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ARTICLE TYPE

Inhibition of Phosphatidylinositol-3,4,5-trisphosphate Binding to AKT Pleckstrin Homology Domain by 4-Amino-1,2,5-oxadiazole Derivatives

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Aberrant regulation of phosphatidylinositol-3-kinases (PI3K) dependent cell signalling pathways is directly linked with different human cancers. AKT is a phosphatidylinositol (PIP) binding serine/threonine kinase enzyme and a key component of PI3K-signalling pathway. Several efforts have been made to perturb the activities of AKT enzyme; however, selectivity issue remains a key challenging point. Pleckstrin homology (PH) domain of AKT strongly interacts with the membrane localized PIPs and activates the enzyme. Several attempts are underway for the development of small molecules, which can specifically inhibit the PIP/PH-domain interaction to ascertain an alternate approach for the development of drug(s) for PI3K-AKT-signalling pathway. This proposed approach is highly beneficial because of easy synthesis of small molecules and low side effects. In this study, we used a series of small molecule antagonists (**PI**s) for PI(3,4,5)P_3/PH-domain interaction and determined their inhibitory effect by using competitive-surface plasmon resonance (SPR) analysis (IC_{50} ranges from 1.85 to 16.35 μM for PI(3,4,5)P_3/AKT1 PH-domain binding assay). To elucidate their binding selectivity, we also used PI(3,4,5)P_3, PI(3,4)P_2, PI(4,5)P_2 specific PH-domains. For further understanding of their PH-domain inhibition mechanism, we also performed various physicochemical analyses. The results showed that these water-soluble compounds do not significantly interact with the model membranes. The oxadiazole and N′-hydroxy moieties of the compounds are essential for their exothermic interaction with the PH-domains and their bindings do not alter the secondary structure of the PH-domain. Potent compounds show moderate effect on phosphothreonine (308) level of AKT enzyme. Overall, our studies demonstrate that these compounds could interact with the PIP-binding PH-domains and inhibit their membrane recruitment. These studies also illustrate the feasibility of further development of 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide moiety-based small molecule antagonists for PI3K-signalling pathway.

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Introduction

The phosphatidylinositol-3-kinases (PI3K) dependent cell signalling pathway is directly linked with several human diseases. Upregulation of PI3K-pathway plays an important role in carcinogenesis, whereas down-regulation is linked with type-II diabetes. In this regard, PI3K-signalling pathway is considered as potential therapeutic target for the treatment of these diseases.[1-4] In responses to growth factor and hormone stimulation the PI3K enzyme phosphorylate phosphtidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) to generate phosphtidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) at the plasma membrane. The $PI(3,4,5)P_3$ generation recruits several proteins to the plasma membrane through their interaction with lipid-binding modules. The $PI(3,4,5)P_3$ signalling is terminated due to the dephosphorylation of $PI(3,4,5)P_3$ by the phosphatase and tensin homolog (PTEN) protein. $PI(3,4,5)P_3$-dependent membrane association of the lipid binding modules are necessary and sufficient for activation and proper functioning of the effector proteins, including AKT. These $PI(3,4,5)P_3$ binding proteins are generally cytosolic in unstimulated cells and recruited to the cellular membranes through PIP-binding module mediated binding to newly generated $PI(3,4,5)P_3$ lipid.[2-6]

To date there are a number of PIP-binding modules, including phox homology (PX), pleckstrin homology (PH) domain and others that have been reported as binding partners of $PI(3,4,5)P_3$.[7, 8] PH-domains are one of the systematically characterized PIP-binding modules and exhibit a broad range of binding specificities. Interestingly, majority of the PH-domains of the effector proteins bind to the membrane containing $PI(3,4,5)P_3$, $PI(3,4)P_2$ and $PI(4,5)P_2$ lipids with high affinity. $PI(3,4,5)P_3$ selectively regulates several PH-domain containing proteins including AKT, GRP and BTK that mediate a wide verity of cellular process.[9, 10] In particular, the AKT protein plays an important role in cell proliferation, survival, metabolism and others. Activation of AKT is achieved through $PI(3,4,5)P_3$-binding to its PH domain followed by PDK dependent phosphorylation at the membrane. Intramolecular interaction between lipid binding domain and kinase domain is also important in maintaining cellular level of the effector protein in its inactive state. For an example, AKT activation proceeds after PIP-binding at the inner plasma membrane that dislodges the PH-domain from kinase domain.[11-13]

In general direct inhibition/activation of enzyme through its catalytic domain is considered as potential approach for drug development. However, the activities of the enzymes should be systematically regulated in the cells, since direct activation/inhibition could also incite side-effects by perturbing the other up/down-stream pathways.[14, 15] As an example the development of AKT kinase inhibitors (for catalytic domain) to target and treat cancers are effective but experience selectivity problems resulting in an unwanted side effects, including myelosuppression, skin rash and stomatitis and others.[15, 16] In the mean time mechanistic studies described that highly specific PIP/PH-domain interactions can regulate activities of the effector proteins.[2, 4, 15, 17] It is also described that small molecules can alter the protein-lipid interactions, because of the presence of well defined binding pocket of most of the lipid binding modules of the effector proteins. It is also reported that small molecule inhibitors for protein-lipid interactions are advantageous over usual approaches due to lower side-effects and rational design with comparatively simple structures and functions. In this regard, selective PH domains with true lipid specificity regarded as an alternate approach in designing selective inhibitors for enzymes.[2, 15, 18-20]

However, regulations of enzyme activities by inhibiting the PIP/PH domain interactions have not been considerably described yet. We have recently demonstrated that development of regulators for diacylglycerol/C1-domain interactions can be considered as an alternative target to regulate the activities of the PKC enzymes.[21-23] PH-domain targeting lipid-based drug, 3-deoxy phosphatidylinositol ether lipid (DPIEL) and PHT-427 showed usefulness for the treatment of cancer.[16, 19] PITENINs inhibit the $PI(3,4,5)P_3$ binding of AKT1/PDK1-PH-domains and down-regulate PI3K-PDK1-AKT1 pathways. PITENINs also inhibit the membrane localization and $PI(3,4,5)P_3$ binding of the ARNO/GRP1 PH-domain due to its inhibition of ARF6 activation, an important mediator of cytoskeleton and cell motility. The PITENINs also suppressed tumour growth in a mouse cancer model.[2, 24] Therefore, inhibition of PIP/PH domain interactions is considered as a potential approach to regulate the target protein activates.

