



# Insights into the inhibitory mechanism of triazole-based small molecules on phosphatidylinositol-4,5-bisphosphate binding pleckstrin homology domain



Sukhamoy Gorai<sup>a</sup>, Prasanta Ray Bagdi<sup>a</sup>, Rituparna Borah<sup>a</sup>, Debasish Paul<sup>b</sup>, Manas Kumar Santra<sup>b</sup>, Abu Taleb Khan<sup>c</sup>, Debasish Manna<sup>a,\*</sup>

<sup>a</sup> Department of Chemistry, Indian Institute of Technology Guwahati, Assam 781039, India

<sup>b</sup> National Center for Cell Science, Pune 411007, Maharashtra, India

<sup>c</sup> Alia University, DN 18, 8th Floor, Sector V, Kolkata 700091, India

## ARTICLE INFO

### Article history:

Received 22 April 2015

Received in revised form

18 May 2015

Accepted 19 May 2015

Available online 27 May 2015

### Keywords:

Pleckstrin homology domains

Phosphatidylinositols

1,2,3-Triazol-4-yl methanol substituent

Inhibition of protein–lipid interactions

Surface plasmon resonance-based competitive binding assay

Cellular displacement assay

## ABSTRACT

**Background:** Phosphatidylinositol 4,5-bisphosphate [PI(4,5)P<sub>2</sub>] is an important regulator of several cellular processes and a precursor for other second messengers which are involved in cell signaling pathways. Signaling proteins preferably interact with PI(4,5)P<sub>2</sub> through its pleckstrin homology (PH) domain. Efforts are underway to design small molecule-based antagonist, which can specifically inhibit the PI(4,5)P<sub>2</sub>/PH-domain interaction to establish an alternate strategy for the development of drug(s) for phosphoinositide signaling pathways.

**Methods:** Surface plasmon resonance, molecular docking, circular dichroism, competitive Förster resonance energy transfer, isothermal titration calorimetric analyses and liposome pull down assay were used.

**Results:** In this study, we employed 1,2,3-triazol-4-yl methanol containing small molecule (CIPs) as antagonists for PI(4,5)P<sub>2</sub>/PH-domain interaction and determined their inhibitory effect by using competitive-surface plasmon resonance analysis (IC<sub>50</sub> ranges from 53 to 159 nM for PI(4,5)P<sub>2</sub>/PLCδ1-PH domain binding assay). We also used phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P<sub>2</sub>], PI(4,5)P<sub>2</sub> specific PH-domains to determine binding selectivity of the compounds. Various physicochemical analyses showed that the compounds have weak affect on fluidity of the model membrane but, strongly interact with the phospholipase C δ1 (PLCδ1)-PH domains. The 1,2,3-triazol-4-yl methanol moiety and nitro group of the compounds are essential for their exothermic interaction with the PH-domains. Potent compound can efficiently displace PLCδ1-PH domain from plasma membrane to cytosol in A549 cells.

**Conclusions:** Overall, our studies demonstrate that these compounds interact with the PIP-binding PH-domains and inhibit their membrane recruitment.

**General significance:** These results suggest specific but differential binding of these compounds to the PLCδ1-PH domain and emphasize the role of their structural differences in binding parameters. These triazole-based compounds could be directly used/further developed as potential inhibitor for PH domain-dependent enzyme activity.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

In response to specific stimuli, various cytosolic proteins are reversibly recruited to the cellular membranes and form dynamic signaling complexes with specific lipid molecules, including phosphatidylinositols (PIPs). The PIP lipids have drawn considerable

attention due to their various cellular functions through a plethora of effector proteins [1–4]. A range of human diseases which are directly or indirectly linked with PIP-binding/metabolism have been identified and become an exciting therapeutic target in biomedical research [2,3,5–8]. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>) is the most abundant PIP in the plasma membrane (PM). Hydrolysis of PI(4,5)P<sub>2</sub> to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP<sub>3</sub>) by activated phospholipase C (PLC) enzymes is one of the crucial cellular signaling pathways. IP<sub>3</sub> regulates Ca<sup>2+</sup> release from

\* Correspondence author. Fax: +91 361 258 2349.  
E-mail address: [dmanna@iitg.ernet.in](mailto:dmanna@iitg.ernet.in) (D. Manna).

endoplasmic reticulum (ER) and DAG is the activator of protein kinase C (PKC) enzymes. Various membrane receptors including G-protein-coupled receptors (GPCRs) strongly interact with PLC enzyme and regulate PI(4,5)P<sub>2</sub> turnover and consequent downstream cell signaling pathways. PI(4,5)P<sub>2</sub> regulates several proteins including PLC $\delta$ , Ras GTPase activating protein (RasGAP) and pleckstrin, which also mediate a wide variety of cellular processes [9–12]. Improper cellular functions of these effector proteins are associated with disorder such as neurodegenerative, cardiovascular diseases and others [13,14]. The PI-kinase I/II and SH2-containing Inositol-5'-Phosphatase (SHIP), phosphatase and tensin homolog (PTEN) enzymes phosphorylate phosphatidylinositol-4-phosphate (PI(4)P)/phosphatidylinositol-5-phosphate (PI(5)P) and dephosphorylate phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>), respectively to generate PI(4,5)P<sub>2</sub> at the inner PM in responses to various stimuli [15,16]. PI(4,5)P<sub>2</sub> lipid is phosphorylated by class I PI3-kinase to form PI(3,4,5)P<sub>3</sub>, which regulates cellular functions of several crucial signaling proteins including AKT.

The PI(4,5)P<sub>2</sub> generation recruits several proteins to the PM through their interaction with pleckstrin homology (PH)- or other PIP-binding modules. This PI(4,5)P<sub>2</sub>-dependent membrane association of the lipid binding modules is necessary and sufficient for activation and proper functioning of the effector proteins; including PLC $\delta$ . Activation of PLC $\delta$  proceeds only after PI(4,5)P<sub>2</sub>-binding of the PH domain at the inner PM that reorient the EF-hands-C2 domain-TIM barrel unit so that the catalytic domain is in a productive orientation relative to the membrane. Recent studies reported that in addition to PH domain, C2 domain of PLC $\delta$  also interacts with the membrane and plays an important role in PLC $\delta$  activation [17,18].

In general catalytic domains of the proteins are considered as drug target to down/up-regulate the cellular activities by direct inhibition/activation mechanism. However, the catalytic activities of the proteins should be effectively regulated in the cells, because direct inhibition/activation could also induce side-effects by disrupting the other up/down-stream of the cellular pathways [19–21]. Detailed mechanistic studies demonstrated that highly specific PIP/PH-domain interactions can regulate activities of the effector proteins. It is also important to note that the PH-domains contain a conserved structure with well-defined binding site for "prototypic" small molecule-protein interaction studies [22]. It is also depicted that protein-lipid interactions are readily targetable by small molecules due to the small and defined binding site of the proteins for specific lipid molecules, whereas protein-protein interactions normally need extended flat protein surfaces, that are difficult to disrupt by small molecules. It is also demonstrated that development of small molecules based inhibitors for lipid-protein interactions is advantageous over typical approaches in the aspect of side-effect and rational design because of relatively simple structures and functions [5,20,23,24]. For these reasons, the PH-domains of the effector proteins can be considered as an attractive alternate target in designing selective inhibitors for its interaction with PIPs.

However, development of inhibitors for PIP/PH-domain interactions to regulate enzyme activities had not been substantially described yet. Using the similar hypothesis, we recently demonstrated that development of DAG-responsive C1 domain based activator can be considered as an alternative target to regulate the activities of the PKC enzymes [25–27]. Recently developed PH-domain targeting lipid-based compound, 3-deoxy phosphatidylinositol ether lipid (DPIEL) and PHT-427 were described as potential drug candidates for the treatment of cancer and other human diseases [21,28]. Small molecules like PITENINs were also demonstrated as the inhibitor of PI(3,4,5)P<sub>3</sub> binding of AKT1/PDK1-PH-domains and down-regulator of PI3-Kinase/PDK1/AKT1 pathways [8]. There are only a few PLC regulators that play a significant role in understanding the PI(4,5)P<sub>2</sub> mediated cellular signaling

pathways [17,29]. Neomycin is known as a regulator of PI(4,5)P<sub>2</sub>-PLC enzyme interactions and PLC induced PI(4,5)P<sub>2</sub> hydrolysis at the cellular membranes. However neomycin directly interacts with the PI(4,5)P<sub>2</sub> molecules present at the membrane through an electrostatic interaction and blocks PLC $\delta$  enzyme activity [29]. However, there is no report of PH domain specific PLC regulator.

In this regard our current study describes the development of 1,2,3-triazol-4-yl methanol-based antagonists, CIPs for PI(4,5)P<sub>2</sub>/PLC $\delta$ 1-PH domain binding. These compounds at lower concentrations showed certain degree of selective inhibitory effect towards different PH-domains used for the current study. The 1,2,3-triazol-4-yl methanol moiety and nitro group of the compounds play a crucial role in distinguishing the PH-domains. Potent compound, CIP-4, can competitively interact with the PLC $\delta$ 1-PH domain and displace it from PM to cytoplasm. We believe that these non-lipid potent compounds would be able to inhibit PI(4,5)P<sub>2</sub> targeted PIP-binding proteins/enzyme under cellular environment and regulate its cellular signaling pathways.

## 2. Experimental methods

### 2.1. General Information

All chemicals and reagents were purchased from Sigma (St. Louis MO), SRL (Mumbai, India), Merck (Mumbai, India) and used for the synthesis without further purification. Phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P<sub>3</sub>), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P<sub>2</sub>) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>) were purchased from Cayman Chemicals (Ann Arbor, MI). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (PS), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dPE) were purchased from Avanti Polar Lipids. Octyl glucoside was purchased from Fisher. The Pioneer L1 sensor chip was purchased from Biacore AB (Piscataway, NJ). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers. Compounds were first dissolved in DMSO and then diluted in working buffer so that overall DMSO concentration was < 5% (v/v).

### 2.2. Protein purification

The AKT1 (homo sapiens; 1-121 amino acids), GRP1 (mus musculus, 1-127 amino acid), TAPP1 (homo sapiens; 180-305 amino acid) and PLC $\delta$ 1 (rattus norvegicus, 1-131 amino acid) PH-domains were expressed in *Escherichia coli* cells (BL21-DE3) and purified using methods similar to those reported earlier [22]. The plasmids were generous gift from Prof. Wonhwa Cho (University of Illinois at Chicago, IL, USA).

