J. Microbiol. Biotechnol. (2018), 28(8), 1401–1411
https://doi.org/10.4014/jmb.1804.04051

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Regulation of AKT Activity by Inhibition of the Pleckstrin Homology Domain-PtdIns(3,4,5)P_3 Interaction Using Flavonoids S

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Received: May 2, 2018
Revised: June 15, 2018
Accepted: July 3, 2018

First published online
June 22, 2018

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

The serine-threonine kinase AKT plays a pivotal role in tumor progression and is frequently overactivated in cancer cells; this protein is therefore a critical therapeutic target for cancer intervention. We aimed to identify small molecule inhibitors of the pleckstrin homology (PH) domain of AKT to disrupt binding of phosphatidylinositol-3,4,5-trisphosphate (PIP3), thereby downregulating AKT activity. Liposome pulldown assays coupled with fluorescence spectrometry were used to screen flavonoids for inhibition of the AKT PH-PIP3 interaction. Western blotting was used to determine the effects of the inhibitors on AKT activation in cancer cells, and in silico docking was used for structural analysis and optimization of inhibitor structure. Several flavonoids showing up to 50% inhibition of the AKT PH-PIP3 interaction decreased the level of AKT activation at the cellular level. In addition, the modified flavonoid showed increased inhibitory effects and the approach would be applied to develop anticancer drug candidates. In this study, we provide a rationale for targeting the lipid-binding domain of AKT, rather than the catalytic kinase domain, in anticancer drug development.

Keywords: PtdIns(3,4,5)P_3, pleckstrin homology (PH) domain, AKT, flavonoid, in silico docking

Introduction

The cell membrane acts as a platform for diverse cellular processes by recruiting a wide variety of signaling proteins. Recruitment of signaling proteins at the cell membrane is dependent on interactions between various lipids and lipid-binding domains, and dysfunction of these lipid-mediated signaling pathways is known to cause severe human diseases, including neurodegenerative diseases, diabetes, and cancer [1, 2]. Phosphatidylinositol phosphates (PIPs), generated by the activity of phosphatidylinositol kinases, comprise a small proportion of phospholipids in the cell membrane, but play critical roles in cell signaling [3–5]. The PIPs phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-trisphosphate (PIP3), which are interconverted by kinases and phosphorylases, are involved in many signaling pathways, including the PI3K/PDK1 and AKT (PKB)/mTOR pathways [4, 6–8]. Kinases such as PI3K, PDK1, and AKT interact with PIP3 on the plasma membrane via theirlipid-binding domains, resulting in activation; downstream signaling then results in activation of various cellular processes. Over-activation of these proteins related in cellular signaling pathways is regarded as a potential cause of cancer [6, 9, 10]; therefore, these proteins are well established as therapeutic targets for anticancer drugs, and several inhibitors of kinase activity, including trastuzumab (Herceptin) and imatinib (Gleevec), have been used for cancer treatment [9, 11], However, drugs that reduce the activity of kinases by targeting their catalytic domains have several shortcomings, such as drug resistance; it is therefore necessary to identify alternative targets for reducing the activity of kinases.

The serine-threonine kinase AKT (also known as PKB) is an effector protein involved in lipid-mediated signaling, and is regulated by PIP3 [12, 13]. Activation of AKT is dependent on the specific interaction between the pleckstrin homology (PH) domain of AKT and PIP3 in the plasma membrane. PIP3 is produced from PIP2 by PI3K, a key upstream regulator of the AKT pathway [9, 14]. PDK1 is bound to PIP3 in plasma membrane via its PH domain, and then phosphorylates Thr308 on AKT. AKT is activated after the phosphorylation of Ser473 by mTORC2. AKT then activates mTOR, GSK3β, and FKHR, inducing cell proliferation and differentiation and inhibiting apoptosis; thus, overactivation of the PI3K/AKT pathway can result in cancer [9, 10, 15].

Because AKT is involved in a variety of cell signaling pathways, dysfunction of AKT (for example, hyperactivation) is observed in several diseases, including cancer. Many attempts to develop anticancer drugs by regulation of AKT activity have been reported, and indeed, AKT inhibitors have been shown to have anticancer activity [11, 16]. However, previous work has mostly aimed to regulate the kinase activity of AKT by targeting its catalytic domain, neglecting the interaction between AKT and PIP3 on the plasma membrane, which is necessary for AKT activation, as a possible target. Therefore, in this work, we used liposome pulldown assays together with fluorescence spectrometry to identify flavonoid derivatives that downregulate AKT activity by inhibiting binding of PIP3 to its PH domain. We also evaluated the inhibitory effects of selected flavonoids in cancer cells. Furthermore, we used structure-activity relationship (SAR) analysis to provide information for further structural optimization of flavonoid AKT inhibitors.

Materials and Methods

Materials

Lipids used for liposome pulldown assays, including 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and phosphatidylinositol-3,4,5-trisphosphate (PIP3), were purchased from Avanti Polar Lipids (USA) and Cayman Chemical (USA). Flavonoids and derivatives were purchased from INDOFINE Chemical Company (USA). The structural information regarding all flavonoids tested in this study is provided in Table S1.

