



**HAL**  
open science

## Exploring the specificity of the PI3K family inhibitor LY294002

Severine I Gharbi, Marketa J Zvelebil, Stephen J Shuttleworth, Tim Hancox,  
Nahid Saghir, John F Timms, Michael D Waterfield

► **To cite this version:**

Severine I Gharbi, Marketa J Zvelebil, Stephen J Shuttleworth, Tim Hancox, Nahid Saghir, et al..  
Exploring the specificity of the PI3K family inhibitor LY294002. *Biochemical Journal*, 2007, 404 (1),  
pp.15-21. 10.1042/BJ20061489 . hal-00478676

**HAL Id: hal-00478676**

**<https://hal.science/hal-00478676>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Title:**

Exploring the specificity of the PI3K family inhibitor LY294002

**Authors:**

Severine I. Gharbi<sup>\*</sup>, Marketa J. Zvelebil<sup>†</sup>, Stephen J. Shuttleworth<sup>‡</sup>, Tim Hancox<sup>‡</sup>, Nahid Saghir<sup>‡</sup>, John F. Timms<sup>\*§</sup> and Michael D. Waterfield<sup>\*†¶</sup>

*<sup>\*</sup>Ludwig Institute for Cancer Research, Proteomics Unit, Cruciform Building, Gower Street, London WCE1 6BT, UK. <sup>†</sup>Ludwig Institute for Cancer Research, Bioinformatics Group, 91 Riding House Street, London W1W 7BS, UK. <sup>‡</sup>Piramed, 957 Buckingham Avenue, Slough, Berkshire, SL1 4NL, UK. <sup>§</sup>Transitional Research Laboratory, Institute of Women's Health, University College London, Huntley Street, London, UK*

**¶Corresponding Author:** Professor M D Waterfield FRS, Email: [mikew@ludwig.ucl.ac.uk](mailto:mikew@ludwig.ucl.ac.uk).

**Abbreviations**

**EAS**, Epoxy-activated sepharose; **CK2**, casein kinase 2; **GSK3**, Glycogen synthase kinase 3; **IC<sub>50</sub>**, Half maximal inhibitory concentration; **LC-MS/MS**; Liquid chromatography-tandem mass spectrometry; **MS**; Mass spectrometry; **PIP3**, Phosphatidylinositol (3,4,5) triphosphate; **PI3K**, Phosphoinositide 3-kinase; **Wm**, Wortmannin

The Phosphatidylinositol 3-kinases (PI3Ks) regulate cellular signaling networks that are involved in processes linked to the survival, growth, proliferation, metabolism and specialized differentiated functions of cells. The subversion of this network is common in cancer and has also been linked to disorders of inflammation. The elucidation of PI3K physiological function has come from pharmacological studies, which use the enzyme inhibitors Wortmannin and LY294002, and from PI3K genetic knockout models of the effects of loss of PI3K function. Several reports have shown that LY294002 is not exclusively selective for the PI3Ks, and could in fact act on other lipid kinases and additional apparently unrelated proteins. Since this inhibitor still remains a drug of choice in numerous PI3K studies (over 500 in the last year), it is important to establish the precise specificity of this compound. We report here the use of a chemical proteomic strategy in which an analogue of LY294002, PI828, was immobilized onto epoxy-activated sepharose beads. This affinity material was then used as a bait to fish-out potential protein targets from cellular extracts. Proteins with high affinity for immobilized PI828 were separated by 1D-gel electrophoresis and identified by LC-MS/MS. This study reveals that LY294002 not only binds to class I PI3Ks and other PI3K-related kinases, but also to novel targets seemingly unrelated to the PI3K family.

## Introduction

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases responsible for the phosphorylation of phosphatidylinositols on the D3 position of their inositol ring. The evolution of this family of enzymes and its critical role in the life, death, growth, proliferation, metabolism and in differentiated cell function has been recently reviewed [1]. The family is made up of 14 enzymes separable into four classes of which Class I, II & III are lipid kinases and class IV are related protein kinases [2, 3]. The most studied are the class I PI3Ks, which are sub-divided into class IA and class IB. Following receptor activation, the class I PI3Ks are recruited to the membrane where they generate a pool of the lipid second messenger, phosphatidylinositol (3,4,5) triphosphate (PIP3). The

transient production of PIP3 initiates a cascade of events starting with the recruitment and activation of effector proteins which carry PIP3 binding domains (PH, FYVE, ENTH) [4]. PDK1 and Akt, for example regulate a multitude of targets [5]. Due to the pivotal role of the PI3K/Akt axis in cellular physiology, its perturbation has been directly linked to various diseases, most notably cancer, immune disorders, inflammatory disease and diabetes [6]. Indeed, over-expression of PI3K [7] with recent evidence of oncogenic and probably activating mutations of p110 $\alpha$  [8, 9] and loss of or reduced activity of the tumor suppressor PIP3 phosphatase PTEN [10] are reported during tumorigenesis. Many other genes in the PI3K pathway are also tumor suppressors which validates the importance of this pathway in cancer and the PI3K network as a target for cancer therapy [11, 12].