### 2.3. Surface plasmon resonance (SPR) assay

All surface plasmon resonance (SPR) measurements were performed (at 25 °C, in 20 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, flow rate of 30  $\mu$ L/min) using a lipid-coated L1 sensorchip in the Biacore-X100 (GE Healthcare) system as described earlier [22,30]. Vesicles for SPR analysis were prepared at a concentration of 0.5 mg/ml in 20 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, and were vortexed vigorously and passed through a 100-nm polycarbonate filter using an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) according to the manufacturer's protocol. After washing the sensor chip surface with the running buffer (20 mM HEPES, pH 7.4, containing 0.16 M KCl) PC/PE/PS/PI(4,5)P<sub>2</sub>

or PC/PE/PS/PI(3,4)P<sub>2</sub> or PC/PE/PS/PI(3,4,5)P<sub>3</sub> (57:20:20:3) and PC/PE/PS (60:20:20) vesicles were injected at 5  $\mu\text{L}/\text{min}$  to the active surface and the control surface, respectively, to achieve similar response unit (RU) values (3500–4000 RU). To minimize non-specific adsorption the control surface was also coated with 40  $\mu\text{L}$  of BSA (0.1 mg/ml in the running buffer) at a flow rate of 5  $\mu\text{L}/\text{min}$ , and equilibrated for 20 min, before the injection of protein. The competitive inhibitory effects of each compound were determined by measuring the change in response unit (RU) of the SPR sensorgrams of PH-domains (500 nM) in the absence/presence of compounds (0–20  $\mu\text{M}$ ) at a flow rate of 30  $\mu\text{L}/\text{min}$ . For inhibition studies of **CIP-4** with different PH domains the range of concentration was 0–200  $\mu\text{M}$ . The compounds were equilibrated with respective PH-domain for 30 min before any SPR measurements. The decrease in RU value of each sensorgram with various compound concentrations was measured to calculate % of inhibition efficiency. The inhibition potencies were calculated as  $(1 - (\text{RU of protein mixed with chemicals}/\text{RU of protein only})) \times 100\%$ . The RU value after 180 s of injection was considered for % of inhibition efficiency calculations. The IC<sub>50</sub> values for this SPR analysis were calculated using nonlinear least square fit analysis (using an equation,  $R_{\text{eq}} = R_{\text{max}}/(1 + K_d/A_0)$ ) considering a Langmuir-type binding model between the protein (A) and vesicles (B) i.e.  $A + B \leftrightarrow AB$ . Each experiment was repeated more than three times.

#### 2.4. In-silico molecular docking analysis

Computational docking and scoring studies of the interaction of the compounds with PH-domains were performed using Molegro Virtual Docker (version 4.3.0, Molegro ApS, Aarhus, Denmark) and AutoDock 4 (The Scripps Research Institute, La Jolla, USA) software with essentially the same results [25,27]. The crystal structure of AKT1-PH (1–116 amino acids, PDB ID: 1H10) and PLC $\delta$ 1-PH (1–131 amino acids, PDB ID: 1MAI) was utilized for docking analyses [31,32]. To generate apo-protein, the ligands were first removed from the co-crystal structures and then they were processed by energy minimization. In the meantime energy minimized three-dimensional structures of ligands were prepared by using the GlycoBioChem PRODRG2 Server (<http://davapc1.bioch.dundee.ac.uk/prodrg/>). The GRONINGEN Machine for Chemical Simulations (GROMACS) library of three-atom combination geometries employing a combination of short molecular dynamics simulations and energy minimizations were utilized for the conversion of 2D molecular structures to 3D structures. The original blind-docking parameters (selected binding site for docking/grid was chosen to cover the entire PH domain; Grid coordinates were  $X=22$ ,  $Y=16$ ,  $Z=12$ ,  $r=30$ ) were used in combination with an evaluation scheme based on Gibbs free energy change ( $\Delta G$ ). In each docking run, 100 docked structures were generated for individual ligand. Energetically favored docked conformations were evaluated on the basis of moledock and re-rank score. The docking poses were exported and examined with PyMOL software (The PyMol Molecular Graphics System, Version 1.0r1, Schrödinger, LLC.). The residues surrounding the compounds were also analyzed using LigPlot provided by the European Bioinformatics Institute.

#### 2.5. Circular dichroism studies

The circular dichroism (CD) spectroscopy was studied on a JASCO J-815 CD spectropolarimeter at room temperature [33]. CD spectra of PLC $\delta$ 1-PH domain (1  $\mu\text{M}$ ) in the absence and presence of compounds (1:3 molar ratio of the PLC $\delta$ 1-PH domain to the compounds) were obtained in the wavelength range of 190–245 nm in 10 mM phosphate – containing 10 mM NaCl buffer (pH 7.2).

#### 2.6. Anisotropy measurement

Fluorescence anisotropy measurements were performed on a Fluoromax-4 spectrofluorometer at 25 °C. The anisotropies of 1,6-diphenyl-1,3,5-hexatriene (DPH) and NBD-PE under liposomal environment were measured according to the reported procedure [25,27,34]. The fluorescence probe DPH was incorporated into the PC/PE/PS (60:20:20) liposome (100  $\mu\text{L}$  of 0.5 mg/mL of total lipid) by adding the dye dissolved in tetrahydrofuran (THF, 1 mM) to vesicles up to a final concentration of 1.25  $\mu\text{M}$ . The NBD-PE probe was incorporated to the PC/PE/PS/NBD-PE (59:20:20:1) liposome using our earlier mentioned procedure [27]. After 30 min of incubation at room temperature, DPH ( $\lambda_{\text{ex}}=355$  nm;  $\lambda_{\text{em}}=430$  nm) and NBD ( $\lambda_{\text{ex}}=460$  nm;  $\lambda_{\text{em}}=535$  nm) fluorescence anisotropies were measured. The concentration of compounds was 15  $\mu\text{M}$ . The degree of anisotropy in the DPH/NBD fluorescence of the probes was calculated using Eq. (1), at the peak of the fluorescence spectrum, where  $I_{\text{VV}}$  and  $I_{\text{VH}}$  are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and  $G=I_{\text{VH}}/I_{\text{HH}}$  is the instrumental grating factor.

$$r = \frac{(I_{\text{VV}} - GI_{\text{VH}})}{(I_{\text{VV}} + 2GI_{\text{VH}})} \quad (1)$$

#### 2.7. Zeta-potential measurement

The zeta-potential measurements of the liposomes in the absence and presence of the compounds were carried out in aqueous medium (at 25 °C, in 20 mM Tris–HCl buffer, pH 7.4, containing 0.16 M NaCl) using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) light scattering spectrometer equipped with a He–Ne laser working at 4 mW ( $\lambda_0=632.8$  nm) [35,36]. Unilamellar vesicles composed of PC/PE/PS/PI(3,4,5)P<sub>3</sub> (57:20:20:3) lipids were prepared in 20 mM Tris–HCl buffer, pH 7.4, containing 0.16 M NaCl by vigorous vortexing and extruding through a polycarbonate filter (100-nm) using an Avanti Mini-Extruder. 100  $\mu\text{L}$  of liposomes from 0.5 mg/mL of total lipid was used for zeta-potential measurements. All the measurements were performed three times per sample and averaged to give the final value.

#### 2.8. Förster resonance energy transfer (FRET) measurements

Analysis of protein-to-membrane Förster resonance energy transfer (FRET) based binding assay was used to detect the selectivity of the compounds for PH-domain binding through PIP-binding site and detect the effectiveness of the compounds under liposomal environment [25,27]. The vesicles composed of PC/PE/PS/dPE (59:20:20:1) and PC/PE/PS/dPE/PI(4,5)P<sub>2</sub> (56:20:20:1:3) were used as control and for ligands, respectively. In this assay, PLC $\delta$ 1-PH domain was allowed to bind to the liposome and then efficacy of the compounds to displace the protein from membrane surface was tested by monitoring the change in FRET signal in the presence of the compounds. The FRET signal due to PIP-dependent protein binding to the liposomes was corrected with non-specific fluorescence signal originated from control liposome (PC/PE/PS/dPE) and protein interactions. The stock solution of compounds was titrated into the sample containing PLC $\delta$ 1-PH domain (1  $\mu\text{M}$ ) and excess liposome (100  $\mu\text{M}$  total lipid) in a buffer solution (20 mM Tris–HCl buffer, pH 7.4, containing 0.16 M NaCl) at room temperature. The competitive displacement of protein from the membrane was monitored using protein-to-membrane FRET signal ( $\lambda_{\text{ex}}=280$  nm and  $\lambda_{\text{em}}=505$  nm). Control experiments were performed to measure the dilution effect under similar experimental condition and the increasing background emission arising

from direct dPE excitation. Effect of direct compound binding to the protein and compound binding to the dPE lipid (present in liposome) was also measured as control experiment. Protein-to-membrane FRET signal values as a function of compound concentration were subjected to nonlinear least-squares-fit analysis using Eq. (2) to calculate apparent equilibrium inhibition constants ( $K_i(\text{Compound})_{\text{app}}$ ) for compounds, where  $[x]$  represents the total compound concentration and  $\Delta F_{\text{max}}$  represents the calculated maximal fluorescence change

$$F = \Delta F_{\text{max}} \left( 1 - \frac{[x]}{[x] + K_i(\text{Compound})_{\text{app}}} \right) + C \quad (2)$$

### 2.9. Isothermal titration calorimetric (ITC) measurements

Thermodynamic parameters of protein–ligand interactions were measured using an ITC-200 micro-calorimeter from Microcal (Northampton, MA, USA). PLC $\delta$ 1-PH domain (200  $\mu\text{M}$ ), after dialysis with 20 mM HEPES buffer, pH 7.4, containing 0.16 M KCl, was titrated against compounds (2 mM) dissolved in the final dialysate [37]. A typical titration involved injecting 20 injection volumes (2  $\mu\text{L}$ ) of compound into the sample cell containing PLC $\delta$ 1-PH domain (201.6  $\mu\text{L}$ ) at 2.0 min intervals with continuous stirring (at 25  $^{\circ}\text{C}$  with stirring speed of 500 rpm). The heat of dilution data corresponding to individual injections was analyzed using a sequential binding model with one binding site considering both IP3 and PS-binding sites per PLC $\delta$ 1-PH domain monomer with the system running Microcal Origin 7.0 software. The  $\Delta H$  and  $\Delta S$  values were obtained using a nonlinear least-square fit of the data. Gibbs free energy ( $\Delta G$ ) was calculated by using Gibbs equation:  $\Delta G = \Delta H - T\Delta S$ .

