specific antibodies for phospho-GSK3α/β, and detected using a Supersignal West Femto Chemiluminescence Western blotting detection system (Pierce Biotech., U.S.A.).

Cytotoxicity Test

A human cancer cell line, HCT116, was grown to 90% (v/v), divided into a 96-well plate to a concentration of 10^4 cells/100 μl per well, and incubated for 1 day at 37°C in a CO_2 incubator. One μl of the isolated compound was then added to each well and the cells further incubated for 3 days. After incubation, the cells were treated with 15 μl of 5 mg/ml MTT for 4 h and 100 μl of a solubilization buffer (0.01 N HCl/10% SDS) was added. After 14 h of incubation, the remaining viable cells were measured using a spectrophotometer at both 570 and 652 nm.

NMR Spectroscopy

The ^{1}H-NMR and ^{13}C-NMR spectra were obtained using a Jeol JNM-LA300 NMR spectrometer (300 MHz).

Results and Discussion

Application of Nonradioactive Protein Kinase Assay System

Akts are a family of highly conserved serine/threonine kinases, including Akt1, Akt2, and Akt3. These kinases are activated in response to a wide variety of growth factors through PI3K. Activated phospho-Akt mediates cell survival by phosphorylating several downstream targets, such as BAD, caspase-9, telomerase, and GSK1. Moreover, the specific amino acid sequence (xxRxRxx(S/T)xx, with the hydrophobic amino acid underlined) that can be phosphorylated by an Akt kinase has already been characterized from all known Akt kinase substrate proteins [9]. Thus, a flourescein-conjugated (the amino terminus of the peptide was conjugated with fluorescein isothiocyanate) insulin receptor substrate-2 (IRS-2) (^{349}VR*RS*RTDSLA^{358}) oligopeptide was synthesized and used for the nonradioactive protein kinase assay. Since

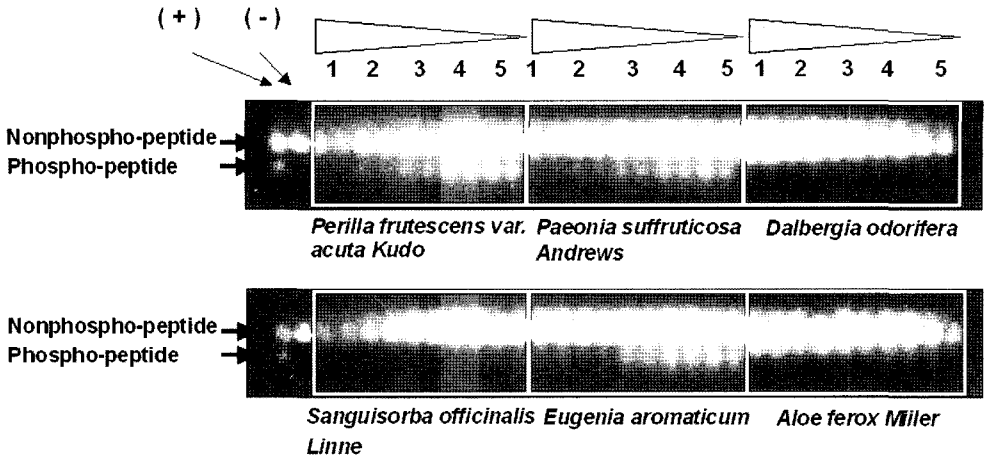

Fig. 1. Application of a nonradioactive protein kinase assay system for effective and rapid screening of protein kinase inhibitors.
The reaction mixtures were prepared with an FITC-labeled synthetic peptide as described in Materials and Methods, and herb extracts added to each reaction mixture in two-fold dilutions. 1, 20 μg; 2, 10 μg; 3, 5 μg; 4, 2.5 μg; 5, 1.25 μg; (+), positive control containing kinase source without herb extract; (−), negative control without kinase source. The arrow indicates the phosphorylated peptide that migrated faster than the nonphosphorylated form in agarose gel electrophoresis.

the IRS-2 was effectively phosphorylated by certain kinases of *Streptomyces*, the crude cell extract of *S. griseus* was used as the source of the IRS-2 kinase [6, 23].