Gene Cloning and Expression

To test the inhibitory effects of flavonoids on AKT-PH and PIP3 interaction, the recombinant AKT PH-eGFP used for liposome-pulldown assay was obtained. Briefly, the segment of the *AKT1* gene encoding the N-terminal PH domain of AKT (residues 1-125) was amplified by PCR. The resulting PCR product was cloned into the BamHI/EcoRI site of the pET21a plasmid (Invitrogen, USA), and then *egfp*, encoding enhanced green fluorescent protein (eGFP), was cloned into EcoRI/XhoI site of the same plasmid. The resulting plasmid, designated pET_AKT-PH_eGFP, was used for the expression of recombinant AKT PH-eGFP in *Escherichia coli*. Additionally, PDK1 PH-eGFP was obtained by using a plasmid described previously [17]. For overexpression of recombinant proteins, *E. coli* BL21 cells were transformed with the appropriate plasmid, incubated at 37°C overnight in Lysogeny Broth (LB) supplemented with 100 μg/ml ampicillin, and then inoculated into fresh LB medium. The cells were cultured until the optical density at 600 nm (OD_{600}) reached 0.6, induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with 24 h of incubation at 24°C. Both of the recombinant proteins (eGFP-tagged AKT PH and PDK1 PH) were purified using Ni-NTA resin (Qiagen, USA).

Liposome Pulldown Assays Coupled with Fluorescence Spectrometery

The liposomes used for liposome pulldown assays were composed of POPC/POPE/POPS/PIP3 in a molar ratio of 60-*x*:20:20:*x* (*x* = 0 or 1); liposomes with 1 mol % PIP3 were used for flavonoid screening, while those with 0 mol % PIP3 were used as a negative control. Lipid mixtures were dried under nitrogen gas, resuspended at a concentration of 1 mg/ml in 50 mM Tris-HCl (pH 7.4) containing 160 mM NaCl and 50% (w/v) sucrose and then sonicated, resulting in the formation of homogenized liposomes, which were used for pulldown assays. Because the liposomes were loaded with 50% (w/v) sucrose solution, they were easily precipitated by low-speed centrifugation. Flavonoids were screened for inhibition of the AKT PH-PIP3 interaction using liposome pulldown assays, as described previously. Briefly, 10 μM AKT PH-eGFP and 30 μg of the liposome mixture were incubated with 0–100 μM of the relevant flavonoid for 10 min, and then the liposomes and liposome-bound AKT PH-eGFP were precipitated by centrifugation at 5,000 ×*g*. The coprecipitate was washed and resuspended in Tris buffer, and then the amount of liposome-bound AKT PH-eGFP was measured by fluorescence spectrometry (FC-2, Scinco, Korea). The excitation wavelength was 480 nm, and fluorescence emission was recorded between 500 and 600 nm. The excitation and emission bandwidths were 5 nm. The inhibitory effect of each flavonoid was quantified as the difference in eGFP fluorescence intensity at 510 nm between dimethylsulfoxide (DMSO) and flavonoid-treated liposomes, divided by the eGFP fluorescence intensity of the DMSO-treated liposomes. Data are represented as the mean ± s.d. of at least three independent replicates.

Western Blot Analysis

Human breast cancer cell line, MDA-MB-231, was obtained from the American Type Culture Collection (USA), and maintained

in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Cellgro, USA). The cells were serum-starved in DMEM containing 0.5% FBS for 24 h, pretreated with 20 μM AKTi (AKT inhibitor; Sigma-Aldrich, USA), 100 μM DM-PIT-1 (Calbiochem, USA), or 100 μM of the selected flavonoid for 30 min, and then stimulated with 20 ng/ml epidermal growth factor (EGF). After 20 min, the cells were collected and lysed in 20 mM HEPES (pH 7.2) containing 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein samples (20 μg each) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The blots were incubated with a primary antibody against phospho-Akt (Ser473; Cell Signaling Technology, USA) or GAPDH (Santa Cruz Biotechnology, USA) and developed using an enhanced chemiluminescence detection system (GE Healthcare, USA).

Structure-Activity Relationships (SARs)

To investigate the SARs between flavonoid structure and inhibition of the AKT PH-PIP3 interaction, the three-dimensional (3D) structures of 27 compounds, including 21 flavone derivatives and seven flavanone derivatives, were modeled using Sybyl 7.3 software (Tripos, USA). Initial models were built based on the structures of myricetin (PDB: 3HBF) and naringenin (PDB: 2BRT) for flavone and flavanone derivatives, respectively, and the structures were energy minimized using the Tripos force field and Gästeiger-Hückel charges until the total energy gradient reached 0.05 kcal/(mol Å).

In Silico Docking

The in silico docking experiments were employed to analyze interactions between the flavonoids and the AKT PH domain. The crystal structure of the AKT PH domain (PDB: 1UNQ) was prepared for docking by the removal of small molecules, including inositol-1,3,4,5-tetrakisphosphate (IP_4) and crystallographic water molecules, and the addition of hydrogen atoms [18]. The residues that comprised the binding pocket were identified using LigPlot software [19]. In silico docking was performed using our previously described procedure, with minor modifications [20]. An Intel Core 2 Quad Q6600 (2.4 GHz) Linux PC with Sybyl 7.3 software was used for all docking experiments [21]. The docking method was validated by docking IP_4, the native ligand of the AKT PH domain, into the structure. The 30 poses of IP_4 generated by this procedure were well conserved with the pose observed in the crystal structure. Ligplot and Chimera software were used to analyze and visualize the docking poses [22].