### 2.10. Liposome binding assay

Inhibition of PI(4,5) $P_2$ /PLC $\delta$ 1-PH domain interaction by the compounds was further determined by liposome pull-down assay according to the reported procedures [26,38,39]. Liposomes of PC/PE/PS (60:20:20) and PC/PE/PS/PI(4,5) $P_2$  and PC/PE/PS/PI(3,4,5) $P_3$  (57:20:20:3) in 50 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 170 mM sucrose, pH 7.4 buffer solution were prepared by sonication, followed by extrusion through 0.1 mm pore size polycarbonate filters. The suspension was pelleted down at 100,000g, 4  $^{\circ}\text{C}$ , for 20 min and resuspended in binding buffer (20 mM Tris buffer, pH 7.4, containing 100 mM KCl, 5 mM MgCl $_2$ ). Purified PLC $\delta$ 1- and AKT1 PH domains (50  $\mu\text{M}$ ) in the absence or presence of compounds were incubated with sucrose-loaded vesicles (40  $\mu\text{g}$  in 100  $\mu\text{L}$ ) for 30 min in the presence of binding buffer containing 0.3 mg mL $^{-1}$  BSA. Control experiment of PLC $\delta$ 1 binding to PC/PE/PS liposome was also performed using the same method. Membrane-bound protein was separated from free protein by centrifugation at 100,000g for 30 min at 4  $^{\circ}\text{C}$ . Protein of supernatant and pellet fractions was analyzed by SDS-PAGE gel.

### 2.11. Cellular translocation measurements

Human lung cancer cells (A549) were cultured according to the American Type Culture Collection (ATCC) instructions. Cells were grown in RPMI-1640 medium on cover slip supplemented with 10% heat inactivated FBS along with penicillin and streptomycin at 37  $^{\circ}\text{C}$  with 5% CO $_2$ . After 24 h of seeding the cells, GFP-tagged PLC $\delta$ 1-PH domain construct was transfected using polyethylenimine transfection reagent in Opti-MEM media, according to the manufacturer's instructions. Transfected media was changed with complete media after 24 h of transfection. Just before the compound treatment (compounds were first dissolved in DMSO

and then diluted in Opti-MEM media, so that DMSO concentration was < 5% (v/v)), the media were removed and the cells were washed and overlaid with serum free media. Cells were treated with different concentration (5, 10, 20, 100  $\mu\text{M}$ ) of **CIP-1** and **CIP-4** for 30 min. For immunofluorescence study, cells were first washed three times with phosphate-buffered saline (PBS) to remove the media completely. Then, cells were fixed using 4% paraformaldehyde solution at room temperature for 15 min. Fixed cells were washed three times with PBS to remove the extra paraformaldehyde and were then permeabilized using 0.1% Triton X-100 solution for 10 min at room temperature. Following permeabilization, the cells were again washed three times with PBS and mounted with mounting media containing DAPI on a glass slide. Confocal images of the fixed cells were collected on Zeiss LSM 510 NLO confocal microscopes (Carl Zeiss, Inc.) using 63  $\times$  1.4 NA oil objective. For excitation of GFP and the green membrane marker, the argon laser at 488 nm was used.

## 3. Results and discussion

Phosphatidylinositol signaling pathway is considered as one of the most commonly deregulated pathways in several human diseases including cancer [5,20,40,41]. For this reason development of potent and specific inhibitors for the effector proteins associated with this PI-kinase/phosphatase pathway is highly demanding in the drug discovery and related research fields [42]. However, targeting the catalytic domain of these enzymes to develop inhibitor remained quite challenging. For example, several potent inhibitors of AKT1 enzyme turn out to be relatively toxic, presumably due to the inhibition of other ser/threonine kinases [20,43]. On the other hand, small molecules such as DPIEL and perifosine developed for the inhibition of PIP/PH-domain interaction are comparatively nontoxic and offer a better therapeutic strategy than inhibitors for ATP-site [21,41]. In recent years, studies have been carried out to identify phosphate or nonphosphate containing small molecules as being inhibitors for PIP-binding domains [8,20,44,45].

It is well documented that stimulated PLC $\delta$ 1 enzyme hydrolyzes PI(4,5) $P_2$  to DAG and IP3, which acts as second messengers. Dysregulation of PLC enzyme activity is related with diverse diseases including cancer, cardiovascular diseases [46–48]. Therefore, development of small molecule-based inhibitors is considered as potential pharmacological tools to investigate the roles PLC enzyme in diseases and can be used as candidates for drug discovery. The interaction of PH domain with PI(4,5) $P_2$  at the inner PM is essential for PLC $\delta$  enzyme to hydrolyze PI(4,5) $P_2$  [17,29,49]. In this regard, we have synthesized 1,2,3-triazol-4-yl methanol derivatives targeting the PI(4,5) $P_2$  binding PH-domain of PLC $\delta$ 1 (Fig. 1). The compounds were synthesized according to the reported procedure using CuO nanoparticles as catalyst [50–52]. Typically, all molecules were designed such a way, that there must be 1,2,3-triazol-4-yl methanol moiety, which are critical in forming more

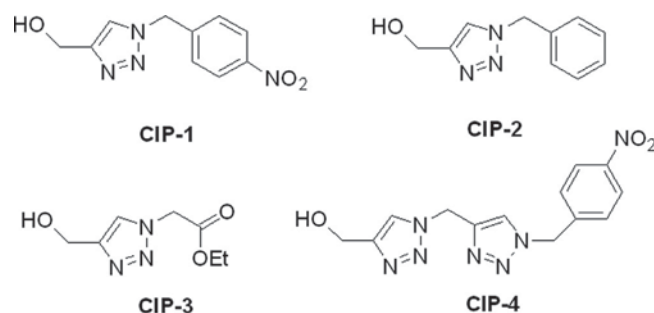
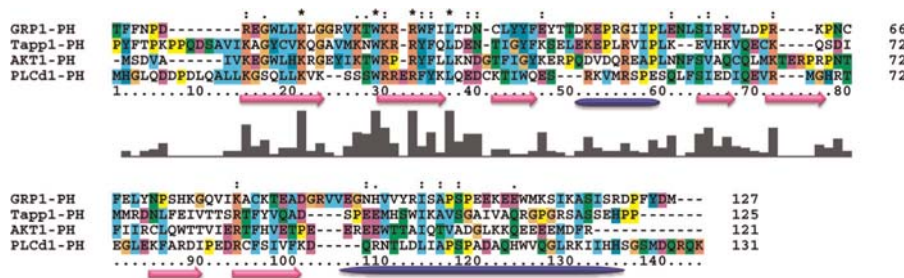


Fig. 1. Structures of the compounds (CIPs) used in the present study.

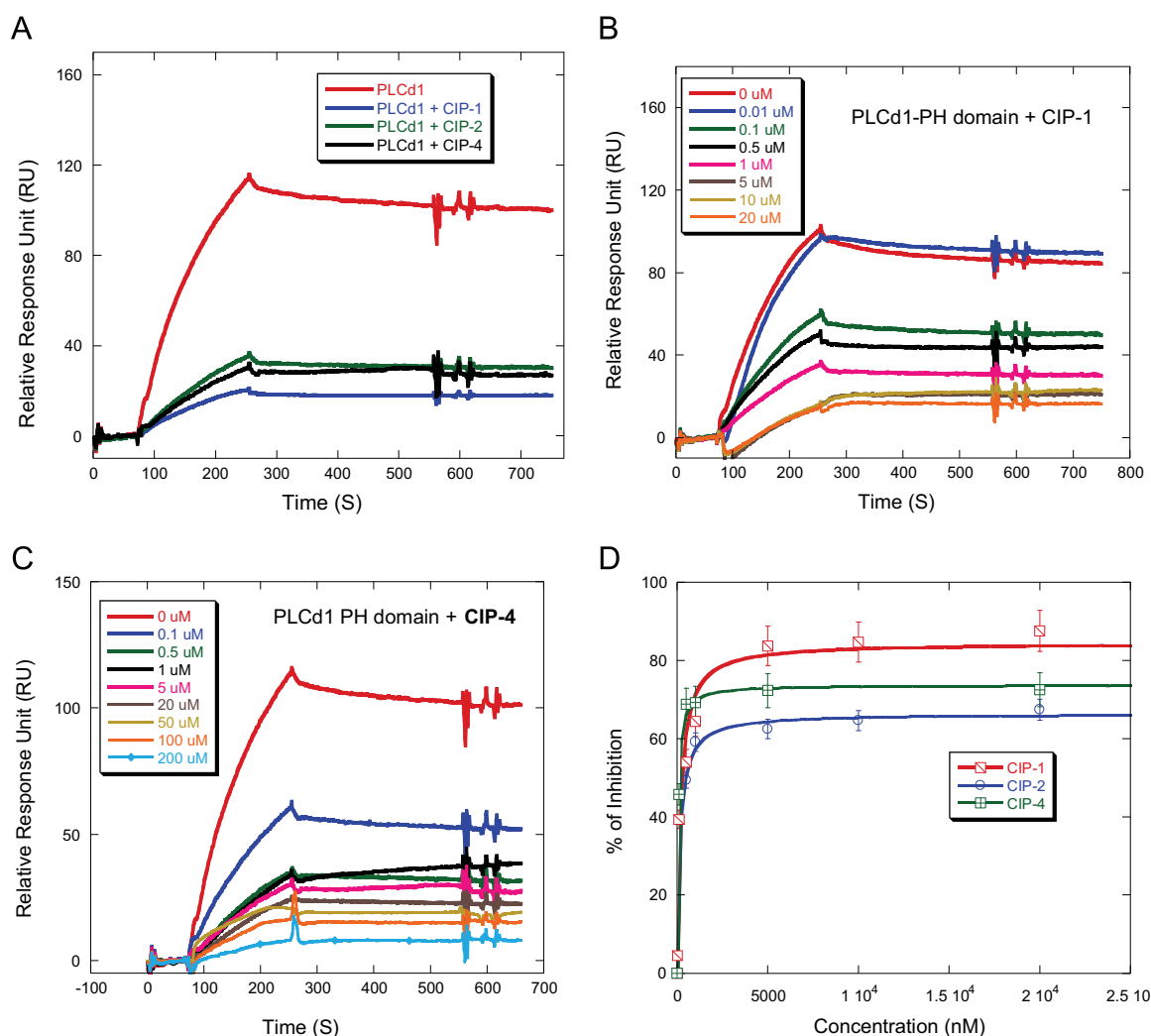


**Fig. 2.** Amino-acid sequence alignment of the AKT1, GRP1, TAPP1, PLCδ1-PH domains is shown using Clustal X. Secondary structural elements of the PH domains are shown below the alignments with colored pink ( $\beta$ -sheets) or blue ( $\alpha$ -helices). PH domain residues forming the cationic patch at its base are labelled with chocolate squares.

hydrogen bond with their binding partners and can be fitted inside the shallow positively charged binding pocket of the PLC $\delta$ 1-PH domain (Fig. 2). The nitro group and an additional triazole ring were installed to understand their impact in hydrogen bond formation with the amino acid residues within the PH-domain binding pocket. Thus, the impact of this study is not only inhibition constant measurement but also elucidate their binding mechanism.