Plant extracts prepared from 330 kinds of herb were added to the above nonradioactive protein kinase reaction to a final concentration of 1 mg/ml. The primary screened 64 candidates were rescreened through the same assay system and the herb extracts added in a serial 2-fold dilution (Fig. 1). Three candidates gave an MIC_{50} value lower than 62.5 μg/ml in the nonradioactive protein kinase assay system.

Application of Cell-Based Paper Disc Assay

Streptomyces includes unique cell differentiation features and produces various secondary metabolites during its life cycle. Previous observations have suggested that various protein kinases, such as serine/threonine kinases and phosphatases similar to those found in eukaryotes, control secondary metabolite formation or morphogenesis in *Streptomyces*.

Many compounds, such as staurosporine [21], K-252a [10], herbimycin [26], and radicicol [24], have already been listed as inhibitors of various types of eukaryotic protein kinase. In contrast to the profound effects of these kinase inhibitors on eukaryotic regulatory systems both *in vitro* and *in vivo*, the inhibition of aerial mycelium formation, sporulation, and secondary-metabolites formation without killing the cell have been observed in *Streptomyces* at a relatively higher concentration [7]. Thus, it was concluded that the inhibitory effects of eukaryotic protein kinases inhibitors on the morphogenesis of *Streptomyces* could be used as an indicator of the presence of some protein kinase inhibitors in a sample, and a cell-based paper disc assay was designed.

The herb extracts selected by the nonradioactive protein kinase assay system were also applied to the cell-based paper disc assay, and the *in vivo* effects of the extracts on the morphological differentiation of *S. griseus* examined. A paper disc containing each extract was placed on YMPD agar plates seeded with spores and the mycelial morphology observed (Fig. 2). The extracts of *Paeonia suffruticosa Andrews* and *Eugenia aromaticum* exhibited a strong killing effect around the disc, indicating the presence of some strong antibiotic substances in the extracts. The extracts of *Perilla frutescens var. acuta Kudo* and *Aloe ferox Miiler* also had strong killing effects on strain IFO13350 around the disc. However, when the extracts of *Sanguisorba officinalis Linne* and *Dalbergia odorifera* that gave the strongest inhibitory activities (MIC_{50}<62.5 μg/ml) in the nonradioactive protein kinase assay, no growth inhibition was observed, even in the area close to the disc, plus sporulation was inhibited, indicating that the extracts inhibited cellular differentiation without affecting vegetative growth (Fig. 2).

In particular, the extract of *Dalbergia odorifera* (2–20 μg/disc) caused the formation of a normally sporulating zone around the disc, producing a different effect depending on its concentration. A ring was also formed outside the sporulating zone where no spores were formed. As similar results were reported in the case of K-252a, a strong serine/threonine/tyrosine kinase inhibitor [7], it was assumed that some strong kinase inhibitor(s) may be contained in the extract of *Dalbergia odorifera.* Thus, *Dalbergia odorifera* was finally selected for further study.

Isolation of Compounds from *Dalbergia odorifera*

The methanol extract of *Dalbergia odorifera* was further fractionated with *n*-hexane, dichloromethane, ethylacetate,

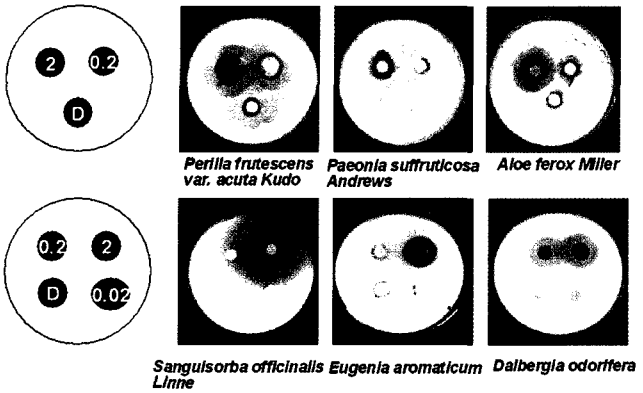

Fig. 2. Application of a cell-based paper disc assay system for effective and rapid screening of protein kinase inhibitors.
Each paper disc, containing the extract at a concentration of 0.02, 0.2, and 2 μg (as indicated in the empty circle), was placed on a YMPD plate seeded with spores of *S. griseus* IFO13350. The photographs were taken after 3 days of incubation at 28°C. D, DMSO.