Synthesis of Flavonoid Derivatives

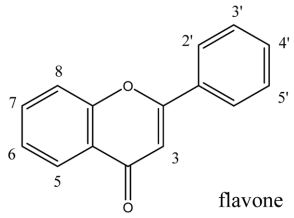
Based on in vitro and in silico analysis, thirteen new flavonoid derivatives were chemically synthesized to enhance the inhibitory activity. Detailed synthetic procedures for these flavonoid derivatives are described in Supplementary Methods and summarized in Fig. S1. The compounds were obtained in several steps using hydroxyketones and nitro-substituted benzaldehydes as starting materials. The structure of each compound was confirmed by NMR and mass spectroscopy analysis (data not shown); structural information is summarized in Table S2. The inhibitory effect of each synthetic compound on the AKT PH-PIP3 interaction was measured as same experimental conditions as flavonoids described above.

Results and Discussion

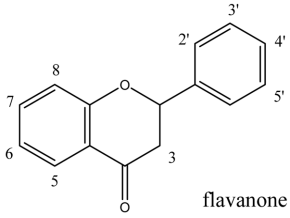
Screening of Flavonoids for Inhibiting AKT PH-PIP3 Interaction

The PH domain of AKT fused to eGFP (AKT PH-eGFP) was recombinantly expressed in *E. coli* BL21 cells and purified using Ni-NTA resin. The concentration of the protein was determined using the bicinchoninic acid (BCA) assay, and its purity was confirmed by SDS-PAGE. We used a liposome pulldown assay to evaluate binding of the AKT PH domain to PIP3. To validate the capability of this assay, we tested binding of AKT PH-eGFP to liposomes with different compositions: PC/PE/PS (60:20:20 mol %), PC/PE/PS/PIP2 (59:20:20:1 mol %), and PC/PE/PS/PIP3 (59:20:20:1 mol %). As expected, a three-fold increase in AKT PH-eGFP binding was observed when PIP3-containing liposomes were used rather than PIP2-containing liposomes, and a basal level of eGFP was detected when liposomes without PIPs were used (data not shown). These results confirmed that the liposome pulldown assay was capable of reporting interactions between PIP3 and the AKT PH domain, as we have shown previously. To identify flavonoids that inhibit the interaction between the PH domain of AKT and PIP3, 99 flavonoids, including 40 flavones, 40 flavanones, and 19 isoflavones, were screened using the liposome pulldown assay (Table S1). Of these flavonoids, 21 flavones and 9 flavanones showed inhibitory effects (Table 1, Fig. 1). The inhibition rate was calculated as the percentages of [decreased eGFP intensity by flavonoid treatment]/[eGFP intensity without flavonoid treatment]. The inhibitory effect of each flavonoid varied widely depending on its structure; however, flavonoid no. **15**, **21**, **22**, and **30**, corresponding to 3,6-dihydroxyflavone, 6,2′-dihydroxyflavone, 3,7,3′,4′,5′-pentahydroxyflavanone, and 3,5,7,3′,4′,5′-hexahydroxyflavanone, showed substantial inhibitory effects (>25%) at a concentration of 10 μM (Fig. 1). These compounds were even stronger inhibitors of the AKT PH-PIP3 interaction than DM-PIT-1, a known antagonist of interactions between PH domains and PIPs [14, 23].

Table 1. Chemical structures, nomenclature, and inhibitory effects of 32 compounds tested in this study.



flavone



flavanone

Flavonoid No.		Name	Activity	SD
Flavone	1	2′,3′-dihydroxyflavone	13.67	13.51
	2	2′,4′-dihydroxyflavone	10.37	8.03
	3	3′,4′-dihydroxyflavone	13.33	11.97
	4	2′,3′-dimethoxyflavone	8.05	12.16
	5	3′,4′-dimethoxyflavone	3.53	10.94
	6	4′-hydroxy-3′-methoxyflavone	1.92	6.95
	7	3-hydroxyflavone	4.79	8.65
	8	3,2′-dihydroxyflavone	11.19	7.23
	9	3,3′-dihydroxyflavone	7.00	4.96
	10	3,4′-dihydroxyflavone	9.30	5.98
	11	3,3′-dimethoxyflavone	18.05	13.58
	12	3,4′-dimethoxyflavone	2.95	4.90
	13	3-hydroxy-2′-methoxyflavone	6.50	1.49
	14	3-hydroxy-3′-methoxyflavone	5.47	5.25
	15	3,6-dihydroxyflavone	38.52	10.42
	16	3,6-DIMETHOXYFLAVONE	8.81	4.56
	17	3,7-DIMETHOXYFLAVONE	6.67	6.43
	18	5,3′-DIHYDROXYFLAVONE	2.27	8.75
	19	5,4′-DIHYDROXYFLAVONE	11.63	5.55
	20	5,2′-DIMETHOXYFLAVONE	13.07	15.83
	21	6,2′-DIHYDROXYFLAVONE	49.04	4.73
Flavanone	22	3,7,3′,4′,5′-pentahydroxyflavanone	27.46	14.20
	23	6-methoxyflavanone	4.21	4.85
	24	2′,3′-dimethoxyflavanone	3.07	3.12
	25	3′,4′-dimethoxyflavanone	11.65	9.52
	26	Eriodictyol-7-mtinoside	6.39	4.22
	27	Narigenin-7-neohesperidoside	9.04	1.65
	28	4′-methoxyflavanone	1.14	3.70
	29	4′,5,7-trimethoxyflavanone	7.65	0.93
	30	3,5,7,3′,4′,5′-Hexahydroxyflavanone	26.78	9.26
		COMPOUND **12** (5-methoxy-3′-nitroflavone)	27.69	7.91
Inhibitor		DM-PIT-1	15.74	5.60

The inhibitory activity of each compound on AKT PH domain-PIP3 interaction was calculated from more than three replicates and SD represents the standard deviation.