### 3.1. Surface plasmon resonance based-competitive binding assay

We first measured the efficiency of the compounds in disrupting the PIP/PH domain interaction, by using SPR-based competitive binding assay, a useful technique for quantitative determination of binding and/or inhibition affinities in real-time [20,30]. All SPR measurements were performed using two parallel flow-channel systems (control and active channel). Liposomes of PC/PE/PS (60:20:20) and PC/PE/PS/PI(4,5)P<sub>2</sub> or PC/PE/PS/PI(3,4)P<sub>2</sub>



**Fig. 3.** Screening of PI(4,5)P<sub>2</sub>/PLC $\delta$ 1-PH domain (500 nM) interaction selectivity by compounds (5  $\mu$ M) (A). Surface plasmon resonance sensorgrams of PI(4,5)P<sub>2</sub> binding PLC $\delta$ 1-PH domain in the presence of increasing concentration of compounds **CIP-1** (B) and **CIP-4** (C). Selectivity analysis (% of inhibition) of the compounds for PI(4,5)P<sub>2</sub>/PLC $\delta$ 1-PH domain inhibition (D). PC/PE/PS/PI(4,5)P<sub>2</sub> (57:20:20:3) and PC/PE/PS (60:20:20) vesicles were used as active and the control surface, respectively. Values represent the mean  $\pm$  SD from triplicate measurements. PLC $\delta$ 1 stands for PLC $\delta$ 1.

**Table 1**  
Relative inhibitory activity of the compounds measured by competitive-surface plasmon resonance analysis.

Protein	Relative inhibitory activity (%)				IC <sub>50</sub> values (nM)			
	CIP-1	CIP-2	CIP-3	CIP-4	CIP-1	CIP-2	CIP-3	CIP-4
PLCδ1-PH	83 ± 4	62 ± 3	–	72 ± 3	113 ± 7	159 ± 11	–	53 ± 9
TAPP1-PH	18 ± 2	41 ± 4	–	21 ± 2	647 ± 33	106 ± 8	–	1112 ± 78
AKT1-PH	44 ± 2	55 ± 2	4 ± 1	33 ± 3	175 ± 21	315 ± 32	NM	3799 ± 98
GRP1-PH	56 ± 3	58 ± 5	–	52 ± 4	145 ± 11	216 ± 9	–	789 ± 35

Protein, 500 nM; % of inhibition was calculated using 5 μM compound concentration; measurements were performed in 20 mM HEPES buffer at pH 7.4 containing 160 mM KCl; NM, not measured; liposome composition of PC/PE/PS/PIP (57:20:20:3) was used. The inhibition of PI(4,5)P<sub>2</sub>/PLCδ1-PH, PI(3,4,5)P<sub>3</sub>/AKT1-PH, PI(3,4,5)P<sub>3</sub>/GRP1-PH and PI(3,4)P<sub>2</sub>/Tapp1-PH interactions was measured in the presence of compounds. Values represent the mean ± SD from triplicate measurements.

or PC/PE/PS/PI(3,4,5)P<sub>3</sub> (57:20:20:3) were coated on control and active channel, respectively. For all measurements only protein or protein equilibrated with CIP compounds were passed over control channel to active channels. To remove any non-specific binding of protein on the liposomal surface, RU of control channel was subtracted from RU of active channel. PIP selectivity, of the respective PH domains was also tested under similar experimental conditions. PLCδ1-PH domain does not bind to the PC/PE/PS/PI(3,4,5)P<sub>3</sub> (57:20:20:3) surface. The calculated % of inhibition values from SPR measurements showed that compounds CIP-1 and CIP-4 strongly inhibit the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction under liposomal environment (Fig. 3A). The compounds CIP-1 and CIP-4 with 5 μM concentration showed 83% and 72% inhibitory effect for PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interactions, respectively (Table 1). For further understanding of this inhibitory effect of the compounds on PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction, we also carried out SPR measurements in a concentration dependent manner (Fig. 3B and C). The analysis clearly showed that the relative RU values of the SPR sensorgrams of PLCδ1-PH domain decrease as a function of concentration of the compounds. These results clearly suggest that the compounds, CIP-1 and CIP-4 could affect the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction, either by blocking the binding site or by altering the protein conformation.

The calculated IC<sub>50</sub> values for CIP-1 and CIP-4 compounds were 113 and 53 nM, respectively for the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction, indicating their strong binding pattern under experimental conditions. PLCδ1-PH domain is reported as cellular marker of PI(4,5)P<sub>2</sub> lipid [2,9,15,29]. To determine the selectivity of potent compounds in inhibiting the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction, we also measured their inhibitory effect on other PIPs binding PH-domains (Fig. S1). It is well documented that AKT1-PH domain strongly interacts with both PI(3,4,5)P<sub>3</sub> and PI(3,4)P<sub>2</sub>, whereas GRP1 and TAPP1-PH domains selectively interact with PI(3,4,5)P<sub>3</sub> and PI(3,4)P<sub>2</sub> respectively [22]. We measured the inhibitory effects of compounds CIP-1 and CIP-4 on PI(3,4,5)P<sub>3</sub>/AKT1-PH domain (IC<sub>50</sub> values were 175 and 3799 nM, respectively), PI(3,4,5)P<sub>3</sub>/GRP1-PH domain (IC<sub>50</sub> values were 159 and 789 nM, respectively) and PI(3,4)P<sub>2</sub>/TAPP1-PH domain (IC<sub>50</sub> values were 647 and 1112 nM, respectively) interactions (Table 1). Concentration dependent % of inhibition values of CIP-4 for different PIP/PH domain interactions are shown in Table S1. We also used compound (1-methyl-1H-1,2,3-triazol-4-yl)methanol as negative control for competitive SPR measurements (data not shown). This compound contain triazole ring as of our tested compounds (CIPs) but did not affect the PLCδ1-PH domain binding to the PC/PE/PS/PI(4,5)P<sub>2</sub> liposome under similar experimental conditions. This clearly states that certain structural units of the triazole ring are essential for significant inhibition of PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interactions. We also performed SPR analysis with only compounds (in the absence of protein) on liposome coated surface to examine the effect of compounds on this competitive-SPR analysis (Fig. S2). The sensorgrams showed almost no significant binding of the compounds on liposome surface (PC/PE/PS/PI(4,5)P<sub>2</sub>). This

suggests that only compound do not have any effect on the RU of the PLCδ1-PH domain binding to the PI(4,5)P<sub>2</sub> containing liposome.

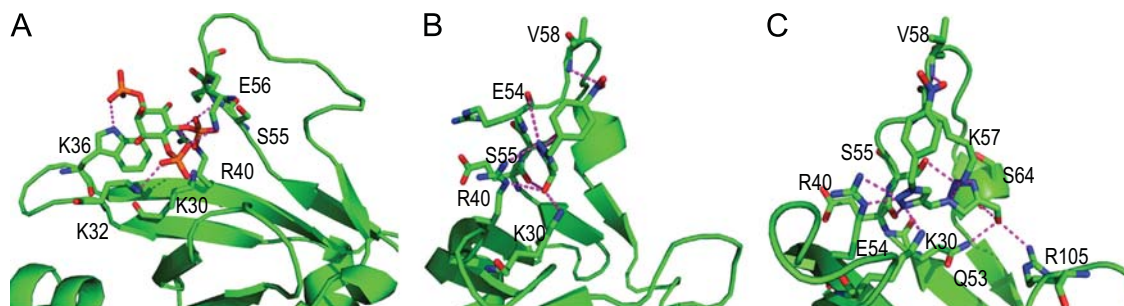
These results suggest that compound CIP-4 has certain degree of selectivity for PI(4,5)P<sub>2</sub> binding PLCδ1-PH domain over PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> binding PH-domains. Differential inhibitory effects of the compounds on PIP/PH-domain interactions could be due to their interaction pattern with the amino acid residues within and/or outside the PIP-binding pocket, stability of the compound-PH-domain complexes, effect of compound on membrane bilayer or their synergistic effect. Hence, additional studies are required to determine the specificities and understand their binding mechanism of these compounds for diverse PIP-binding PH-domains.

### 3.2. Molecular docking analysis

The SPR-based competitive binding assay showed that the compounds differentially inhibit the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction under the similar experimental conditions. The relative inhibitory activity of compound CIP-4 for PI(4,5)P<sub>2</sub>/PLCδ1-PH domain was much higher than other PIP-binding PH-domains, which indicate that CIP-4 was able to distinguish different PH-domains tested under the similar experimental conditions. Structural analysis revealed that all these tested compounds contain 1,2,3-triazol-4-yl methanol moiety, consequently to elucidate the probable binding mode for different inhibitory efficacies, molecular docking analysis was performed.

The reported crystal structure of PLCδ1-PH domain (1MAI) in complex with inositol 1,4,5-triphosphate (IP3) provides a detail insight into the mode of ligand interactions within the binding pocket [32]. IP3 is the only headgroup of PI(4,5)P<sub>2</sub> lipid molecule. Structural analysis and structure-activity studies showed that phosphate groups attached to myo-inositol ring of IP3 preferably interact with the K30, K32, W36, R40, S55, R56 and R57 residues through hydrogen bond formation [10,15,29,32]. More than 90% of the docking poses of the blind-molecular docking analysis (grid covers the whole protein) revealed that CIP-1 and CIP-4 ligands preferably interact with the PLCδ1-PH domain through its IP3 binding site. For further analysis best docking poses were selected based on moldock score, rerank score and position within the IP3-binding site of the protein. The calculated in-silico interaction energies between the ligands and PLCδ1-PH domain protein are of high negative values, suggesting acceptable docking poses for further analysis (Table S2). The triazole ring, hydroxy and nitro groups of the compounds could be mainly responsible for their interaction with the cationic groove of the PLCδ1-PH domain (Fig. 4). The docking poses showed that the pharmacophores of compounds CIP-1 and CIP-4 could form hydrogen bonds with the side chain or backbone carbonyls/amide protons of PLCδ1-PH domain through its IP3 binding site (Fig. 4 and Fig. S3).

However, the docking results did not show any significant difference in their number of interactions or interacting residues. CIP-1 and CIP-4 showed 5 and 6-hydrogen bond formation with



**Fig. 4.** Structure of PLC $\delta$ 1-PH domain (1MAI) in complex with IP<sub>3</sub> (A). Model structures of ligands **CIP-1** (B) and **CIP-4** (C) docked into the PIP-binding site of the PLC $\delta$ 1-PH domain (1MAI). Residues involved in interactions through hydrogen bond formation are shown using dashed lines (pink).