In order to develop drugs that modulate PIP3 levels it is crucial to delineate the specific biological functions of each isoform of the different classes of the PI3K family. Several approaches have been used to alter protein function and recent specific genetic animal models are uncovering isotype selectivity [13-15]. However, complementary chemical biology studies are required to help further elucidate specific class I PI3K isoform functions. Most of the small molecule PI3K inhibitors developed to date are ATP-competitive inhibitors. The first described PI3K inhibitors which were considered as relatively specific were Wortmannin (Wm) [16, 17], a naturally occurring metabolite of *Penicillium funiculosum*, and LY294002 [18], that was derived from the flavonoid quercetin (Figure S1). Although these compounds are invaluable tools to study the PI3K pathway, they have a broad inhibitory profile across different classes of PI3Ks. Indeed, LY294002 was shown to inhibit mTOR and DNA-PK [19] but also other protein kinases, such as casein kinase 2 (CK2) and Pim-1 [20, 21]. Furthermore, other PI3K-independent effects directly attributed to LY294002 have been reported [22-27]. For example, LY294002 was directly implicated in the inhibition of Ca<sup>2+</sup> signaling [27] and transcription factors such as NF- $\kappa$ B [24]. However, the direct targets of LY294002 involved in these processes have not been defined and we suspect that several targets may be involved. Currently, the validation of inhibitor specificity remains closely linked to previous knowledge of potential targets through modeling analysis or *in vitro* activity assays. Recent reports have revealed a new approach to assess drug specificity by direct immobilization of the small molecule inhibitors onto a solid phase and subsequent

identification of bound proteins using optimized proteomic methods [28, 29]. We describe here the use of an LY294002-derived matrix to isolate and identify its direct molecular targets and to further understand reported off-target effects of this compound.

## Materials and Methods

### *Reagents*

Tissue culture media and fetal calf serum were from Invitrogen, Gibco, UK. Antibodies to p110 $\alpha$  and p110 $\delta$  were a kind gift from Dr Bart Vanhaesebroeck (LICR, London, UK). Antibodies to p85 and VCP were from Santa Cruz Biotechnology (CA, US). The antibody to ALDH was from BD Transduction Laboratories (CA, US). The antibody to mTOR was from Cell Signaling Technology (MA, US). LY294002 and LY303511 were from Merck Biosciences Ltd. (CA, US). Synthesis of 8-bromochromenone was carried out by Charnwood Molecular. PI828 and PI103 were synthesized by Piramed Ltd. All other reagents were from Sigma-Aldrich (MO, US) unless otherwise stated.

### *Synthesis of PI828*

The precursor to LY294002 and PI828, 8-bromo-2-morpholin-4-yl-chromen-4-one was prepared as previously described [30]. To prepare PI828 (Fig.1A), a suspension of 161 mM 8-bromo-2-morpholin-4-yl-chromen-4-one, 177 mM 4-(4,4,5,5-tetramethyl-[31]dioxaborolan-2-yl)-phenylamine, 484 mM sodium carbonate in toluene/Ethanol (EtOH) was flushed with argon. 8 mM Dichlorobis(triphenylphosphine) palladium (II) was added and the mixture heated in a microwave reactor for 1h at 120°C. The crude mixture was partitioned between dichloromethane and water. The combined organic layers were washed with brine, separated and dried (MgSO<sub>4</sub>). The crude product was evaporated onto silica and purified by flash column chromatography (5%→10% methanol:dichloromethane) to give a yellow solid which was further purified by trituration in ether:ethyl acetate to furnish the title compound as a pale yellow solid (177 mg, 49%).