Interestingly, the flavonoids with the strongest inhibitory effects were the di-substituted flavones 6,2′-dihydroxyflavone and 3,6-dihydroxyflavone, respectively. This result was unexpected because flavonoids with multiple hydroxyl

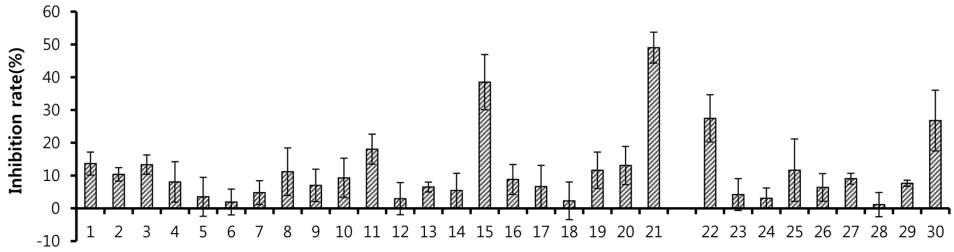


Fig. 1. Inhibitory effects of flavonoids on the interaction between PIP3 and the AKT PH domain.
Inhibitory effects of flavone derivatives (compounds 1–21) and flavanone derivatives (compounds 22–30) were quantified as [eGFP emission signal difference between DMSO and flavonoid treatment]/[eGFP emission signal from DMSO treatment]. The treated concentration of flavonoids was 10 μM.

groups on the C-ring, such as 3,7,3′,4′-tetrahydroxyflavone (fisetin) and 3,5,7,8,3′,4′-hexahydroxyflavone (gossypetin), were found to be the strongest inhibitors of the interaction between PIP3 and the PH domain of PDK1 in our previous report [17]. Although flavonoids with a high level of hydroxyl group substitution, such as 3,7,3′,4′,5′-pentahydroxyflavanone and 3,5,7,3′,4′,5′-hexahydroxyflavanone, had inhibitory effects of ~25% on the interaction between the AKT PH domain and PIP3, flavonoids with a hydroxyl group at the 6-position were superior inhibitors. Further investigation is needed to determine the structural basis for this observation. Our results imply that the PIP3 binding site of AKT PH domain is different to that of PDK1, even though both proteins are activated upon binding to PIP3 on the plasma membrane.

Characterization of the Inhibitory Effects of Flavonoids

Among the tested flavonoids, four flavonoids exhibiting >25% inhibition of binding of PIP3 to the AKT PH domain were selected for further characterization. To confirm that the inhibitory effect of each compound was dose-dependent, we measured the inhibitory effects of the four selected flavonoids, as well as fisetin and DM-PIT-1 in the 0–50 μM concentration range using liposome pulldown assays. The inhibitory effect of each flavonoid on the AKT PH-PIP3 interaction increased in a concentration-dependent manner, and flavonoid **21** (6,2′-dihydroxyflavone) showed the highest inhibitory effect, in agreement with the screening results (Fig. 2A). In the case of fisetin, the inhibitory effect on the AKT PH domain was less pronounced than those of flavonoids with a hydroxyl group at the 6-position, but was similar to those of flavonoid **22** and **30**. This result provided further evidence that the structural bases for the interactions between PIP3 and the PH domains of AKT and PDK1 are different, suggesting that these PH domains could be selectively targeted by antagonists. Next, the

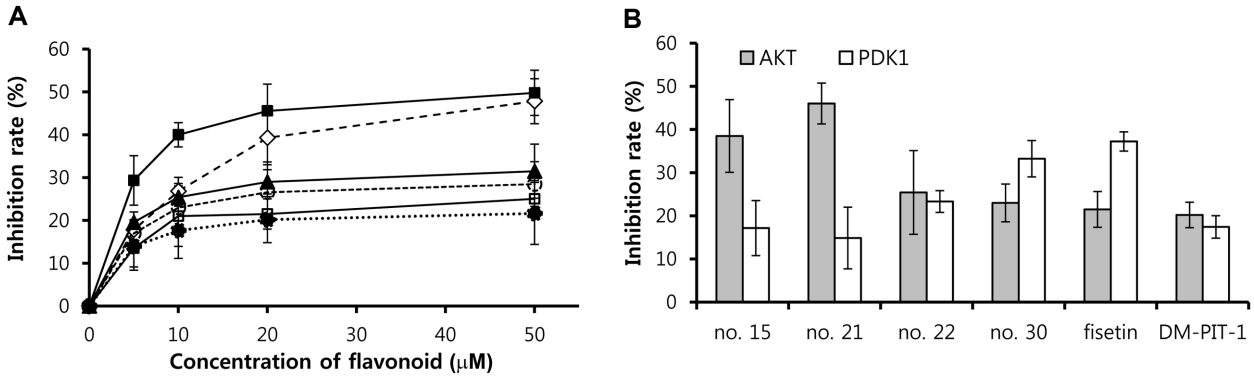


Fig. 2. Inhibitory effects of selected flavonoids on binding of PIP3 to the PH domains of AKT and PDK1.
(**A**) Concentration dependence of the inhibitory effects of selected flavonoids on the AKT PH-PIP3 interaction at concentrations between 0 and 50 μM. Solid squares, compound 21 (6,2′-dihydroxyflavone); open diamonds, compound 15 (3,6-dihydroxyflavone); solid triangles, compound 22 (3,7,3′,4′,5′-pentahydroxyflavanone); open circles, compound 30 (3,5,7,3′,4′,5′-hexahydroxyflavanone); open squares, fisetin; solid circles, DM-PIT1. (**B**) Comparison of the inhibitory effects of selected flavonoids on binding of PIP3 to the PH domains of AKT and PDK1. Although the PH domains of both proteins interact with PIP3, they exhibited differential inhibition by flavonoids. The treated concentration of flavonoids was 10 μM.