In this study, we describe the development of 4-Amino-1,2,5-oxadiazole-based antagonists, **PI**s for $PI(3,4,5)P_3$-PH-domain binding. 1,2,5-Oxadiazole substituents have been used as a building block for several biologically active small molecules for the treatment of cancer, arthritis and others disorders. The **PI**s are water soluble non-lipid compounds with polyamine functionalities. Our studies demonstrate that **PI**s strongly interact with PH-domain and inhibit the $PI(3,4,5)P_3$-protein binding under liposomal environment. These compounds at lower concentrations showed certain degree of selective inhibitory effect towards different PH-domains used for the current study. The hydroxyl group and oxadiazole moiety of the compounds play a crucial role in distinguishing the PH-domains. Our studies also demonstrate that the potent compounds do not show significant cytotoxicity and induce reduction of phosphorylated AKT expression level in MDA-MB-231 cancer cell lines. Therefore, we developed a new class of nonlipid antagonist for $PI(3,4,5)P_3$/AKT-PH domain interactions. These results give us strong encouragement for this PH-domain directed drug discovery strategy.

Results

Identification of Potent Compounds as PI(3,4,5)P_3 Antagonist — To screen the efficiency of the synthesized compounds (Figure 1) in inhibiting the PI(3,4,5)P_3/PH-domain interaction, we used surface plasmon resonance (SPR)-based competitive binding assay, an practical method for quantitative measurement of binding or inhibition parameters.[4, 15, 25, 26] SPR measurements

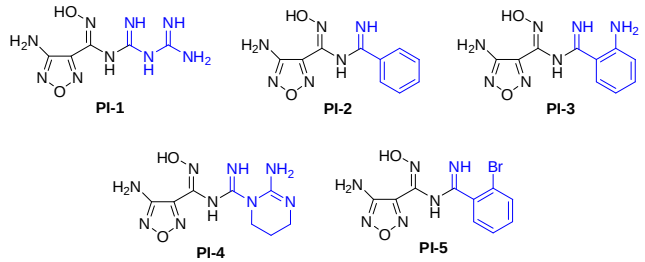

Figure 1: Structures of the compounds (**PI**s) used in the present study.

were carried out using parallel two-flow-channel systems and proteins were passed over control channel to active channels (at a rate of 30 µL/min) coated with PC/PE/PS (60:20:20) and PC/PE/PS/PIP (57:20:20:3) liposome, respectively. Control experiments were performed to remove the effect of non-specific binding of proteins on the sensor surface and effect of only compound binding on the liposome surface (sensograms are not shown). PI(3,4,5)P_3 selectivity of the PH domains were tested using these experimental conditions. The efficacy of the compounds in inhibiting PI(3,4,5)P_3/AKT1-PH domain interactions was first determined by calculating their percent of inhibition. The compounds **PI-1** and **PI-4** with 50 µM concentration showed 95% and 92% inhibitory effect, respectively. The results suggest that compounds **PI-1** and **PI-4** strongly inhibit the interaction of AKT1 PH-domain with the PI(3,4,5)P_3 containing liposome (Table 1 and Figure 2). Whereas, other compounds show moderate competitive inhibitory effect for PI(3,4,5)P_3/AKT1 PH-domain interaction (Figure S1). For better understanding of their inhibition efficacies, the SPR-based competitive binding analyses were also performed in a concentration (0-200 µM) dependent manner (Figure 2). Relative response unit (RU) of the sensorgrams for AKT1 PH-domain decreases in the presence of increasing concentration of the compounds. Calculated IC_{50} values of **PI-1 (**1.85 µM) and

Table 1: Relative Inhibitory Activity of the Compounds Measured by Competitive-Surface Plasmon Resonance Analysis

Compound	Relative inhibitory activity (%)			IC_{50} values (µM)		
	AKT1-PH	GRP1-PH	BTK-PH	AKT1-PH	GRP1-PH	BTK-PH
PI-1	95 ± 3	56 ± 3	40 ± 3	1.85 ± 0.12	24.92 ± 1.02	10.91 ± 0.89
PI-2	54 ± 2	55 ± 3	-	7.19 ± 0.26	11.51 ± 0.69	-
PI-3	74 ± 3	59 ± 5	79 ± 5	8.61 ± 0.21	16.63 ± 0.58	3.18 ± 0.29
PI-4	92 ± 4	67 ± 3	11 ± 2	3.23 ± 0.16	11.54 ± 0.31	46.51 ± 1.59
PI-5	80 ± 2	-	-	16.35 ± 0.35	-	-

Protein, 500 nM; % of inhibition was calculated using 50 µM compound concentration; measurements were performed in 20 mM HEPES buffer at pH 7.4 containing 160 mM KCl; NM, not measured. Values represent the mean ± SD from triplicate measurements.