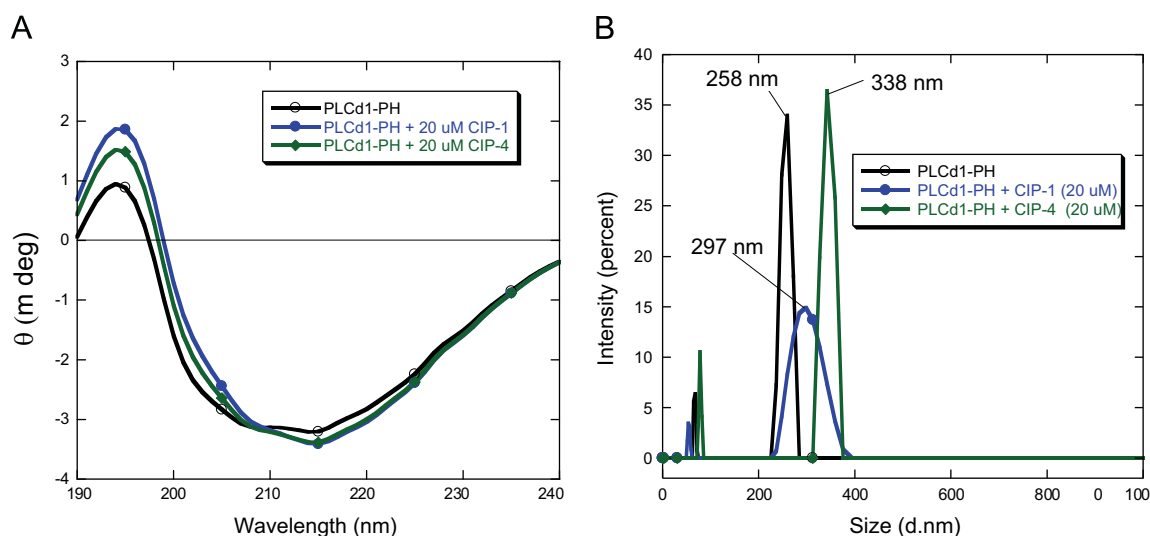
the PLC $\delta$ 1-PH domain, respectively (Fig. S3). Calculated IC<sub>50</sub> values of the compounds showed that **CIP-4** has approximately 2-fold stronger binding affinity than **CIP-1**. Therefore, strength of interactions and surface area of the compounds may be important criterion in showing stronger binding properties. We also performed molecular docking analysis of these ligands with AKT1-PH domain (1H10) [31]. The docking results showed that the ligands specifically interact with the AKT1-PH domain through its IP<sub>4</sub>-binding site in a similar pattern as with PLC $\delta$ 1-PH domain. In particular, compound **CIP-4** forms only 3-hydrogen bonding with AKT1-PH domain, which was also reflected in its IC<sub>50</sub> values. Therefore, the molecular docking analysis predicts that these ligands interact with the PH-domain preferably through their PIP-binding site.

### 3.3. Structural change measurement

Lower IC<sub>50</sub> values of the compounds could be due to the structural change of the PH-domains in the presence of the compounds that could prohibit the PH-domains to interact with the PIP containing membranes. In this regard, we performed circular dichroism (CD) spectral analysis to understand whether interactions of compounds with the PH-domains alter the structural integrity or not. The CD spectra of the PLC $\delta$ 1-PH domain (1  $\mu$ M) in the absence/presence of compounds **CIP-1** and **CIP-4** are shown in Fig. 5A. The negative ellipticity in the range of 225–200 nm and the positive ellipticity around 195 nm are the characteristic of protein secondary structural features such as  $\alpha$ -helix and  $\beta$ -sheet.

The spectral data showed that in the presence of compounds **CIP-1** and **CIP-4** there is almost no change in their secondary structural pattern of PLC $\delta$ 1-PH domain in the far UV-CD spectrum. The change in beta sheet content is little bit higher in the presence of **CIP-4** than **CIP-1**, which is in correlation with the IC<sub>50</sub> values. However, larger change in  $\beta$ -sheet content of the PLC $\delta$ 1-PH domain indicates that the compounds interact with the PH-domain through its IP<sub>3</sub>-binding site which is composed of seven  $\beta$ -sheets and connecting loop regions and stabilizes the PLC $\delta$ 1-PH domain structure than in free dynamical structure. It is important to mention that any increase in helical content of the PLC $\delta$ 1-PH domain upon ligand binding would be small and that any increase in CD-signal in the range of 225–200 nm might be obscured by the change in contribution from high  $\beta$ -sheet content of the PH-domain structure. Therefore CD analyses clarify that these compounds neither induce protein aggregation nor significantly destabilize the 3D-structure of the protein.

In order to ascertain that ligand binding does not induce any significant structural change (such as oligomerization) of the PLC $\delta$ 1 PH-domain protein (1  $\mu$ M), we also performed dynamic light scattering (DLS) measurements in aqueous solution (at 25  $^{\circ}$ C, in 20 mM Tris-HCl buffer, pH 7.4, containing 0.16 M NaCl). Fig. 5B shows that in the presence of compounds sizes (diameter) of the particles are slightly different. Therefore, our CD and DLS measurements clearly showed that the ligand binding does not significantly alter the secondary structure of the PLC $\delta$ 1-PH domain and no oligomerization was observed under the experimental conditions, respectively.



**Fig. 5.** Effect of compounds on the secondary structural content of the PLC $\delta$ 1-PH domain. Far-UV CD spectra of PLC $\delta$ 1-PH domain (1  $\mu$ M) in the absence and presence of compounds (20  $\mu$ M) in 10 mM phosphate – containing 10 mM NaCl buffer (pH 7.2) (A). Size distribution of PLC $\delta$ 1-PH domain (1  $\mu$ M) in the absence or presence of compounds (20  $\mu$ M, at  $t = 5$  min) in aqueous solution (PBS buffer pH 7.0) at 25  $^{\circ}$ C (B). PLC $\delta$ 1 stands for PLC $\delta$ 1.

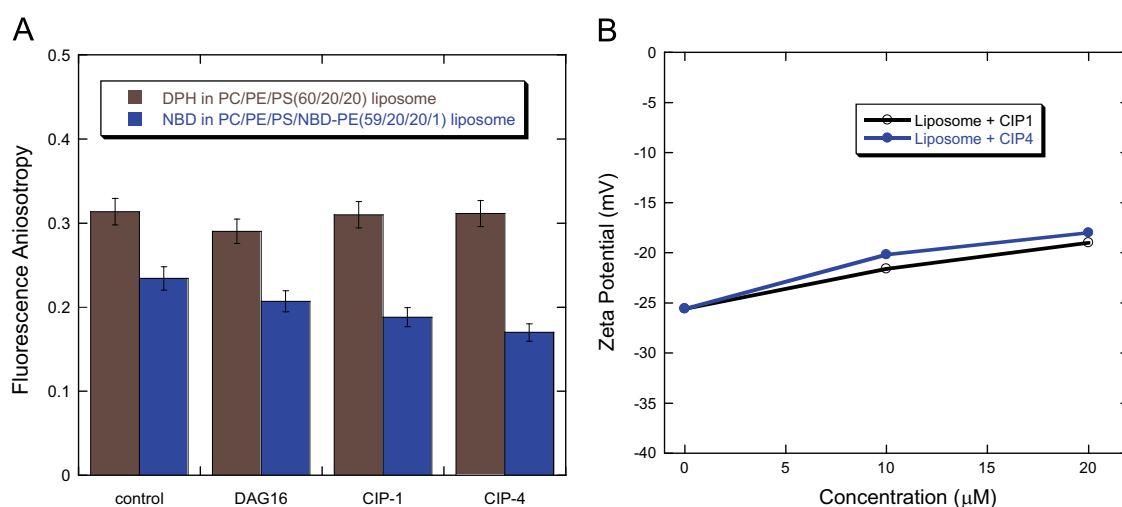
### 3.4. Membrane interaction measurement

Another possibility of the lower  $IC_{50}$  values of the compounds could be also due to their interaction with the membrane as was seen in the case of neomycin [29]. It can be assumed that direct interaction of the compounds with the PIP-containing liposomes can alter the membrane dynamics and inhibit the PH-domains to interact with the membranes. Therefore, to understand whether the compounds alter the membrane dynamics of lipids and fluidity of the lipid bilayer, we measured the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) under liposomal environment [27,34]. The membrane composition used for the SPR studies contain anionic lipids like PS and PIP. We presume that the interaction of the triazole and nitro groups of the compounds with the anionic lipids could alter the membrane organization by blocking the protein binding under the liposomal environment and allowing the competitive-SPR measurements to show high inhibitory effect by the compounds. The DPH molecules generally embedded within the hydrophobic core of the lipid bilayer, NBD of NBD-PE is presumed to be mainly localized at the membrane interface (Fig. S4) therefore changes in fluorescence anisotropy values can be useful in evaluating the modulation of lipid-bilayer fluidity induced by membrane-active compounds. We used membrane localized DAG<sub>16</sub> molecule as a positive control for this measurement. Fig. 6 and Table S3 represent the change in anisotropy values of DPH and NBD-PE molecules in the presence of anionic hybrid lipids under liposomal environment. NBD fluorescence anisotropy was also measured in the presence of different compound concentrations (Fig. S5). The variations in DPH fluorescence anisotropy values affected by the compounds are very small, indicating almost no change in ordering of the core of lipid bilayer. However, NBD fluorescence anisotropy values get affected only at higher concentrations of the compounds. This indicates that ordering of the interfacial region of the lipid bilayer gets affected by the compounds only at higher concentrations. The anisotropy measurements were performed at 25 °C, which is much above the phase transition temperature of PC/PE/PS (60:20:20) lipid bilayers. Therefore, the lipid mixture is in liquid-crystal (LC) phase at 25 °C [27,53]. We also measured the change in surface potential of the liposomes (PC/PE/PS/PI(4,5)P<sub>2</sub>) in the absence and presence of the compounds, to determine whether their interaction could alter the surface charge of the liposomes. The results clearly showed that the compounds have

weak effect on the surface potential values of the liposomes, indicating their weak interaction pattern with the negatively charged membrane surface (Fig. 6). Therefore, the stronger inhibitory effects of the compounds could be predominantly due to its stronger binding with the respective PH-domains.