inhibitory effects of each flavonoid on the AKT PH-PIP3 and the PDK PH-PIP3 interactions were compared. Using the same experimental conditions as for the liposome pulldown assay, the PH domains of AKT and PDK1 were treated with 10 μM fisetin, DM-PIT-1, and four selected flavonoids. As expected, the flavonoids had distinct inhibitory effects on each protein. As shown in Fig. 2B, the AKT PH domain showed stronger binding to 6,2′-dihydroxyflavone and 3,6-dihydroxyflavone, while the PDK1 PH domain showed stronger binding to flavonoids with multiple hydroxyl groups on the C-ring. DM-PIT-1 had similar inhibitory effects on both domains (Fig. 2B), implying that this inhibitor has broad specificity, in accordance with previous reports [2, 4].

Our results show conclusively that selective inhibition of AKT and PDK1 is possible by exploiting the distinct inhibitory effects of flavonoids on the interactions between PIP3 and the PH domain of each protein. These flavonoids are therefore a valuable source of potential anticancer drugs. Indeed, it has been reported that overactivation of AKT and PDK1 occurs in cancer cells [24–26], and antagonists of these kinases are regarded as anticancer drug candidates. Moreover, flavonoids have been shown to suppress tumor growth in a range of cancer types, including prostate cancer and pancreatic cancer, by targeting the PI3K/AKT and mTOR signaling pathways [27–31]. However, although mTOR has been shown to be downregulated by flavonoids as a result of direct interaction, the mechanism for inhibition of AKT by flavonoids remained unclear. The PH domain of AKT is known to bind PIP3, resulting in recruitment of AKT to the plasma membrane, which enables activation of AKT via phosphorylation by diverse proteins including PDK1 [32–34]. Thus, we hypothesized that inhibition of the interaction between PIP3 and AKT interaction would block recruitment of AKT to the plasma membrane, resulting in suppression of AKT activity.

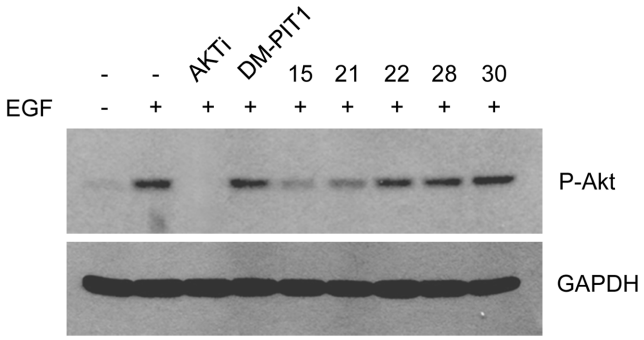


Fig. 3. Effects of selected flavonoids on AKT phosphorylation. MDA-MB-231 cells were starved in 0.5% fetal bovine serum for 24 h, and then treated with 20 μM AKTi, 100 μM DM-PIT-1, or 100 μM of the selected flavonoid. Western blot analysis was performed using total protein extracts and antibodies against phospho-AKT (Ser473). GAPDH was used as an internal control.

Effects of PIP3-PH Domain Interaction on AKT

To test this hypothesis, the flavonoids showing the inhibitory effects were used to treat cancer cells. MDA-MB-231 cells were treated with the four flavonoids that showed an inhibitory effect greater than 25% in the liposome pulldown assays. Cells were induced with 20 ng/ml EGF for 20 min, treated with 100 μM of the selected flavonoid for 30 min, and then harvested, and the level of AKT activation was determined by western blot analysis using a phospho-AKT antibody. Cells were also treated with 20 μM AKTi as a positive control and with DM-PIT-1 for comparison. Treatment of MDA-MB-231 cells with flavonoid **15** and **21** resulted in a reduction in phospho-AKT expression about 3- and 2.5-fold, respectively, with no effect on expression of the housekeeping protein GAPDH (Fig. 3). Although flavonoids such as **22** and **30**, as well as DM-PIT-1, showed inhibitory effects on the AKT PH-PIP3 interaction in vitro, they showed no significant effect on phospho-AKT expression according to the western blot analysis (Fig. 3). However, this result supported that the inhibition of PIP3 binding decreased the activation of AKT.

The concentration of flavonoids used in the cell-based AKT inhibition assay was 100 μM, which was much higher than the concentration used for the screening experiments. A higher concentration was used for the cell-based assays because the delivery efficiency of the inhibitors was not 100%; the effective concentration of each flavonoid acting on the AKT PH domain in cells would have been less than 100 μM and would have varied depending on the physicochemical properties of the flavonoids. Although the exact intracellular concentration of each flavonoid is not known, our results confirm that some flavonoids that disrupt the interaction between AKT and PIP3 in vitro also decrease AKT activation in cells. These results show that regulation of AKT can be achieved by disruption of the lipid-lipid binding domain interaction, suggesting that the lipid-binding domain might be a viable therapeutic target for anticancer drugs. Indeed, numerous effector proteins involved in cell signaling possess lipid-binding domains that are necessary for activation and translocation to their intended targets, suggesting that this strategy could be extended to other effector proteins.