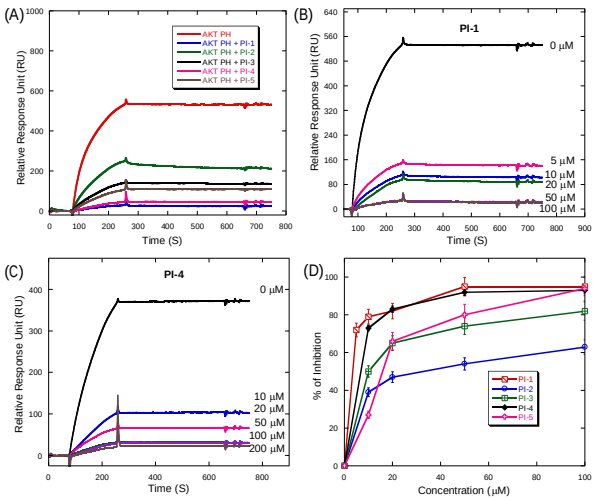

Figure 2: Screening of PI(3,4,5)P_3/AKT1-PH domain (500 nM) interaction selectivity by compounds (50 µM) (A). Surface plasmon resonance sensorgrams of PI(3,4,5)P_3 binding AKT1-PH domain in the presence of increasing concentration of compounds **PI-1** (B) and **PI-4** (C). Selectivity analysis (% of inhibition) of the compounds for PI(3,4,5)P_3/AKT1-PH domain inhibition (D). PC/PE/PS/PI(3,4,5)P_3 (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 ± SD from triplicate measurements.

PI-4 (3.23 µM) suggested strong interaction phenomenon of the compounds with AKT1 PH-domain. To determine the selectivity of the compounds in inhibiting PI(3,4,5)P_3/AKT1 PH-domain interaction, we also measured their inhibitory effect on BTK and GRP1 PH-domains, which are known to interact specifically with the PI(3,4,5)P_3 lipids.[9] We also determined the inhibitory effects of compounds on Tapp1 and PLCδ1-PH domains, which selectively interact with the PI(3,4)P_2 and PI(4,5)P_2 lipids respectively.[9, 11] The IC_{50} values of **PI-1** and **PI-4** for Tapp1-(28 and 5 µM, respectively) and PLCδ1-PH domains (54 and 28 µM, respectively) were relatively much higher (Table S1)**.** Therefore, the competitive SPR analysis suggest that compounds **PI-1** and **PI-4** have certain degree of selectivity towards PI(3,4,5)P_3 binding AKT1 PH-domain over other PI(3,4)P_2 and PI(4,5)P_2 binding PH-domains.

Competitive protein-to-membrane Förster resonance energy transfer (FRET) analyses were also performed for further understanding of the selectivity of these compounds **PI-1** and **PI-4** in inhibiting the PI(3,4,5)P_3/AKT1 PH-domain interactions. Trp-residue (W80) present at the membrane binding surface of the AKT1 PH-domain can act as FRET donor, and membrane-embedded dansyl-PE (dPE) serve as the FRET acceptor for this assay. The FRET-signal at 505 nm wavelength decreased with an increase in compound concentrations. However, detailed spectral

analysis revealed an associated reduction of Trp-fluorescence signal at 340 nm wavelength (Figure S2). Control experiment for the change in Trp-fluorescence signal and dPE fluorescence signal demonstrated that both the compounds interact with AKT1-PH domain in solution and reduce fluorescence signal at 340 nm wavelength (data not shown). Therefore, the reduction in FRET-signal could be primarily due to compound dependent Trp-fluorescence quenching of the protein and subsequent dislocation of protein from bilayer surface. Further detail analyses are required to distinguish their respective contribution in altering the FRET-signal. However, this assay supports that at-least a portion of the compounds in solution specifically interact with the AKT1 PH-domain.

Liposome pull-down assay was also used for qualitative determination of the effect of potent compounds on PI(3,4,5)P_3/AKT1 PH-domain interactions. The binding of GST-tagged AKT1-PH domain (50 μM) to (PC/PE/PS/PI(3,4,5)P_3 (57:20:20:3)) liposome was determined in the absence or presence of compounds (50 μM), **PI-1** and **PI-4**. The SDS-PAGE gel image showed that the binding of AKT1-PH domain to PI(3,4,5)P_3 containing liposomes was almost completely diminished by both the compounds, **PI-1** and **PI-4** and AKT1-PH domain got displaced from PI(3,4,5)P_3 containing liposomes under the experimental conditions (Figure 3).

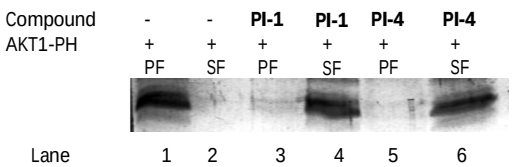

Figure 3: Coomassie blue stained SDS-PAGE gel images of liposome binding assay. PC/PE/PS/PI(3,4,5)P_3 (57:20:20:3) liposome was used for AKT1-PH domain binding study. Lanes 1, 3 and 5 were liposome bound fraction and 2, 4 and 6 were unbound fraction of AKT1-PH domain. Compound **PI-1** and **PI-4** were used as inhibitor. Liposome concentration 100 μM; Protein concentration 8 μM; Compound concentration, 50 μM; PF, palate fraction; SF, soluble fraction.

Additionally, isothermal titration calorimetric (ITC) measurements were performed to understand the direct binding mechanism and thermodynamic parameters of the compounds **PI-1** and **PI-4** with the AKT1 PH-domain. Titration plots of the compounds with AKT1 PH-domain revealed an exothermic reaction with two-step binding mechanism (Figure S3). The higher negative value of the enthalpy change than the entropy change for this PIP/AKT1 PH domain interaction could be due to the hydrogen bond formation and Van der Waals interactions between the compounds and cationic groove of the PIP- and PS-binding sites of the AKT1 PH-domain. Hee-Yong Kim and co-workers recently described that AKT1 PH-domain also contains a phosphatidylserine (PS) binding site next to the IP4-binding site.[27] Therefore, ITC analysis clearly suggests that the compounds in solution can probably interact with the AKT1 PH-domain through both of its PIP and PS-binding sites.