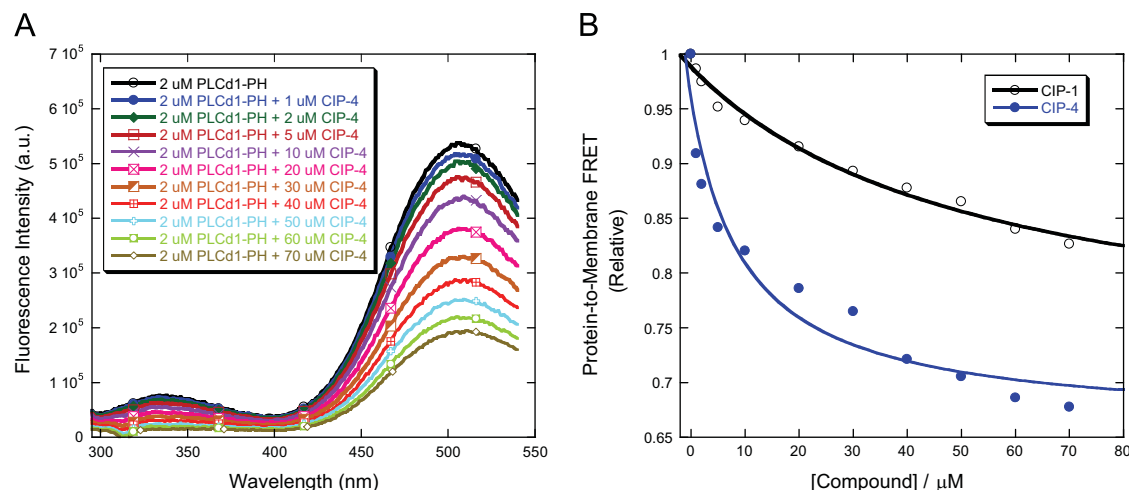
### 3.5. Förster resonance energy transfer analysis

The membrane binding surface surrounding the IP3-binding site of the PH domain allows PLC $\delta$ 1 to interact with the cellular membranes [37]. This PI(4,5)P<sub>2</sub> dependent membrane interaction of the PLC $\delta$ 1-PH domain activates PLC $\delta$ 1 enzyme [18]. Therefore, for further understanding of the selectivity of these compounds in inhibition of the PI(4,5)P<sub>2</sub>/PH-domain interactions, we performed competitive-protein to membrane Förster resonance energy transfer (FRET) analysis. One of the Trp-residues (W27) is localized close to the IP3-binding site of the PLC $\delta$ 1-PH domain and provides as FRET donor, and a low density of membrane-embedded, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dPE) lipid serve as the FRET acceptor. In this assay we first allowed the PH-domain to bind the dPE labeled PI(4,5)P<sub>2</sub> containing liposomes, then the compounds were added to confirm that they interact with the PH domain through its PIP-binding site. The PC/PE/PS/dPE (59:20:20:1) and PC/PE/PS/dPE/PI(4,5)P<sub>2</sub> (56:20:20:1:3) liposomes were used as control and for ligands, respectively. The FRET signal due to PI(4,5)P<sub>2</sub>-dependent protein binding to the liposomes was corrected with non-specific fluorescence signal originated from control liposome (PC/PE/PS/dPE) during only protein and compound binding. The decrease in fluorescence signal at 505 nm wavelength in the presence of compounds confirmed its binding to PLC $\delta$ 1-PH domain under liposomal environment (Fig. 7). However, detailed spectral analysis showed a concomitant decrease of Trp-fluorescence signal (at 340 nm). We tested the effect of CIP-1 and CIP-4 on Trp- and dasyI fluorescence. Our control experiment showed that the compounds directly interact with the PLC $\delta$ 1-PH domain in solution (data not shown). Therefore, the decrease in protein-to-membrane FRET signal could be predominantly due to compound dependent Trp-fluorescence quenching of the protein. The decrease in FRET signal was also examined to calculate apparent inhibitory constant ( $K_i(\text{Compound})_{app}$ ) using Eq. (2). Calculated  $K_i(\text{Compound})_{app}$  values for CIP-1 and CIP-4 were 60.4 and 9.9  $\mu$ M, respectively, demonstrating that CIP-4 efficiently and specifically inhibits PI(4,5)P<sub>2</sub>/PLC $\delta$ 1-PH domain interactions under



**Fig. 6.** Fluorescence anisotropy of DPH and NBD-PE embedded in PC/PE/PS (60:20:20) and PC/PE/PS/NBD-PE (59:20:20:1) liposomes for cholesterol and compounds (A). Control: no ligand was added to the liposomes. Zeta potential of the PC/PE/PS/PI(4,5)P<sub>2</sub> (57:20:20:3) liposomes in the presence of compounds with different concentrations (B). 100  $\mu$ L of liposomes from 0.5 mg/mL of total lipid was used for all measurements. Values represent the mean  $\pm$  SD from triplicate measurements.





**Fig. 7.** Representative protein-to-membrane FRET experiment under liposomal environment. Addition of increased concentration of compounds, **CIP-4** (A) to PLCδ1-PH domain (2 μM) bound to the active liposome (PC/PE/PS/dPE/PI(4,5)P<sub>2</sub> (56:20:20:1:3)) decreases the FRET signal at 509 nm. Competitive displacement assay for the PLCδ1-PH domains (2 μM) bound to PC/PE/PS/dPE/PI(4,5)P<sub>2</sub> liposome. The bound complex was titrated with the **CIP-1** and **CIP-4**. All the measurements were performed in 20 mM Tris, pH 7.4 containing 160 mM NaCl. Compound concentrations were varied from 0 to 70 μM. PLCδ1 stands for PLCδ1.

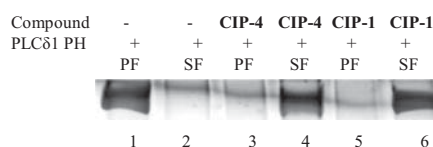
the liposomal environment.

### 3.6. Isothermal titration calorimetric measurements

To better understand the binding mechanism of the compounds, **CIP-1** and **CIP-4** with the PLCδ1-PH and AKT1-PH domains, we performed isothermal titration calorimetric (ITC) measurements. Representative titration plots of both the compounds with PLCδ1-PH domain showed an exothermic reaction with one-step binding mechanism (Fig. S6). This indicates that stronger hydrogen bond formation and van der Waals interactions between the compounds and cationic groove present inside the PIP-binding site of the PLCδ1-PH domain are predominant factors for their interactions. The binding parameters are in accordance with spectroscopic and molecular docking analysis results. Interactions of both the compounds with AKT1-PH domain followed an exothermic reaction with two-step binding mechanism (Fig. S6). However, both the compounds have weaker binding affinity for AKT1-PH domain in comparison with the PLCδ1-PH domain. Therefore, ITC analysis clearly suggests that the compounds in solution interact with the PLCδ1-PH and AKT1-PH domain.

### 3.7. Lipid-pull down assay

The inhibition of PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction under liposomal environment was also qualitatively determined by lipid pull-down assay [26,38,39]. The binding of PLCδ1-PH domain (50 μM) with PI(4,5)P<sub>2</sub> containing liposomes (PC/PE/PS/PI(4,5)P<sub>2</sub> (57:20:20:3)) was measured in the absence or presence of compounds (100 μM), **CIP-1** and **CIP-4** at physiological pH. The coomassie blue staining of the SDS-PAGE gel clearly showed that the PLCδ1-PH domain binding to the PI(4,5)P<sub>2</sub> containing liposomes

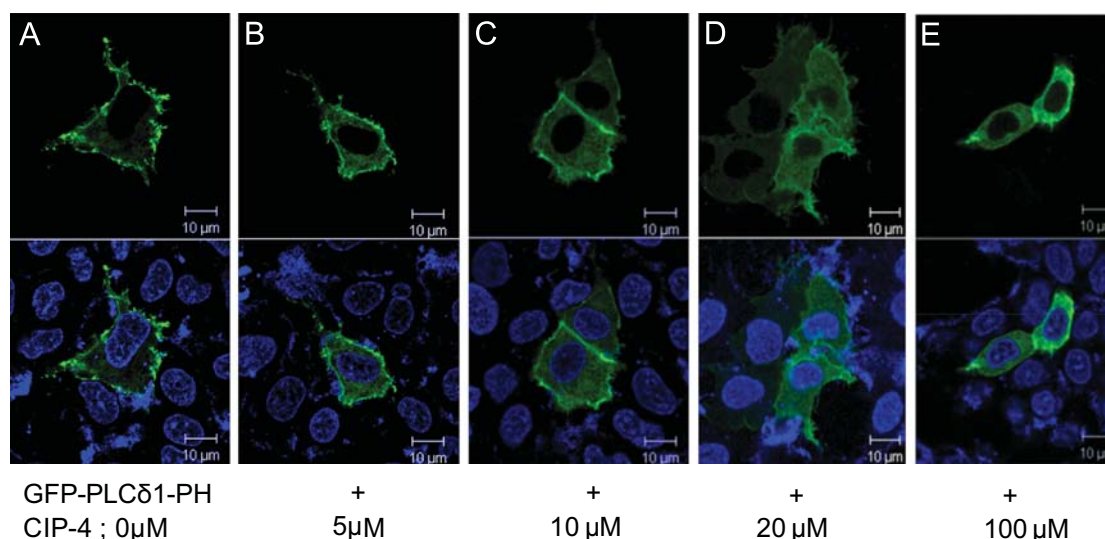


**Fig. 8.** Coomassie blue stained SDS-PAGE gel images of liposome binding assay. PC/PE/PS/PI(4,5)P<sub>2</sub> (57:20:20:3) liposome was used for PLCδ1-PH domain binding study. Lanes 1, 3 and 5 were liposome bound fraction and 2, 4 and 6 were unbound fraction of PLCδ1-PH domain. Compound **CIP-1** and **CIP-4** were used as inhibitor. Liposome concentration 100 μM; Protein concentration 50 μM; Compound concentration, 100 μM; PF, pellet fraction; SF, soluble fraction.

was almost completely diminished by the compound **CIP-4** (Fig. 8). However, compound **CIP-1** was not that efficient in displacing the PLCδ1-PH domain from its liposome bound state. The inhibition of PI(3,4,5)P<sub>3</sub>/AKT1-PH domain interaction by the potent compounds was also measured using similar methods. Visual inspection of coomassie blue stained SDS-PAGE gel images revealed that compound **CIP-1** inhibits the PI(3,4,5)P<sub>3</sub>/AKT1-PH domain interaction more strongly than compound **CIP-4**, under similar experimental conditions (Fig. S7A). Control experiment showed that PLCδ1-PH domain has very weak binding affinity for PC/PE/PS liposome (Fig. S7B). Hence, the liposome-pull down assay results indicate that the PLCδ1-PH domain strongly interacts with PI(4,5)P<sub>2</sub> containing liposomes and **CIP-4** strongly inhibits the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction.

### 3.8. Cellular translocation of PLCδ1-PH domain

To demonstrate the physiological significance of our in vitro inhibition/binding studies that show potent compounds strongly interact with PLCδ1-PH domain and inhibits its specific interactions with PI(4,5)P<sub>2</sub> under liposomal environment, we measured membrane displacement of GFP-tagged PLCδ1-PH domain in A549 cells. As reported earlier PI(4,5)P<sub>2</sub> is mostly present at the inner PM of cells, hence expression of cDNA of GFP-tagged PLCδ1-PH domain allows this protein to be localized at the PM [9,15,54]. Freshly prepared compound solutions were added after 24 h of transfection of cDNA. Cells were treated with compounds for 30 min and then fixed with 4% paraformaldehyde for immunofluorescence study. All measurements were performed for minimum three times with more than 10 cells were monitored for each measurement. In general, more than 80% of cell population showed similar behaviors with respect to PM displacement of PLCδ1-PH domain. The extent of displacement of PLCδ1-PH domain from PM was monitored in the presence of compounds **CIP-1** and **CIP-4** (0–100 μM) in a concentration dependent manner (Figs. 9 and S8). Confocal microscopic images clearly showed that external addition of **CIP-4** can efficiently displace localized PLCδ1-PH domain from PM, whereas extent of displacement of PLCδ1-PH domain from PM is lowered by compound **CIP-1**, which is accordance with their in vitro binding affinities/IC<sub>50</sub> values. The results indicate that the PI(4,5)P<sub>2</sub>/PLCδ1-PH domain interaction is indeed inhibited by the potent compounds under cellular environment.