Structure-Activity Relationship Analysis

The flavonoids evaluated in this study were structurally similar but varied with respect to the number and type of substituents, *e.g.*, hydroxyl and methyl groups (Table 1). Thus, SAR analysis was necessary to understand the relationship between the structures of the flavonoids and their inhibitory effects on the interaction between the AKT PH domain and PIP3. Compounds 21 (6,2′-dihydroxyflavone) and 15 (3,6-dihydroxyflavone), which had the greatest inhibitory effects on AKT, both have a hydroxyl group at the C6 position. Derivatives with methoxy groups at the C6 position, however, did not show high inhibition activity. For instance, the dihydroxy-substituted compounds 1, 3, 9, and 10 showed higher inhibition activity than the corresponding dimethoxy-substituted compounds 4, 5, 11, and 12, respectively. Although the effects of hydroxyl group substitutions at other positions were smaller, a preference for hydroxyl groups over methoxy groups was observed in most cases, suggesting that the size of the binding pocket of the AKT PH domain may be limited or that the hydroxyl group at the C6 position may be involved in important interactions. Compounds 22 and 30 also exhibited substantial inhibitory effects against the AKT PH domain (27.46% and 26.78%, respectively); these two compounds are not substituted at position C6, but instead share five hydroxyl substituents at positions C3, C7, C3′, C4′, and C5′. In order to understand how hydroxyl groups at specific positions affect the inhibitory effects of flavonoids on binding of PIP3 to the AKT PH domain, in silico docking experiments were used to model the interactions between the flavonoid derivatives and the AKT PH domain.

In Silico Docking Experiments

To elucidate how the flavonoids identified in this study inhibited the binding of PIP3 to the AKT PH domain, in silico docking was performed. Flavonoid no. **15** (3,6-dihydroxyflavone), **21** (6,2′-dihydroxyflavone), **22** (3,7,3′,4′,5′-pentahydroxyflavanone), and **30** (3,5,7,3′,4′,5′-hexahydroxyflavanone) were docked into ligand binding site of the AKT PH domain (PDB: 1UNQ). The optimal pose for each complex, which was selected based on the total binding score, is shown in Fig. 4. Based on analysis using LigPlot software, the residues involved in binding of the AKT PH domain to IP_4 (its native ligand) are Lys14, Gly16, Glu17, Tyr18, Ile19, Arg23, Arg25, Leu52, Asn53, Phe55, and Arg86. The docking poses of **15** and **21** were similar; **15** formed six hydrogen bonds and four hydrophobic interactions with Lys14, Gly16, Glu17, Tyr18, Ile19, Arg23, Arg25, Leu52, and Asn53 (Fig. 5A), while **21** formed six hydrogen bonds with Lys14, Glu17, Tyr18, Ile19, Arg23, and Arg25 (Fig. 5B). The docking poses of both flavonoids exhibited hydrogen bonds between the oxygen atom of the ketone group and the backbone nitrogen atom of Glu17, and between the 6-OH substituent and the side chains of Lys14 and Arg25 (Fig. 4A). Tyr18 formed a backbone hydrogen bonding interaction with the 2′-OH substituent of **21** and with the 2-OH substituent of

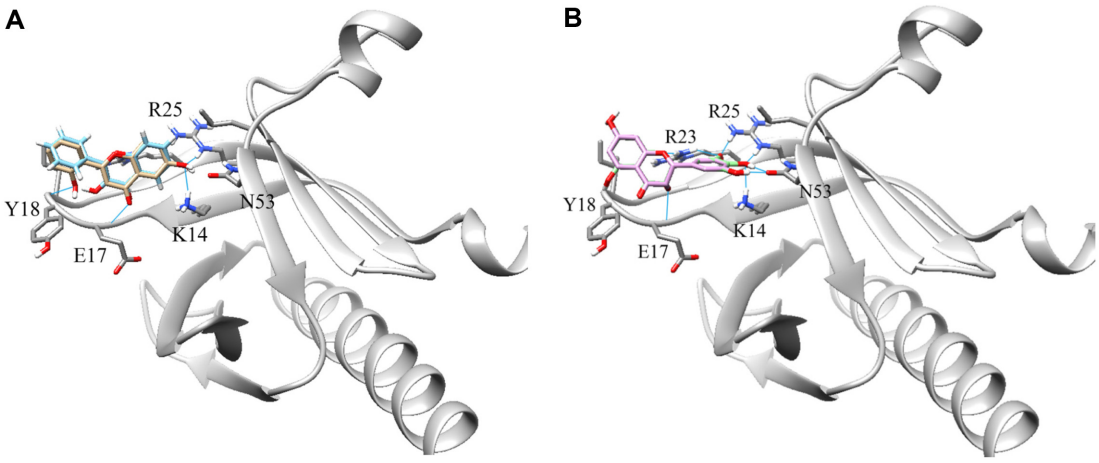


Fig. 4. Docking of selected flavonoids into the AKT PH domain.

(**A**) Docking poses of compounds 15 and 21. The A-ring of each compound was docked into the AKT PH domain, and the compounds partially overlapped. (**B**) Docking poses of compounds 22 and 30. The C-ring of each compound was docked into the AKT PH domain, and the compounds overlapped completely. The AKT PH domain is colored gray. Residues involved in flavonoid binding are labeled and shown in stick representation. Hydrogen bonds are indicated by blue lines.