Molecular Docking Analysis—The competitive inhibition and binding assays clearly revealed that the compounds differentially perturb the PI(3,4,5)P_3/AKT1 PH-domain interaction, suggesting their divergent strength of interaction with the AKT1 PH domain. However, structural analysis showed that all these compounds have 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide moiety, consequently to understand their interaction properties with the PH-domains and clarify the probable binding mechanism for different inhibitory effects, we performed molecular docking analysis. The reported co-crystal structure of AKT1-PH domain (1H10) in complex with inositol 1,3,4,5-tetrakisphosphate (IP_4) presents a detail insight of the ligand interaction modes.[28] Structure-activity relationship studies of the AKT enzyme and isolated PH domain described that the phosphate groups of IP_4-preferably interact with the K14, E17, Y18, I19, R23, R25, N53 and R86 residues through hydrogen bond formation. Molecular docking analysis revealed that the 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide derivatives preferably interact with the IP4 binding site of the AKT1 PH-domain. The oxadiazole ring and N′-hydroxy moiety of the compounds could be responsible for their interaction with the cationic groove of the AKT1 PH-domain (Figure 4 and S5). Calculated in-silico interaction energies between the ligands and AKT1 PH-domain

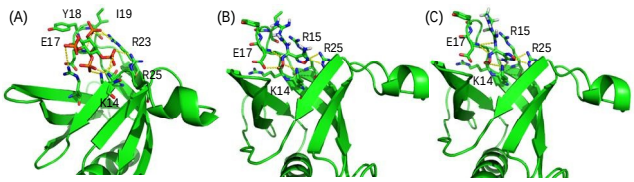

Figure 4: Structure of AKT1-PH domain (1H10) in complex with IP4 (A). Model structures of ligands **PI-1** (B) and **PI-4** (C) docked into the PIP-binding site of the AKT1-PH domain. Residues involved in interactions through hydrogen bond formation are shown using dashed lines (yellow).

protein are of high negative values, suggesting acceptable docking poses for further analysis (Table S2). Further detail analysis of the docking results revealed that the probable binding sites of the ligands are not that specific, as that of IP4 to AKT1 PH-domain. Blind-docking analysis data showed that the 60-70% of the docked poses of the ligands preferentially interact with the AKT1 PH-domain through its IP4-binding site (Figure S5). The docking results showed that the compounds can also interact with the AKT1 PH-domain through its PS-binding site, which is consistent with the two-step binding mechanism determined by the ITC measurements.

Structural Change Measurement— To understand the effect of compounds on the structural integrity of the isolated AKT1-PH domain, circular dichroism (CD) spectral analysis was performed. The CD spectra of the protein in the absence/presence of **PI-1** and **PI-4** are shown in Figure 5A. The spectral analysis revealed that in the presence of compounds there is a weak change in their secondary structural pattern of AKT1 PH-domain in the far-UV CD region. The change in β-sheet contents is little bit higher in the presence of both **PI-1** and **PI-4**, indicating direct interactions of the compounds with AKT1 PH-domain. The PIP and/or PS-binding site of AKT1-PH domain is composed of seven β-sheets and connecting loop regions. Analysis of the crystal structure reveals that AKT1-PH domain is enriched with β-sheet, indicating any change in helical content of AKT1-PH domain would be small and change in CD-signal in the range of 225-200 nm might be suppressed by the change in β-sheet content.

Therefore, our CD analysis clearly showed that the ligand binding partially alter the secondary structure of the AKT1 PH-domain. In order to ascertain that ligand binding does not induce any significant structural change (such as oligomerization) of the AKT1 PH-domain protein, we also performed dynamic light scattering (DLS) measurements in aqueous solution (at 25 °C, in 20 mM Tris–HCl buffer, pH 7.4, containing 0.16 M NaCl). The results showed a monomodal (PDI = 0.158 -0.186) size distribution of AKT1 PH-domain around a mean hydrodynamic diameter (d_h) of 190–240 nm in the presence of varying compound concentrations (Figure 5A). The DLS measurements clearly indicate that compounds **PI-1** and **PI-4** do not induce protein aggregation under the experimental conditions (Figure 5B).

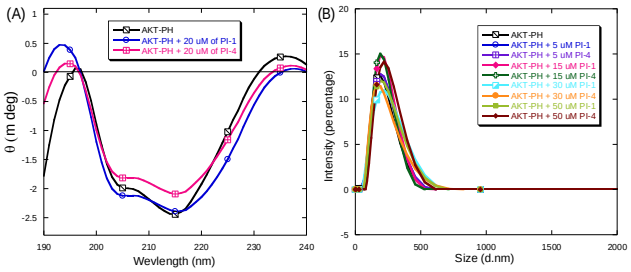

Figure 5: Effect of compounds on the secondary structural content of the AKT1-PH domain. Far-UV CD spectra of AKT1-PH domain in the absence and presence of compounds (20 µM) in 10 mM phosphate–containing 10 mM NaCl buffer (pH 7.2) (A). Size distribution of AKT1-PH domain in the absence or presence of compounds (20 µM, at t = 5 min) in aqueous solution (20 mM Tris–HCl buffer, pH 7.4, containing 0.16 M NaCl) at 25 °C (B).

Membrane Interaction Measurement— The effects of compounds on dynamics and fluidity of the lipid bilayer were measured by 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.[29, 30] The DPH and NBD probes are generally localized within the hydrophobic core and at the membrane interface of the lipid bilayers, respectively. Therefore, changes in fluorescence anisotropy values of DPH and NBD probe would be useful in monitoring the alteration of membrane fluidity influenced by membrane-active compounds. The variations in DPH anisotropy values affected by the compounds are not that significant (Table S3). However, the anisotropy of NBD was affected in the presence of compounds, indicating a weak effect on membrane dynamics and fluidity (Figure 6A). NBD probe of the NBD-PE lipid preferentially localize at the membrane-water interface, hence the change in anisotropy values in the presence of compounds indicate that the compounds weakly interact with the lipid headgroups. Zeta-potential measurements in the absence and presence of the potent compounds indicate that the compounds have a very weak effect on the surface charge of the liposome in aqueous solution (Figure 6B). Therefore, stronger protein binding of the compounds is the predominant factor for their higher inhibitory effect for PI(3,4,5)P_3/AKT1-PH domain interaction