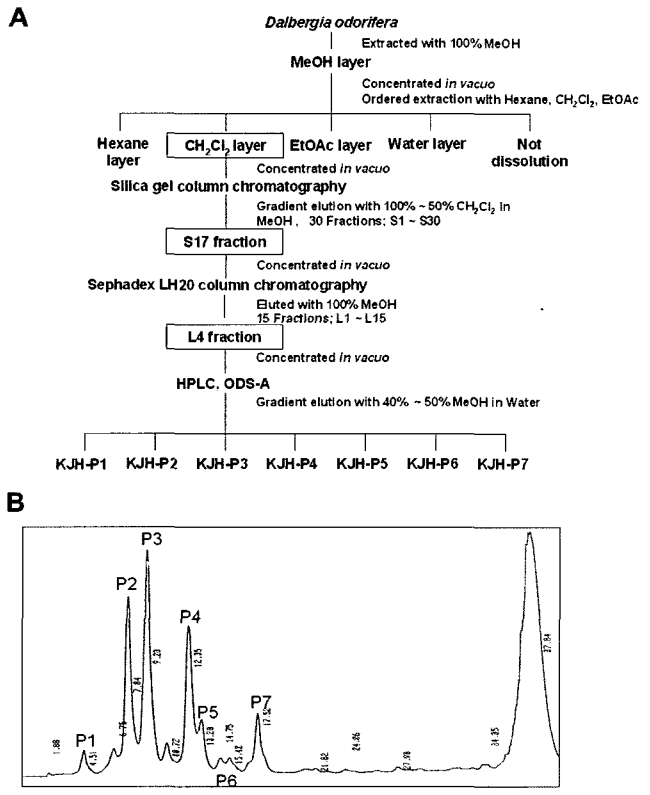

Fig. 3. Procedure for isolation of protein kinase inhibitors (**A**), and HPLC diagram (**B**).
A. The methanol extract of *Dalbergia odorifera* was used to isolate the protein kinase inhibitors based on solvent extraction and many steps of column chromatography, as explained in Materials and Methods. **B**. Preparatory HPLC using an YMC-pack ODS-A column (20×150 mm, YMC Co.) was applied to the active fraction from the Sephadex LH20 column chromatography. Seven peaks were detected at 254 nm, and numbered from KJH-P1 to P7 in the order of increasing retention time.

and water in order of increasing polarity (Fig. 3A), and the dichloromethane extract (KD fraction) exhibited the strongest activity in the nonradioactive protein kinase assay and cell-based paper disc assay among the extracts (data not shown). Thus, silica gel column chromatography and Sephadex LH20 column chromatography were applied to the dichloromethane extract based on tracing the active fraction using a combination of the two assay systems, as explained above. The active fraction from the Sephadex LH20 column chromatography (L4 fraction) was analyzed by