**Fig. 9.** Effect of compound on cellular localization of GFP-tagged PLC $\delta$ 1-PH domain in A549 cells. Representative cellular localization pattern of GFP-tagged PLC $\delta$ 1-PH domain in the absence or presence of compound **CIP-4** with different concentrations, 0 (A), 5 (B), 10 (C), 20 (D) and 100  $\mu$ M (E). The compound was treated in A549 cells for 30 min and then cells were fixed with 4% paraformaldehyde for immunofluorescence study. More than 97% cells showed plasma membrane localization of GFP-tagged PLC $\delta$ 1-PH domain in absence of the compounds, whereas more than 80% (5  $\mu$ M), 83% (10  $\mu$ M), 86% (20  $\mu$ M), and 95% (100  $\mu$ M) of the observed cells showed preferential cytosolic localization of GFP-tagged PLC $\delta$ 1-PH domain at different compound concentrations.

Present study described that compounds with 1,2,3-triazol-4-yl methanol moiety had moderate to strong inhibition of the PI(4,5) $P_2$  binding to the PLC $\delta$ 1-PH domain. Through biophysical analyses demonstrate that the interactions between the potent compounds and lipid bilayer are very weak in nature. The compounds preferably localize in the bulk phase of the solution and its pharmacophores are accessible for PH-domain binding under the experimental conditions. The 1,2,3-triazol-4-yl methanol moiety and nitro group are crucial for their interaction with the PH-domains. These compounds have stronger binding affinity for several PH domains, but their affinity differences are negligible except for compound **CIP-4**. The higher binding affinity of **CIP-4** for PLC $\delta$ 1-PH domain over the other PH- domains including AKT1, TAPP1 and GRP1 proteins could be due to its true selectivity. We hypothesize that stronger binding of the compounds with the PLC $\delta$ 1-PH domain blocks its membrane association and PI(4,5) $P_2$  binding, which is essential for its activity in hydrolyzing PI(4,5) $P_2$  to IP3 and DAG under cellular environment. However, the stronger binding affinity and selectivity for PLC $\delta$ 1-PH domain of compound **CIP-4** over **CIP-1** could be because of the presence of additional triazole ring, which not only provide additional hydrogen bonding with the amino acid residues within the binding pocket but also the surface area of the ligand required for stronger inhibition of PI(4,5) $P_2$ /PLC $\delta$ 1-PH domain interactions.

Recently, neomycin is reported as inhibitor of PI(4,5) $P_2$  binding PLC enzyme, which regulates PI(4,5) $P_2$  hydrolysis at the cellular membranes, but the mechanism of its activity is quite different than compound **CIP-4** [29]. Strong electrostatic interactions between neomycin and PI(4,5) $P_2$  molecules present at the membranes regulate PLC enzyme activity. However, direct binding of neomycin to PI(4,5) $P_2$  can alter biological activities of several other proteins/enzymes including protein kinase C. U73122, ATA are among other reported inhibitors of PLC enzyme, but these compounds targets catalytic site of the enzyme [55,56]. Our experimental results show that **CIP-4** selectively interacts with the PH domain of PLC $\delta$ 1 enzyme. We hypothesize that the surface area of **CIP-4** compound fits properly within the IP3 binding site of PLC $\delta$ 1-PH domain not with other PH domains including that of AKT1 enzyme. Although competitive-SPR and FRET analyses indicate that **CIP-1** and **CIP-4** presumably interact through the IP3 binding site of the PLC $\delta$ 1-PH domain but

further detail analysis including mutation and activity studies required to prove the proper binding site of the compounds, which is beyond the scope of the current study.

In this regard, we hypothesize that selective PLC enzyme activity can be also controlled by these types of compounds, which directly interacts with the PIP-binding domains not with the PIPs. However, further biological studies, including enzyme activity assay, are required to understand the PI(4,5) $P_2$ /PH domain inhibition potency of compound **CIP-4** and regulation of PLC enzyme activity.

#### 4. Conclusion

Taken together, our results show that 1,2,3-triazol-4-yl methanol derivatives strongly interact with PH-domain of PLC $\delta$ 1, AKT1 and GRP1 proteins but weakly interact with the model membranes. Potent compound **CIP-4** efficiently displaces PI(4,5) $P_2$  from its binding sites of the PH-domains, but failed to displace PI(3,4) $P_2$  and PI(3,4,5) $P_3$  effectively from their respective PH-domain binding pockets, indicating its selectivity for PI(4,5) $P_2$ /PH-domain interactions. The interaction of the potent compounds with the PLC $\delta$ 1-PH domain is exothermic and does not alter its secondary structural content. The molecular docking analysis indicates that the presence of two triazole moieties and nitro group is essential for their interaction with the PH-domains. The results suggest that these small molecules seem to act principally by binding to the PH-domain and preventing the recruitment to the cellular membranes, where these effector proteins are primarily activated. Our findings also suggest that the 1,2,3-triazol-4-yl methanol moiety can be used to develop nonphosphate-based potential regulators for PI(4,5) $P_2$  binding PH and other lipid binding domains containing proteins.

#### Acknowledgments

We are thankful to CIF, DBT programme Support (No. BT/01/NE/PS/08) Govt. of India, New Delhi, India, Ministry of Human Resource Development for Centre of Excellence in FAST (5-7/2014-TS-VII) for instrumental support. We like to thank Dr. Mohitosh Dey for his kind help in performing CD analysis.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.05.007>