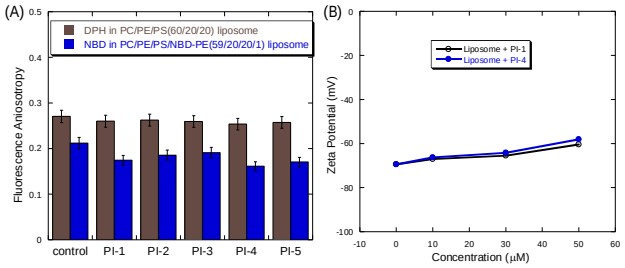

Figure 6: Fluorescence anisotropy of DPH and NBD-PE embedded in PC/PE/PS (60:20:20) and PC/PE/PS/NBD-PE (59:20:20:1) liposomes for compounds (A). Control: no ligand was added to the liposomes. Zeta potential of the PC/PE/PS/PI(3,4,5)P_3 (57:20:20:3) liposomes in the presence of compounds with different concentrations (B). 100 µL of liposomes from 0.5 mg/mL of total lipid was used for all measurements. Values represent the mean ± SD from triplicate measurements.

Biological Activity Studies — To measure cellular activities of the compounds, we first tested their cytotoxic effects using standard MTT assay. Results showed that all the compounds had minimal toxic effect on MDA-MB-231 cells (Figure 7A). For example, 100 µM of **PI-1** inhibited the cell proliferation of MDA-MB-231 only by 10% respectively. All other compounds showed the maximum inhibition of cell proliferation approximately 20% for both the cell lines (Figure 7A). Therefore, the compounds showed minimal cytotoxic effect on MDA-MB-231 cells. We also examined the effect of these compounds on PI3K-AKT signalling pathway. The potent compounds showed preferential binding to the AKT-PH domain, which may alter the activity of AKT enzyme. In this regard, the kinase activity of AKT enzyme was studied by measuring the phosphorylation level and expression level of AKT enzyme in MDA-MB-231 cells. The inhibition in growth of cells by the compounds were less than 25% and almost constant within the concentration range of 0.1 to 100 µM. The kinase activity of the AKT enzyme was initially performed with 25 µM compounds (Figure 7B) and then **PI-1** and **PI-4** were tested in a

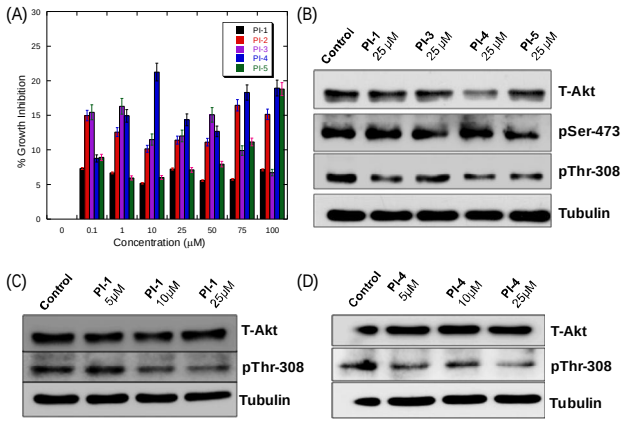

Figure 7: In vitro cytotoxicity of the compounds in human breast (MDA-MB-231) cancer cell line (A). Compounds inhibit AKT signalling pathway in MDA-MB-231 cells. MDA-MB-231 cells were treated with the compounds (25 µM) for 48 h, and the indicated PI(3,4,5)P_3-dependent phosphorylation events were evaluated by immunoblot analysis (B). MDA-MB-231 cells were treated with the **PI-1** and **PI-4** with different concentrations (5, 10 and 25 µM) for 48 h, and the indicated PI(3,4,5)P_3-dependent phosphorylation events were evaluated by immunoblot analysis (C and D). All the immunoblot images were presented in grey scale for better clarity.

concentration dependent manner. Figure 7C and D showed that compound **PI-4** has moderate effect on expression level of phospho threonine-308 of AKT enzyme. The changes in the expression level of phospho threonine-308 of AKT enzyme in the absence and presence of the compounds were semi-quantized by using ImageJ software (Figure S6). These data support that **PI-4** has moderate effect on PI3K-AKT signaling pathway without inducing too much cytotoxic side effects.

Discussion

PI3K-AKT signalling pathway is described as one of the most frequently deregulated pathways and associated with several human diseases including cancer and diabetes. In this regards, AKT has emerged as an attractive therapeutic target.[4, 6, 15, 31, 32] Recent reports showed that, development of specific inhibitors targeting the catalytic domain of the kinase enzymes is considered as quite challenging. There are several protein kinases and their catalytic domain is highly homologous. Several potent inhibitors of AKT enzyme turn out to be relatively toxic, probably due to the uncontrolled inhibition of other ser/threonine kinases.[15, 33] On the contrary, developed inhibitors for the PIP/PH domain interaction of AKT enzyme are comparatively nontoxic and present an improved therapeutic strategy. It is demonstrated that AKT enzyme get fully activated at the membrane interface through its binding with $PI(3,4,5)P_3$ lipid.[2, 4, 5, 16, 19] In this regard, inhibition of $PI(3,4,5)P_3$/AKT PH domain interactions could offer an alternate and improved therapeutic strategy for several diseases associated with PI3K-signaliing pathways. In recent years few research groups have already identified phosphate or non-phosphate containing small molecules as being potent inhibitors for PIP-interacting AKT1 PH domain. Selective inhibition of $PI(3,4,5)P_3$/AKT PH-domains interactions by small molecules like PITENINs, tirucallic acids, TCN-P, NSC348900 and others down regulate PI3K-PDK1-AKT pathways, suppress tumour growth, inhibits AKT-dependent phosphorylation, induce apoptosis in cancer cells.[2, 4, 15, 17, 34]