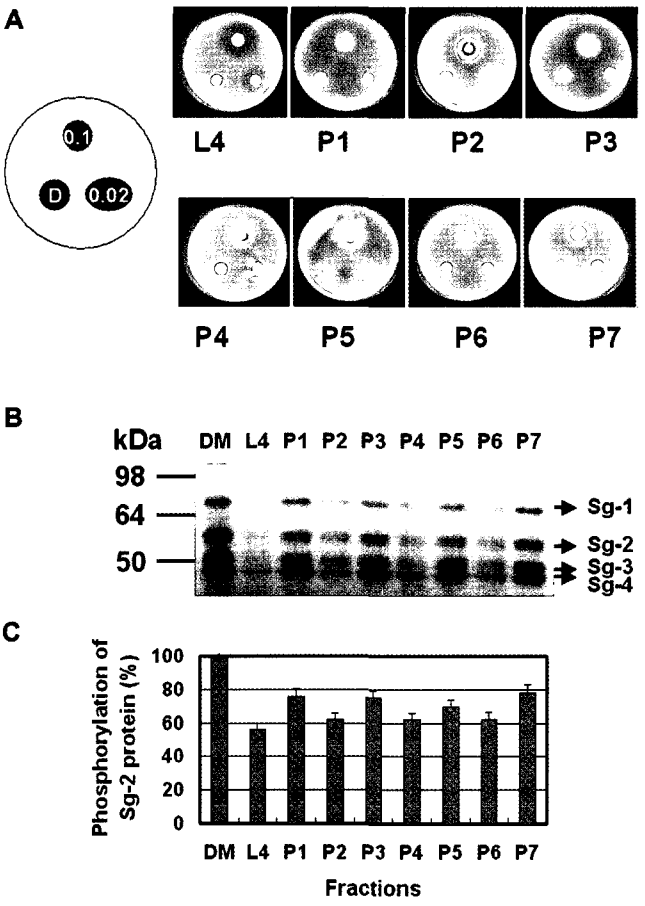

Fig. 4. Effect of isolated compounds on morphogenesis of *S. griseus* (**A**) and phosphorylation of cell-free extract prepared from *S. griseus* (**B**, **C**).
A. Photographs of plates from a cell-based paper disc assay of isolated compounds. Each paper disc, containing the isolated compounds at a concentration of 0.02 and 0.1 μg (as indicated in the empty circle), was placed on a YMPD plate seeded with spores of *S. griseus* IFO13350. The photographs were taken after 3 days of incubation at 28°C. **B**. Autoradiogram showing phosphorylation of cellular proteins of *S. griseus* IFO13350 in the presence of isolated compounds. **C**. Quantitative analysis of the Sg-2 protein phosphorylation in **B**. The intensity of the phosphorylated signals for Sg-2 was measured using a densitometer, where the intensity of the control with DMSO was regarded as 100%. The error bar represents standard deviation for n=3. DM, DMSO only; L4, active fraction from Sephadex LH20 column chromatography; P1 to P7, compounds KJH-P1 to KJH-P7 from preparatory HPLC.

preparative HPLC, as described in Materials and Methods. Seven peaks were detected at 254 nm, and the purity of each peak confirmed to be pure to homogeneity by analytical HPLC (Fig. 3B).

Therefore, the compounds from the seven peaks were numbered from KJH-P1 to P7 in order of increasing retention time. In the nonradioactive protein kinase assay, KJH-P1 and P5 gave MIC_{50} values lower than 12.5 μg/ml, whereas the values for P2, P3, and P4 were 25, 50, and 50 μg/ml, respectively. However, KJH-6 and 7 did not show any significant inhibitory effect (data not shown).

Effect of KJH Compounds on Morphogenesis and Protein Phosphorylation of *S. griseus*

In the cell-based paper disc assay using *S. griseus*, KJH-P1, P2, and P3 induced an abundant formation of spores around the disc, forming a ring outside the sporulating zone where no spores were formed (Fig. 4A). Treatment with KJH-P4 and P5 had strong stimulatory effects on the sporulation without affecting cell growth. In contrast, KJH-P6 and P7 had no significant effect on the morphogenesis of *S. griseus*. Since many protein kinases are networked in a cell and behave in a positive or negative way in the regulatory pathway, the *in vivo* effects of protein kinase inhibitors cannot be strictly predicted, as they appear in different phenotypes depending on the concentration of the inhibitor. Thus, it was concluded that KJH-P1, P2, and P3 had a stimulatory effect at a higher concentration and an inhibitory effect at a lower concentration on the morphogenesis of *S. griseus*.

To study the effects of the KJH compounds on the phosphorylation of the cellular proteins in *S. griseus*, the compounds (50 μg/ml) were added to a reaction mixture containing [γ-^{32}P]ATP and the cell extract of *S. griseus* as the source of phosphoproteins. The ^{32}P-labeled proteins were detected by radioautography and part of the gel is shown in Fig. 4B. For quantification, the intensity of the phosphorylated signals for the protein Sg-2 was measured using the densitometer (Fig. 4C). The Sg-2 protein phosphorylation was decreased to 56% by the active fraction (L4) from the Sephadex LH20 column chromatography, and a 30–40% decrease in the Sg-2 phosphorylation was observed with KJH-P2, P4, and P6. KJH-P1 and P3 also resulted in a 25% decrease of the signal.