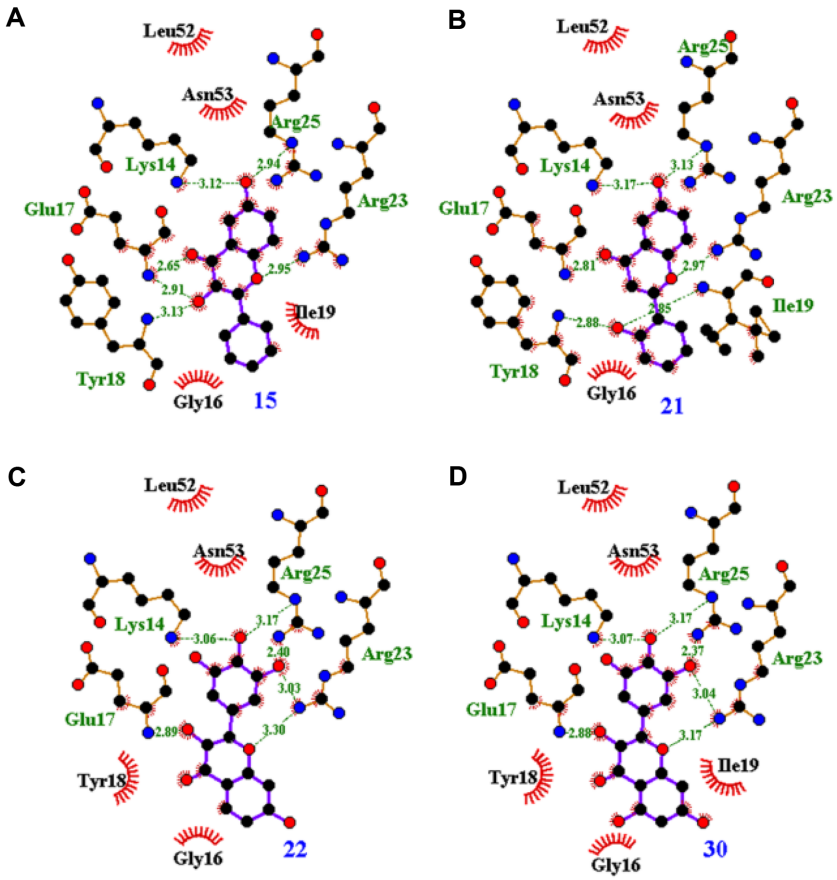


Fig. 5. In silico docking analysis in LigPlot software.
The selected flavonoids showed high inhibitory effects, flavonoid no. **15** (A), **21** (B), **22** (C) and **30** (D), were docked into AKT PH domain by in silico docking, and analyzed by LigPlot software. Residues involved in hydrophobic interactions and hydrogen bonds are shown in black and green, respectively.

15. The main difference between the binding modes of flavonoid **15** and **21** was the conformation of the B-ring, which tilted at different angles in each case.

On the other hand, flavonoid **22** and **30** showed different binding modes (Fig. 4B). The docked structures of **22** and **30** were almost identical, with the flavonoids forming four hydrophobic and six hydrogen bonding interactions and five hydrophobic and six hydrogen bonding interactions, respectively (Figs. 5C and 5D). The backbone nitrogen atom of Glu17, which interacted with the ketone group of **15** and **21**, formed a hydrogen bond with the 2-OH substituent of **22** and **30**, and the side chains of Lys14, Arg25, and Asn53, which interacted with the 6-OH substituent of **15** and **21**, formed hydrogen bonds with the hydroxyl groups of the B-ring. Thus, flavonoid **22** and **30** docked into the AKT PH domain in the opposite orientation compared to **15** and **21**.

Interestingly, the total binding scores of the four docked flavonoids were consistent with the inhibitory effects measured using liposome pulldown assays. Flavonoid **21**, which had the greatest inhibitory effect on the AKT PH-PIP3 interaction, was predicted by in silico docking to form the strongest interaction with AKT PH, with a total energy score of -22.37 kcal/(mol Å), followed by flavonoid **15**, **22**, and **30**, with scores of –20.70, –19.05, and –17.33 kcal/(mol Å), respectively. Flavonoid **21** displayed the best binding score, even though flavonoid no. **22** and **30** were more highly substituted with hydroxyl groups. Despite the differences in binding scores, the selected flavonoids with strong inhibitory effects on the binding of PIP3 to the AKT PH domain shared some similar interactions. LigPlot analysis indicated that Lys14 and Arg25 formed interactions with the 6-OH substituent of **15** and **21**, and similar interactions with the 4′-OH substituent of **22** and **30** (Fig. 5). Moreover,