In this report we describe 4-amino-1,2,5-oxadiazole analogues as a new class of small molecule antagonist for $PI(3,4,5)P_3$ binding PH-domains. All molecules were designed such a way, that there must be 1,2,5-oxadiazole ring and N′-hydroxy moiety which are critical in forming more hydrogen bond with their binding partners and can be fitted inside the shallow binding pocket of the AKT1 PH-domain. The guanidine/biguanide/benzimidamide moieties 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 to elucidate the inhibitory mechanism of $PI(3,4,5)P_3$/PH domain interaction by 4-amino-1,2,5-oxadiazole analogues. The guanidine/biguanide/ benzimidamide moieties are present in several bioactive molecules including metformin which is used as drug for type 2 diabetes. Recent studies identified that metformin also acts as an anticancer agent.[35-37] The 4-amino-1,2,5-oxadiazole moiety containing compounds are potent inhibitor of immunosuppressive enzyme indoleamine 2,3-dioxygenase which are currently in clinical trials.[38]

SPR-based competitive binding study clearly indicates that the potent compounds strongly interacts with the PH-domains through its PIPs binding site. Compound **PI-1** and **PI-4** showed relatively stronger inhibition capabilities for $PI(3,4,5)P_3$/AKT1 PH domain interactions. SPR-based competitive binding analyses of the compounds with other PIP binding PH domain like Tapp1, PLCδ1, BTK and GRP1 clearly showed that **PI-1** and **PI-4** selectively interact with the AKT1-PH domain. We hypothesize that interaction pattern of the compounds with the amino acid residues present inside and/or outside the binding site, stability of the protein-compound complexes, effect of compound on membrane or their cooperative effect could be accountable for this differential inhibitory effect. These compounds can also differentially influence the in vitro $PI(3,4,5)P_3$ interaction properties of the AKT1, BTK and GRP1 proteins under the similar experimental conditions. The higher binding affinity of **PI-1** and **PI-4** for AKT1 PH-domain over the other PH- domains including GRP1, BTK, PLCδ1 and Tapp1 proteins could be due to its true selectivity for proper orientation of oxadiazole ring within the PIP-binding pocket of the AKT1 PH-domain, although its β1/β2 loop region contains fewer basic residues than the BTK and GRP1 PH-domains.

Molecular docking analyses suggest no clear differences in number of hydrogen bond formation between the compounds and AKT1 PH domain. However, SPR and FRET-based competitive binding study, ITC measurements and liposme pull down assay of the potent compounds clearly showed that these ligands strongly interact with the AKT1 PH-domain. We therefore presume that in addition to the 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide moiety the guanidine/biguanide/ benzimidamide moiety of the ligands might be involved in interaction with the amino acid residues of the AKT1 PH-domain through bridging water molecule, which was not considered during the molecular docking analysis. Hence, the difference in their binding affinity values could be due to the strength of direct hydrogen bonding through oxadiazole ring and N′-hydroxy moiety and also due to the hydrogen bonding through the bridging water molecules.

The ITC measurements, which were used to understand the binding parameters for direct protein-ligand interactions, pointed out that compounds **PI-1** and **PI-4** followed a two-step binding mechanism for their interactions with AKT1-PH domain. We hypothesizes that, the ligands are interacting with AKT1 PH domain through its IP4- and PS-binding sites. In-silico interaction energies for ligands with the PIP-binding site of AKT1 PH-domain protein are of higher negative values than that of with the PS-binding site. However, this non-specific ligand binding to the AKT1 PH-domain could also change the protein-conformation allowing it to show stronger competitive inhibition of the $PI(3,4,5)P_3$/AKT1 PH-domain interaction than the expected ones. Molecular docking analysis of PIT-1 (PITenins), a reported strong antagonist of $PI(3,4,5)P_3$/AKT1 PH-domain interactions also showed probable interaction through both IP4- and PS-binding sites.[2] Therefore, **PI-1** and **PI-4** could bind to both IP4- and PS-binding sites of the AKT1 PH-domain but with preference for IP4- binding site at lower compound concentrations. Recent studies demonstrated that AKT activation require not only $PI(3,4,5)P_3$ but also membrane localized PS

lipid. The PS lipid supports $PI(3,4,5)P_3$ binding of AKT PH-domain and involved in $PI(3,4,5)P_3$-dependent interdomain conformational change for Thr-308 and Ser-473 phosphorylation. Perturbation of PS binding to the PH-domain alters the AKT signalling pathways.[27] Therefore we presume that binding of these compounds to the PS-binding site of the AKT1-PH domain could alter its $PI(3,4,5)P_3$ binding and AKT-phosphorylation activities. However, further detailed analyses including mutational studies are required to understand the exact binding site of these compounds.

Stronger binding of the compounds to protein can also alter the structure of AKT1 PH-domain, which could forbid the protein to interact with the $PI(3,4,5)P_3$ containing membranes.[39] CD and DLS measurements showed that stronger binding of the potent compounds have a very weak effect on the secondary structure of AKT1 PH domain and there was no protein aggregation under the experimental conditions. It is important to note that the inhibition/binding properties of the compounds were measured under liposomal environments. Stronger binding affinity values of the compounds could be also as a result of their direct interaction with the liposomes, which can alter the membrane dynamics and inhibit the PH-domains to interact with the membranes. The membrane composition used to measure the efficacy of the compounds in inhibiting respective PIP/PH domain interactions contains anionic lipids PS and PIP. There is a possibility that guanidine/biguanide/benzimidamide moiety of the compounds could interact with the anionic lipids present in the membrane. This interaction could block the protein binding to the membrane and allow the *in vitro* measurements to show high inhibitory effect by the compounds in a concentration dependent manner. Anisotropy and surface potential measurements demonstrate that theses water soluble compounds weakly interact with the interfacial region of the liposome and preferably localize in the bulk phase of the solution and its pharmacophores are accessible for PH-domain binding under the experimental conditions. Therefore, stronger binding of the potent compounds with the AKT1 PH domain perturbs its $PI(3,4,5)P_3$-dependent membrane association, which is essential for it activity in initiating a range of local responses, like accumulating signalling complexes, initiating protein kinase cascades and others.