Effect of KJH Compounds on Akt Activity and Cytotoxicity

As the IRS-2 peptide used in the nonradioactive protein kinase assay was designed from the conserved sequences phosphorylated by Akt, the effect of the KJH compounds on the phosphorylating activity of Akt was studied. The degree of phosphorylation of GSK3 by Akt was compared using Western blotting analysis, and an anti-phosphoGSK3α/β antibody used to detect the phosphoserine in the substrate (Fig. 5A).

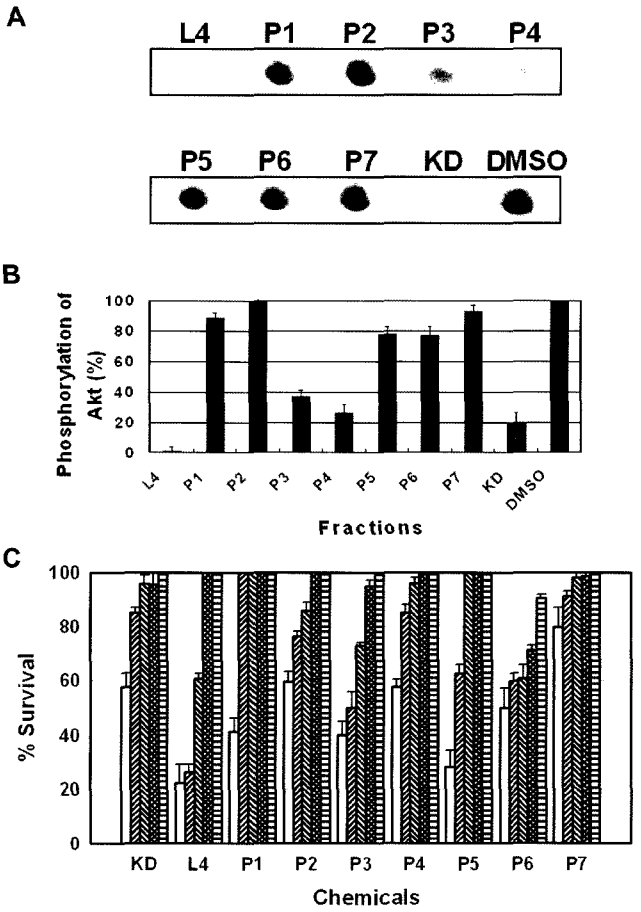

Fig. 5. Inhibition of Akt activity (**A**, **B**) and cytotoxic effect (**C**) of isolated compounds.

A. Western blot analysis of the phosphorylated form of GSK3 by Akt protein. The GSK protein, a substrate protein of Akt, was phosphorylated by Akt, transferred onto PVDF membranes, and detected using a Western blot analysis, as explained in the text. **B**. Quantitative analysis of GSK3 protein phosphorylation in (**A**). The intensity of the phosphorylated signals was measured using a densitometer, where the intensity of the control with DMSO was regarded as 100%. **C**. Cytotoxic effects of isolated compounds on human cancer cell line HCT116. The cells were treated with each sample to final concentrations of 6.25 (▤), 12.5 (▩), 25 (▧), 50 (▨), and 100 (□) μg/ml, respectively. The survival rate when treated with DMSO was regarded as 100%. KD, dichloromethane extract; L4, active fraction from Sephadex LH20 column chromatography; P1 to P7, compounds KJH-P1 to KJH-P7 from preparatory HPLC. The error bar represents standard deviation for n=3.

The phosphorylation of GSK3 by Akt was almost completely inhibited by the active fraction from the Sephadex LH20 column chromatography (L4), and severely inhibited by the dichloromethane extract (KD) of *Dalbergia odorifera* (Fig. 5B). KJH-P3 and P4 inhibited the phosphorylation signal to 37 and 26%, respectively.

The cytotoxicity of the isolated compounds was also examined. After treating a human cancer cell line, HCT116, for three days with the compounds, the viable cells were counted, as explained in Materials and Methods. KJH-P3 exhibited the strongest cytotoxicity with an ED_{50} lower