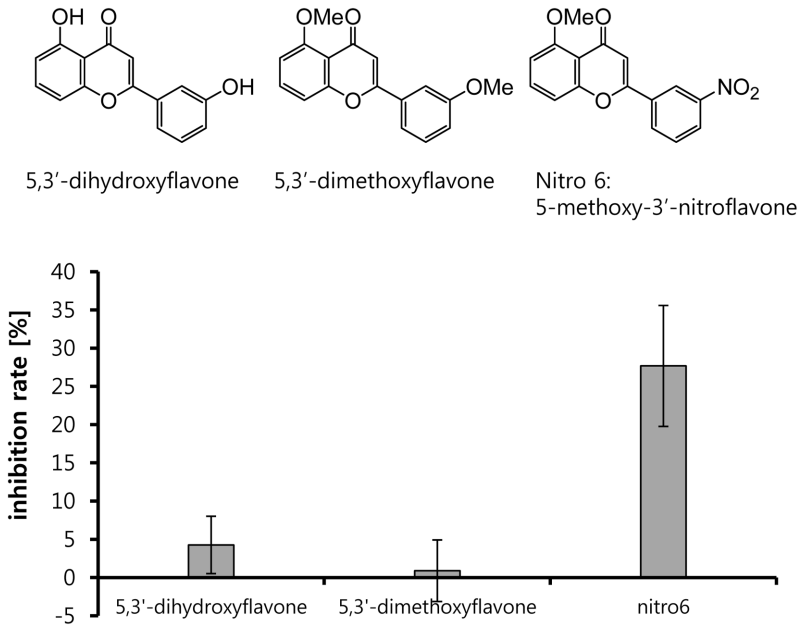


Fig. 6. Effects of functional group substitutions on the PIP3-AKT PH domain interaction.
The tested flavonoids, 5,3′-dihydroxyflavone, 5,3′-dimethoxyflavone, and 5-methoxy-3′-nitroflavone, are structurally analogous, possessing the same substitution pattern with different functional groups. The nitro-substituted flavonoid exhibited six-fold and 20-fold increases in inhibition compared with the dihydroxyflavone and the dimethoxyflavone, respectively. The tested concentration of flavonoids was 10 μM.

hydrogen bonding interactions between Glu17 and the flavonoids were observed in all four cases (Fig. 5).

Our computational analysis suggested several structural modifications that might enhance the inhibitory effects of the flavonoid derivatives on the AKT PH-PIP3 interaction. Firstly, the in silico docking experiments indicated that the hydroxyl groups of the characterized flavonoids are able to interact with positively charged amino acids in the binding pocket of the AKT PH domain. In particular, Lys14, Arg23, and Arg25 appear to be important for strong interactions with flavonoids. We therefore hypothesized that substitution of hydroxyl groups on the flavonoids with negatively charged nitro or carboxylate groups would enable the formation of stronger salt bridges with these positively charged residues in the AKT PH domain, enabling stronger inhibition. In addition, in silico docking indicated that the small flavonoids docked into the binding site of AKT PH domain formed fewer interactions than the head group of the native ligand PIP3. Thus, we hypothesized that the inhibitory effects of the flavonoids could be enhanced by increasing their bulkiness, modifying their hydrophobic regions, and increasing the number of hydrogen bonds through functional group substitutions. We therefore aimed to synthesize nitro (-NO_2)- and methoxy (-OCH_3)-substituted flavonoids and to measure their inhibitory effects on the AKT PH-PIP3 interaction.

Structural Optimization of Flavonoids

A total of 13 new flavonoids, including 5-methoxy-3′-nitroflavone, were chemically synthesized, and the inhibitory effects of these compounds on the AKT PH-PIP3 interaction were tested using liposome pulldown assays (Fig. S1). Of these compounds, only compound **12** (5-methoxy-3′-nitroflavone) exhibited an inhibitory effect on the AKT PH-PIP3 interaction. To elucidate the effect of the nitro substitution on the inhibitory effect of compound **12**, this compound was compared with 5,3′-dihydroxyflavone and 5,3′-dimethoxyflavone. While the hydroxy- and methoxy-substituted flavonoids resulted in little (5%) or no inhibition of the AKT PH-PIP3 interaction, respectively, the 3′-nitro-substituted flavonoid displayed an increased inhibitory effect of 27% (Fig. 6). The observation that methylation of the 3′-OH substituent abolished binding likely results from the increase in the hydrophobicity of the flavonoid and the decrease in capability for hydrogen bonding to the AKT PH domain. As expected, substitution of the 3′-OH with a nitro group resulted in a substantial increase in the inhibitory effect of the flavonoid, likely

because this substitution increased the bulk of the flavonoid and resulted in the formation of new hydrogen bonds with the AKT PH domain. Of course, this result was limited only to the 5,3′-disubstituted flavone, and the modified flavonoid did not have the strongest inhibitory effect among all flavonoids characterized in this study. Thus, functional group substitution should be attempted with a larger number of flavonoids and a larger number of functional groups to obtain optimized structural moieties for anticancer drug candidates. Nonetheless, we believe that the approaches described in the present study are valuable and have provided new insights into the development of antagonists to decrease the activities of target proteins.

Conclusively, the goal of anticancer drug development is typically to identify molecules that decrease the activities of effector proteins overexpressed in cancer cells, usually by inhibiting their catalytic domains directly, and indeed, most anticancer drugs target the catalytic domains of effector proteins. However, these proteins often have regulatory domains that are critical for activation; therefore, it should also be possible to decrease the activity of effector proteins by inhibiting their regulatory domains. In the present study, we identified flavonoid derivatives that disrupt activation of the protein kinase AKT by targeting the interaction between PIP3 and the regulatory PH domain of AKT, which is essential for activation. We showed that flavonoids that inhibited binding of PIP3 to the PH domain of AKT in liposome pulldown assays also decreased the level of AKT activation in cancer cells. Moreover, using computational analysis based on SAR analysis and in silico docking, we were able to enhance inhibition of the AKT PH-PIP3 interaction through rational structural modification of the flavonoids. Our results provide preliminary evidence that targeting of the regulatory domains of effector proteins is a viable strategy for the development of anticancer drug candidates.

Acknowledgment

This study was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2015R1C1A1A02037275 and 2017R1E1A1A01073894 to Y. Y.).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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