Cytotoxicity measurements of the compounds in breast cancer cells (MDA-MB-231) showed that the compounds have low toxicity, which is one of the key requirements in developing selective PIP/protein interactions in controlling the activities of the target enzymes. The kinase activity of the AKT enzyme was moderately altered in the presence of **PI-4** in MDA-MB-231 cells. The change in kinase activity of the enzyme in the presence of compound **PI-4**, correlates well with its PH domain binding or inhibition of PI(3,4,5)P3/AKT-PH domain interaction abilities. However, *in vitro* inhibition efficiency of the compounds is not in the same order with their cellular kinase activities. Additional biological studies are required to understand their potency in inhibiting AKT kinase activity. The 4-amino-1,2,5-oxadiazole moiety provide a good template to develop more potent AKT enzyme inhibitor.

Conclusion

Targeting the specific enzymes of PI3K-signalling pathway, which would show limited side effects, is considered as future direction of anticancer drug discovery. To this end, this study demonstrate the identification of non-lipid based compounds with 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide moiety that strongly interact with the $PI(3,4,5)P_3$ binding of AKT1 PH-domain, but weakly interact with the $PI(3,4)P_2$ and $PI(4,5)P_2$ binding PH domains. The potent compounds also weakly interact with the liposome through its interfacial region. Cellular measurements showed that the potent compounds have weak cytotoxicity and moderate inhibition effect on AKT-kinase activity. These results suggest the inhibition of $PI(3,4,5)P_3$ dependent membrane recruitment and AKT enzyme activity by these potent compounds. Our findings imply that the 4-amino-N′-hydroxy-1,2,5-oxadiazole-3-carboximidamide moiety can be used as a template in developing non-phosphate-based potential inhibitors for PIP-binding proteins.

Experimental Section

General Information: General Information: All chemicals and reagents were purchased from Sigma (USA), SRL (India), Merck (India) and used for the synthesis without further purification. Phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$), phosphatidylinositol-3,4 -bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), 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) 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. DMEM, RPMI1640, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen. Antibody against AKT1, phospho serine-473 and phospho threonine-308 were obtained from Cell Signalling. α-tubulin antibody and all other biochemical were purchased from Sigma.

Protein Purification: The AKT1 (homo sapiens; 1-121 amino acid), BTK (homo sapiens; 2-170 amino acid), GRP1 (mus musculus, 1-127 amino acid), Tapp1 (homo sapiens; 180-305 amino acid) and PLCδ1 (rattus norvegicus, 1-131 amino acid) PH-domains were expressed in E. coli cells (BL21-DE3) and purified using methods similar to those reported earlier.[9, 40] The plasmids were generous gift from Prof. Wonhwa Cho (University of Illinois at Chicago, IL, USA).

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 μL/min) using a lipid-coated L1 sensorchip in the Biacore-X100 (GE Healthcare) system as described earlier.[9, 25, 26, 40] 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 MiniExtruder (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/PIP (57:20:20:3) and PC/PE/PS (60:20:20) vesicles were injected at 5 μL/min to the active surface and the control surface, respectively, to achieve similar response unit (RU) values (3500-4000 RU). To minimize nonspecific adsorption the control surface was also coated with 40 μL of BSA (0.1 mg/ml in the running buffer) at a flow rate of 5 μL/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 (0-200 μM). The compounds were equilibrated with respective PH-domain for 30 minutes 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-(RU of protein mixed with compounds/ RU of protein only)) × 100%. The RU value after 180 sec of injection was considered for % of inhibition efficiency calculations.

In–silico Molecular Docking Analysis: Computational docking and scoring studies of the interaction of the compounds with PH-domains were performed using AutoDock 4 (The Scripps Research Institute, La Jolla, USA) and Molegro Virtual Docker software, version 4.3.0 (Molegro ApS, Aarhus, Denmark) with essentially the same results.[21-23] The crystal structure of AKT1 PH (1-116 amino acids, PDB code: 1H10),[28] BTK PH (1-169 amino acids, PDB code: 1B55)[41] and GRP1 (261-386 amino acids, PDB code: 1FGY)[42] were utilized for docking experiment. To generate apo-protein, the ligands were removed from the co-crystal structures and then they were processed by energy minimization. The energy minimized three-dimensional structure of ligands was 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 were used in combination with an evaluation scheme based on Gibbs free energy change (ΔG). In each docking run, one hundred docked structures were generated for individual ligand. Energetically favoured 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.

Circular Dichroism Studies: The circular dichroism (CD) spectra of the protein samples were measured using JASCO J-815 CD spectropolarimeter at room temperature. CD spectra of AKT1 PH-domain in the absence and presence of complexes (1 : 3 molar ratio of the AKT1 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).[39, 40]

Anisotropy Measurement: Fluorescence anisotropy measurements were performed on a Fluoromax-4 spectrofluorometer at 25 °C. The anisotropies of DPH and NBD-PE under liposomal environment were measured according to the reported procedure.[21, 43] The fluorescence probe DPH was incorporated into the PC/PE/PS (60/20/20) liposome (100 μL of 0.5 mg/mL of total lipid) by adding the dye dissolved in THF (1 mM) to vesicles up to a final concentration of 1.25 μM. The NBD-PE probe was incorporated to the PC/PE/PS/NBD-PE (59/20/20/1) liposome using our earlier mentioned procedure.[22] After 30 min of incubation at room temperature, DPH (λ_{ex} = 355 nm; λ_{em} = 430 nm) and NBD (λ_{ex} = 460 nm; λ_{em} = 535 nm) fluorescence anisotropies were measured. The concentration of compounds was 15 μM. The degree of anisotropy in the DPH/NBD fluorescence of the probes were calculated using equation 1, at the peak of the fluorescence spectrum, where I_{VV} and I_{VH} are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and $G = I_{VH}/I_{HH}$ is the instrumental grating factor.