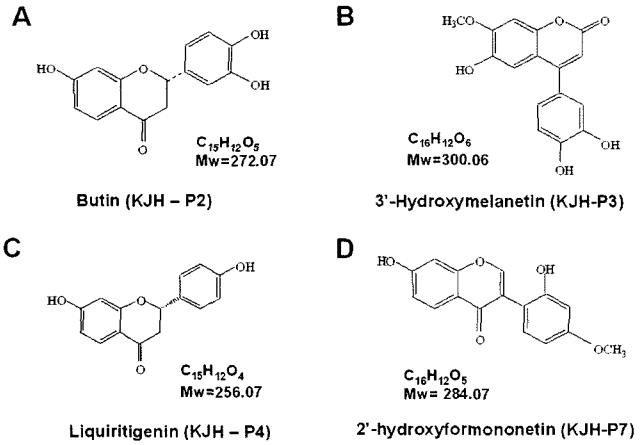

Fig. 6. Chemical structures of KJH-P2, P3, P4, and KJH-P7 compounds.
^{1}H-NMR spectroscopy (300 MHz in D_2O) and the ^{13}C-NMR spectrum were obtained and analyzed to determine the chemical structures.

than 50 μg/ml. The ED_{50} values for P2, P4, P5, and P6 were lower than 100 μg/ml.

Structural Determination of KJH Compounds

The chemical structures of KJH-P2, P3, P4, and P7 were determined by ^{1}H-NMR and ^{13}C-NMR spectroscopies, and identified as butin ($C_{15}H_{12}O_5$, Mw=272.07) [20], 3'-hydroxymelanetin ($C_{16}H_{12}O_6$, Mw=300.06) [4], liquiritigenin ($C_{15}H_{12}O_4$, Mw=256.07) [20], and 2'-hydroxyformononetin ($C_{16}H_{12}O_5$, Mw=284.07) [18], respectively (Fig. 6). All four compounds are flavonoids isolated from several medicinal plants, yet their biochemical activity has not yet been reported, except for liquiritigenin, which has been reported to exhibit cytotoxic activity against five human cancer cell lines [5], anti-allergic activity by inhibiting hyaluronidase activation and the release of histamine [8], and the anti-angiogenesis activity of granuloma [4] and licorice root [14]. All these biological activities of liquiritigenin may be explained by the inhibition of certain protein kinases, like Akt.

It has been suggested that inhibitors of Akt could be effective cancer chemopreventive agents. Deguelin that has been isolated from several plant species, including *Mundulea sericea* (*Leguminosae*), inhibits the PI3K/Akt pathway and decreases the expression of cyclooxygenase-2, which participates in xenobiotic metabolism, angiogenesis, and the inhibition of immune surveillance and apoptosis during tumorigenesis [25]. Importantly, deguelin induces apoptosis in premalignant and malignant human bronchial epithelial cells and exhibits to have cancer chemopreventive activities in colon cancer, melanoma, and lung cancer by blocking Akt activation [17].

7-Hydroxystaurosporine (UCN-01), originally identified from a soil bacterium, is a potent inhibitor of calcium-dependent protein kinase C (PKC). It also potently inhibits the DNA damage response regulatory kinases *chk*1 and *chk*2, phosphatidylinositide-dependent kinase 1 (PDK-1), and many other kinases. Furthermore, it shows antineoplastic activity in a number of preclinical animal models and induces clinical insulin resistance by blocking Akt activation in Thr^{308} phosphorylation [15].

Based on the present results, two naturally occurring compounds, 3'-hydroxymelanetin and liquiritigenin, were identified as Akt kinase inhibitors with cytotoxic activities against the cancer cell line HCT116. Deguelin and 7-hydroxystaurosporine are potent inhibitors of the PI3K pathway and inhibit the phosphorylation reactions caused by PI3K and PDK1. However, the current results clearly show that 3'-hydroxymelanetin and liquiritigenin are specific inhibitors of Akt kinase in the PI3K pathway. Although the phosphorylation of GSK by Akt kinase was severely inhibited by 3'-hydroxymelanetin and liquiritigenin, further study is needed to elucidate the exact inhibitory mechanism, such as whether it involves blocking the Akt phosphorylation or blocking the subsequent phosphorylating reaction of GSK by normally phosphorylated Akt.

In addition, the present data showed that the combined application of the nonradioactive protein kinase assay system and cell-based assay system can be used for the rapid and efficient screening of protein kinase inhibitors. The specificity of the screened kinase inhibitor depends on the source of the FITC-labeled peptide designed from consensus sequences of phosphorylating domains, thereby broadening the possibility for identifying specific kinase inhibitors.

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