## References

- [1] W. Cho, Building signaling complexes at the membrane, *Sci. STKE* 2006 (2006) pe7.
- [2] W. Cho, R.V. Stahelin, Membrane–protein interactions in cell signaling and membrane trafficking, *Annu. Rev. Biophys. Biomol. Struct.* 34 (2005) 119–151.
- [3] M.A. Lemmon, Membrane recognition by phospholipid-binding domains, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 99–111.
- [4] J.C. Holthuis, T.P. Levine, Lipid traffic: floppy drives and a superhighway, *Nat. Rev. Mol. Cell Biol.* 6 (2005) 209–220.
- [5] T.L. Yuan, L.C. Cantley, PI3K pathway alterations in cancer: variations on a theme, *Oncogene* 27 (2008) 5497–5510.
- [6] P. Malaney, R.R. Pathak, B. Xue, V.N. Uversky, V. Dave, Intrinsic disorder in PTEN and its interactome confers structural plasticity and functional versatility, *Sci. Rep.* 3 (2013) 2035.
- [7] S. Srisankarajah, N. Hamblin, S. Worsley, A.R. Calver, E.M. Hessel, A. Amour, Targeting phosphoinositide 3-kinase delta for the treatment of respiratory diseases, *Ann. N. Y. Acad. Sci.* 1280 (2013) 35–39.
- [8] B. Miao, I. Skidan, J. Yang, A. Lugovskoy, M. Reibarkh, K. Long, T. Brazell, K. A. Durugkar, J. Maki, C.V. Ramana, B. Schaffhausen, G. Wagner, V. Torchilin, J. Yuan, A. Degterev, Small molecule inhibition of phosphatidylinositol-3,4,5-triphosphate (PIP3) binding to pleckstrin homology domains, *Proc. Natl. Acad. Sci. USA* 107 (2010) 20126–20131.
- [9] K. Itsuki, Y. Imai, H. Hase, Y. Okamura, R. Inoue, M.X. Mori, PLC-mediated PI(4,5)P2 hydrolysis regulates activation and inactivation of TRPC6/7 channels, *J. Gen. Physiol.* 143 (2014) 183–201.
- [10] T.J. Kubiseski, Y.M. Chook, W.E. Parris, M. Rozakis-Adcock, T. Pawson, High affinity binding of the pleckstrin homology domain of mSos1 to phosphatidylinositol (4,5)-bisphosphate, *J. Biol. Chem.* 272 (1997) 1799–1804.
- [11] B. Sot, E. Behrmann, S. Raunser, A. Wittinghofer, Ras GTPase activating (Ras-GAP) activity of the dual specificity GAP protein Rasal requires colocalization and C2 domain binding to lipid membranes, *Proc. Natl. Acad. Sci. USA* 110 (2013) 111–116.
- [12] J. Gomez-Rodriguez, E.A. Wohlfert, R. Handon, F. Meylan, J.Z. Wu, S. M. Anderson, M.R. Kirby, Y. Belkaid, P.L. Schwartzberg, Itk-mediated integration of T cell receptor and cytokine signaling regulates the balance between Th17 and regulatory T cells, *J. Exp. Med.* 211 (2014) 529–543.
- [13] J. Shi, L. Birnbaumer, W.A. Large, A.P. Albert, Myristoylated alanine-rich C kinase substrate coordinates native TRPC1 channel activation by phosphatidylinositol 4,5-bisphosphate and protein kinase C in vascular smooth muscle, *FASEB J.* 28 (2014) 244–255.
- [14] L. Trovo, T. Ahmed, Z. Callaerts-Vegh, A. Buzzi, C. Bagni, M. Chuah, T. Vandendriessche, R. D'Hooge, D. Balschun, C.G. Dotti, Low hippocampal PI(4,5)P(2) contributes to reduced cognition in old mice as a result of loss of MARCKS, *Nat. Neurosci.* 16 (2013) 449–455.
- [15] A. Gericke, N.R. Leslie, M. Losche, A.H. Ross, PtdIns(4,5)P2-mediated cell signaling: emerging principles and PTEN as a paradigm for regulatory mechanism, *Adv. Exp. Med. Biol.* 991 (2013) 85–104.
- [16] L.S. Ehrlich, G.N. Medina, S. Photiadis, P.B. Whittredge, S. Watanabe, J. W. Taraska, C.A. Carter, Tsg101 regulates PI(4,5)P2/Ca(2+) signaling for HIV-1 Gag assembly, *Front. Microbiol.* 5 (2014) 234.
- [17] S. Scarlata, R. Gupta, P. Garcia, H. Keach, S. Shah, C.R. Kasireddy, R. Bittman, M. J. Rebecchi, Inhibition of phospholipase C-delta 1 catalytic activity by sphingomyelin, *Biochemistry* 35 (1996) 14882–14888.
- [18] J.H. Hurley, J.A. Grobler, Protein kinase C and phospholipase C: bilayer interactions and regulation, *Curr. Opin. Struct. Biol.* 7 (1997) 557–565.
- [19] K.D. Courtney, R.B. Corcoran, J.A. Engelman, The PI3K pathway As drug target in human cancer, *J. Clin. Oncol.* 28 (2010) 1075–1083.
- [20] D. Mahadevan, G. Powis, E.A. Mash, B. George, V.M. Gokhale, S. Zhang, K. Shakalya, L. Du-Cuny, M. Berggren, M.A. Ali, U. Jana, N. Ihle, S. Moses, C. Franklin, S. Narayan, N. Shirahatti, E.J. Meuillet, Discovery of a novel class of AKT pleckstrin homology domain inhibitors, *Mol. Cancer Ther.* 7 (2008) 2621–2632.
- [21] E.J. Meuillet, N. Ihle, A.F. Baker, J.M. Gard, C. Stamper, R. Williams, A. Coon, D. Mahadevan, B.L. George, L. Kirkpatrick, G. Powis, In vivo molecular pharmacology and antitumor activity of the targeted Akt inhibitor PX-316, *Oncol. Res.* 14 (2004) 513–527.
- [22] D. Manna, A. Albanese, W.S. Park, W. Cho, Mechanistic basis of differential cellular responses of phosphatidylinositol 3,4-bisphosphate- and phosphatidylinositol 3,4,5-triphosphate-binding pleckstrin homology domains, *J. Biol. Chem.* 282 (2007) 32093–32105.
- [23] C.R. McNamara, A. Degterev, Small-molecule inhibitors of the PI3K signaling network, *Future Med. Chem.* 3 (2011) 549–565.
- [24] B. Vanhaesebroeck, L. Stephens, P. Hawkins, PI3K signalling: the path to discovery and understanding, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 195–203.
- [25] N. Mamidi, R. Borah, N. Sinha, C. Jana, D. Manna, Effects of ortho substituent groups of protocatechualdehyde derivatives on binding to the C1 domain of novel protein kinase C, *J. Phys. Chem. B* 116 (2012) 10684–10692.
- [26] N. Mamidi, S. Gorai, J. Sahoo, D. Manna, Alkyl cinnamates as regulator for the C1 domain of protein kinase C isoforms, *Chem. Phys. Lipids* 165 (2012) 320–330.
- [27] D. Talukdar, S. Panda, R. Borah, D. Manna, Membrane interaction and protein kinase C-C1 domain binding properties of 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues, *J. Phys. Chem. B* 118 (2014) 7541–7553.
- [28] E.J. Meuillet, S. Zuohe, R. Lemos, N. Ihle, J. Kingston, R. Watkins, S.A. Moses, S. Zhang, L. Du-Cuny, R. Herbst, J.J. Jacoby, L.L. Zhou, A.M. Ahad, E.A. Mash, D. L. Kirkpatrick, G. Powis, Molecular pharmacology and antitumor activity of PHT-427, a novel Akt/phosphatidylinositol-dependent protein kinase 1 pleckstrin homology domain inhibitor, *Mol. Cancer Ther.* 9 (2010) 706–717.
- [29] C. Wang, X.N. Du, Q.Z. Jia, H.L. Zhang, Binding of PLCdelta1PH-GFP to PtdIns(4,5)P2 prevents inhibition of phospholipase C-mediated hydrolysis of PtdIns(4,5)P2 by neomycin, *Acta Pharmacol. Sin.* 26 (2005) 1485–1491.
- [30] Y. Yoon, Small chemicals with inhibitory effects on PtdIns(3,4,5)P3 binding of Btk PH domain, *Bioorg. Med. Chem. Lett.* 24 (2014) 2334–2339.
- [31] C.C. Thomas, M. Deak, D.R. Alessi, D.M. van Aalten, High-resolution structure of the pleckstrin homology domain of protein kinase b/akt bound to phosphatidylinositol (3,4,5)-triphosphate, *Curr. Biol.* 12 (2002) 1256–1262.
- [32] K.M. Ferguson, M.A. Lemmon, J. Schlessinger, P.B. Sigler, Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain, *Cell* 83 (1995) 1037–1046.
- [33] C.C. Milburn, M. Deak, S.M. Kelly, N.C. Price, D.R. Alessi, D.M. Van Aalten, Binding of phosphatidylinositol 3,4,5-triphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change, *Biochem. J.* 375 (2003) 531–538.
- [34] O. Raifman, S. Kolusheva, M.J. Comin, N. Kedeei, N.E. Lewin, P.M. Blumberg, V. E. Marquez, R. Jelinek, Membrane anchoring of diacylglycerol lactones substituted with rigid hydrophobic acyl domains correlates with biological activities, *FEBS J.* 277 (2010) 233–243.
- [35] R. Borah, D. Talukdar, S. Gorai, D. Bain, D. Manna, Bilayer interaction and protein kinase C-C1 domain binding studies of kojic acid esters, *RSC Adv.* 4 (2014) 25520–25531.
- [36] N. Mamidi, S. Gorai, B. Ravi, D. Manna, Physicochemical characterization of diacyltetrol-based lipids consisting of both diacylglycerol and phospholipid headgroups, *RSC Adv.* 4 (2014) 21971–21978.
- [37] M.A. Lemmon, K.M. Ferguson, R. O'Brien, P.B. Sigler, J. Schlessinger, Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain, *Proc. Natl. Acad. Sci. USA* 92 (1995) 10472–10476.
- [38] J.E. Johnson, J. Giorgione, A.C. Newton, The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain, *Biochemistry* 39 (2000) 11360–11369.
- [39] O. Lorenzo, S. Urbe, M.J. Clague, Analysis of phosphoinositide binding domain properties within the myotubularin-related protein MTMR3, *J. Cell Sci.* 118 (2005) 2005–2012.
- [40] R. Katso, K. Okkenhaug, K. Ahmadi, S. White, J. Timms, M.D. Waterfield, Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer, *Annu. Rev. Cell Dev. Biol.* 17 (2001) 615–675.
- [41] L. Catley, T. Hideshima, D. Chauhan, P. Neri, P. Tassone, R. Bronson, W. Song, Y. T. Tai, N.C. Munshi, K.C. Anderson, Alkyl phospholipid perifosine induces myeloid hyperplasia in a murine myeloma model, *Exp. Hematol.* 35 (2007) 1038–1046.
- [42] P. Workman, P.A. Clarke, E.I. Raynaud, R.L. van Montfort, Drugging the PI3 kinase: from chemical tools to drugs in the clinic, *Cancer Res.* 70 (2010) 2146–2157.
- [43] C.C. Kumar, V. Madison, AKT crystal structure and AKT-specific inhibitors, *Oncogene* 24 (2005) 7493–7501.
- [44] M. Hussein, M. Bettio, A. Schmitz, J.S. Hannam, J. Theis, G. Mayer, S. Dosa, M. Gutschow, M. Famulok, Cypecksins are covalent inhibitors of the pleckstrin homology domain of cytohesin, *Angew. Chem. Int. Ed. Engl.* 52 (2013) 9529–9533.
- [45] A.C. Estrada, T. Syrovets, K. Pitterle, O. Lunov, B. Buchele, J. Schimana-Pfeifer, T. Schmidt, S.A. Morad, T. Simmet, Tirucallic acids are novel pleckstrin homology domain-dependent Akt inhibitors inducing apoptosis in prostate cancer cells, *Mol. Pharmacol.* 77 (2010) 378–387.
- [46] W. Huang, M. Barrett, N. Hajicek, S. Hicks, T.K. Harden, J. Sondek, Q. Zhang, Small molecule inhibitors of phospholipase C from a novel high-throughput screen, *J. Biol. Chem.* 288 (2013) 5840–5848.
- [47] G. Sala, F. Diturì, C. Raimondi, S. Previdi, T. Maffucci, M. Mazzeo, C. Rossi, M. Iezzi, R. Lattanzio, M. Piantelli, S. Iacobelli, M. Brogginì, M. Falasca, Phospholipase Cgamma1 is required for metastasis development and progression, *Cancer Res.* 68 (2008) 10187–10196.
- [48] L. Zhang, S. Malik, G.G. Kelley, M.S. Kafiloff, A.V. Smrcka, Phospholipase C epsilon scaffolds to muscle-specific A kinase anchoring protein (mAKAPbeta) and integrates multiple hypertrophic stimuli in cardiac myocytes, *J. Biol. Chem.* 286 (2011) 23012–23021.
- [49] J.H. Hurley, S. Misra, Signaling and subcellular targeting by membrane-binding domains, *Annu. Rev. Biophys. Biomol. Struct.* 29 (2000) 49–79.
- [50] C. Deraedt, N. Pinaud, D. Astruc, Recyclable catalytic dendrimer nanoreactor for part-per-million Cu(I) catalysis of “click” chemistry in water, *J. Am. Chem. Soc.* 136 (2014) 12092–12098.
- [51] P.R. Bagdi, R.S. Basha, P.K. Baruah, A.T. Khan, Copper oxide nanoparticle mediated ‘click chemistry’ for the synthesis of mono-, bis- and tris-triazole derivatives from 10,10-dipropargyl-9-anthrone as a key building block, *RSC Adv.* 4 (2014) 10652–10659.

- [52] H.A. Stefani, M.F.Z.J. Amaral, F. Manarin, R.A. Ando, N.C.S. Silva, E. Juaristi, Functionalization of 2-(*S*)-isopropyl-5-iodo-pyrimidin-4-ones through Cu(I)-mediated 1,3-dipolar azide-alkyne cycloadditions, *Tetrahedron Lett.* 52 (2011) 6883–6886.
- [53] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 112–124.
- [54] D. Manna, N. Bhardwaj, M.S. Vora, R.V. Stabelin, H. Lu, W. Cho, Differential roles of phosphatidylserine, PtdIns(4,5)P<sub>2</sub>, and PtdIns(3,4,5)P<sub>3</sub> in plasma membrane targeting of C2 domains. Molecular dynamics simulation, membrane binding, and cell translocation studies of the PKC $\alpha$  C2 domain, *J. Biol. Chem.* 283 (2008) 26047–26058.
- [55] N.E. Wilsher, W.J. Court, R. Ruddle, Y.M. Newbatt, W. Aherne, P.W. Sheldrake, N.P. Jones, M. Katan, S.A. Eccles, F.I. Raynaud, The phosphoinositide-specific phospholipase C inhibitor U73122 (1-(6-((17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) spontaneously forms conjugates with common components of cell culture medium, *Drug Metab. Dispos.* 35 (2007) 1017–1022.
- [56] R.R. Klein, D.M. Bourdon, C.L. Costales, C.D. Wagner, W.L. White, J.D. Williams, S.N. Hicks, J. Sondel, D.R. Thakker, Direct activation of human phospholipase C by its well known inhibitor u73122, *J. Biol. Chem.* 286 (2011) 12407–12416.