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

Dynamic Light Scattering Measurement: The dynamic light scattering (DLS) experiment was carried out to determine the degree of aggregation of AKT1 PH-domain protein with varying concentration of compounds 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 (λ_0 = 632.8 nm).[40, 44] The reduced glutathione content in protein samples were removed using a PD-10 desalting column (Sigma, St. Louis MO). Before performing any measurements dust or other particles were removed from sample solutions through syringe filtration technique (0.22 μm) and equilibrated for 10 min before performing any measurement (at least 11 runs were performed for each sample, all measurements were performed in triplicates). The scattering intensity was measured at 90° (right angle) to the incident beam.

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 (λ_0 = 632.8 nm).[45, 46] Unilamellar vesicles composed of PC/PE/PS/PI(3,4,5)P_3 (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 μL 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.

Förster Resonance Energy Transfer (FRET) Measurements: Analysis of protein-to-membrane Förster resonance energy

transfer (FRET) based binding assay was used to detect the specificity of the compounds for PH-domain binding through PIP-binding site. In this assay, membrane-bound AKT1 PH-domain was displaced from liposomes by the addition of the compound. The vesicles composed of PC/PE/PS/dPE (60/15/20/5) and PC/PE/PS/dPE/PIP (57/15/20/5/3) was used as control and for ligands, respectively. 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. Control experiments were also performed to test the effect of only compounds on the fluorescence of dPE. The stock solution of compounds was titrated into the sample containing AKT1 PH-domain (1 μM) and excess liposome (100 μM 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 (λ_{ex} = 280 nm and λ_{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.[21, 22]

Isothermal Titration Calorimetry (ITC) Measurements: Thermodynamic parameters of protein-ligand interactions were measured using ITC-200 microcalorimeter from Microcal (Northampton, MA, USA). AKT1 PH-domain (200 μM), after dialysis with 20 mM HEPES buffer, pH 7.4, containing 0.16 M NaCl, was titrated against compounds (2 mM) dissolved in the final dialysate. A typical titration involved injecting 20 injection volumes (2 μL) of compound into the sample cell containing AKT1 PH-domain (201.6 μL) at 2.0 min intervals with continuous stirring (at 25 °C with stirring speed of 500 rpm). The heat of dilution data corresponding to individual injections were analyzed using a sequential binding model with two binding sites considering both PIP and PS-binding sites per AKT1 PH-domain monomer with the system running Microcal Origin 7.0 software. The data points obtained from the ITC measurements were first fitted with different models and the best fit was obtained using sequential binding model with two binding sites. The ΔH and ΔS values were obtained using a nonlinear least-square fit of the data. Gibbs free energy (ΔG) was calculated by using Gibbs equation: $\Delta G = \Delta H - T\Delta S$.

Liposome Binding Assay: Inhibition of PI(3,4,5)P_3/AKT1-PH domain interaction by the compounds was further determined by liposome pull-down assay according to the reported procedures.[40, 45, 47] Liposomes of PC/PE/PS/PI(3,4,5)P_3 (57:20:20:3) in 20 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 0.1mm pore size polycarbonate filters. The suspension was pelleted down at 100000g, 4 °C, for 20 minutes and resuspended in binding buffer (20 mM Tris buffer, pH 7.4, containing 100 mM KCl, 5 mM $MgCl_2$). Purified AKT1-PH domain (8 μM) was incubated with sucrose-loaded vesicles (40 μg in 100 μL) for 30 min in the presence of binding buffer containing 0.3 mg mL^{-1} BSA. Membrane-bound protein was separated from free protein by centrifugation at 100000*g* for 30 min at 4 °C. Protein of supernatant and pellet fractions was analyzed by SDS-PAGE gel.

***In vitro* Cytotoxicity Assay:** The *in vitro* toxicity of the compounds was determined in human breast cancer cell line (MDA-MB-231). Cells were cultured according to the American Type Culture Collection (ATCC) instructions. MDA-MB-231 cells were grown in RPMI1640 media supplemented with 10% Fetal Bovine Serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO_2 atmosphere under humid condition. Cytotoxic effect of the compounds (**PI-1** to **5**) were determined by 3-(4, 5 dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide (MTT) assay as described previously.[48] Briefly, cells (3×10^3/per well) were seeded one day prior the treatment of the compounds. Then cells were treated with different concentrations (1 – 100 μM) of compounds for 48 hours. At the end of incubation, MTT solution (20 μl of 5 mg/ml of stock) was added to the media and incubated for 4 hours in the CO_2 incubator. Finally media containing MTT solution was replaced with 100 μl of MTT solvent (5 mM HCl and 0.1% Triton X-100 in iso-propanol) and incubated at room temperature for 15 minutes with gentle rocking. Then absorbance was measured at 590 nm using a Thermo pierce plate reader. The percentage of viable cells was calculated as the mean with respect to the vehicle treated cells and considered to be 100%. All the compounds were dissolved in DMSO and maximum 0.01% DMSO was used for the experiments. This experiment was repeated three times. The data from cancer cell lines were acquired from three independent cell passages and the IC_{50} values were calculated from the plot of cell viability *vs* concentration of compounds.

Immunoblot analysis: Cells were collected at the end of treatment and washed with ice cold PBS. Then, whole cell extracts were prepared using lysis buffer (50 mM Tris buffer of pH 7.4, 250 mM NaCl, 50 mM NaF, 0.5 mM Na_3VO_4, 5 mM EDTA and 0.5% Triton-X 100 contain protease inhibitor cocktail). Cells were lysed in ice for 30 minutes and then lysates were clarified by centrifugation at 13000 rpm for 15 minutes at 4 °C. The supernatants were transferred to the fresh tubes and protein concentration was measured by using Bradford reagent as described previously.[2] Then equal amount of whole cell lysates were resolved by SDS-PAGE and transferred to PVDF membrane and probed with indicated antibodies.

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Notes and references

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Inhibition of Phosphatidylinositol-3,4,5-trisphosphate Binding to AKT Pleckstrin Homology Domain by 4-Amino-1,2,5-oxadiazole Derivatives

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