### *Production of Immobilized PI828*

Epoxy-activated Sepharose 6B (EAS) beads (GE Healthcare, 1 g) were incubated overnight at 55°C with 2 volumes of 20 mM PI828 in 50% (v/v) DMF/0.1 M sodium phosphate buffer at pH 6.8, while protected from light and permanently shaking. The resin was then washed in 2 volumes of 50% (v/v) DMF/0.1 M Na<sub>2</sub>CO<sub>3</sub> and incubated for 16 h at 40°C in the dark with 2 volumes of 1 M ethanolamine. Further washes were performed as follows: 3x 50% DMF/0.1M Na<sub>2</sub>CO<sub>3</sub>; 1 x 0.1 M NaHCO<sub>3</sub>/0.5 M NaCl; 1 x 0.1 M NaOAc (pH 4.0) /0.1 M NaCl; H<sub>2</sub>O; 20% (v/v) EtOH. The lilac colored resin was stored in 20% (v/v) EtOH at 4°C in the dark. The control beads (blocked EAS) were generated by incubation with 1 M ethanolamine for 16 h followed by washes as described above.

### *PI3K Activity Assays*

PI3K inhibition by PI828 and LY294002 was determined in a radiometric assay using purified, recombinant enzymes (class IA and class IB) with 1 μM ATP. The kinase reaction was carried out for 1 h at room temperature and was terminated by addition of Phosphate-Buffered Saline (PBS). IC<sub>50</sub> values were subsequently determined using sigmoidal dose-response curve fit (variable slope). CK2 and GSK3β inhibition was established by kinase selectivity screening. 10 μM inhibitor (PI828 and LY294002) was tested against the Upstate panel of kinases in 10 μM ATP (Upstate Kinase Profiler™ version 7.0, www.upstate.com).

### *Cell lysis and affinity purification experiments*

HeLa cells (2.5x10<sup>9</sup> cells, Cilbiotech, Belgium) were lysed in 30ml of lysis buffer A (20 mM Hepes (pH 7.5), 150 mM NaCl, 0.25% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT supplemented with protease and phosphatase inhibitors). WEHI231 cells were grown in suspension in RPMI1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 μM β-mercaptoethanol and antibiotics. For large-scale experiments, 15 mg of total cellular extract were adjusted to 1 M NaCl and incubated with 300 μl of 50% bead slurry for 4 h. For competition assays, lysates were pre-incubated with 1 mM of LY294002 or PI828 in lysis buffer A supplemented with 10 mM ATP, 20 mM MgCl<sub>2</sub>.

Affinity complexes were washed extensively in lysis buffer A adjusted to 1M NaCl and then in lysis buffer A. Complete elution was achieved in 1 x Laemmli sample buffer with boiling. Specific elutions were also carried out with free inhibitor (LY294002, PI828, PI103, LY303511) in lysis buffer A supplemented with 10 mM ATP, 20 mM MgCl<sub>2</sub>.

#### *Protein separation and Mass spectrometry*

Eluted proteins were separated by 1D gel electrophoresis and 1/10<sup>th</sup> of the sample was kept for Western Blotting analysis. Preparative gels were stained with colloidal coomassie blue G-250 (Merck) and the whole lane dissected into smaller bands. An in-gel digestion procedure was followed as previously described [32]. Each sample (1/2 of total peptide mixture) was analyzed by LC-ESI-MS/MS using an Ultimate nano HPLC (Dionex Ltd, UK) with a PepMap C18 75- $\mu$ m inner diameter column (Dionex) at a flow rate of 300 nL/min, linked on line to a QToF-I mass spectrometer (Waters, UK). Processed mass spectra (MassLynx, Waters) were searched against the updated IPI human (HeLa) or mouse (WEHI 231) databases using Mascot version 2.0.02 (Matrix Sciences, UK). Identifications were accepted when a minimum of 2 individual peptide masses matched a protein (mass error of  $\pm 100$  mmu allowing 1 missed cleavage), MOWSE scores were higher than the threshold value ( $P = 0.05$ ), and the predicted protein mass agreed with the gel-based mass.

#### *Expression of recombinant VCP and binding assays*

The wild type (WT) and ATP hydrolysis mutant (AA) p97/VCP constructs were a kind gift from Dr. Y Ye (NIDDK/NIH, MD, US) [33]. Full length mouse His-tagged p97/VCP was cloned into a pQE9 vector (Qiagen Ltd.) [33, 34] and expressed in *E.coli* following induction with 0.5 mM IPTG. Recombinant proteins were purified as described [34]. 2  $\mu$ g of WT and AA VCP were incubated with 10  $\mu$ l of beads slurry and pull-downs were carried out as described in the large-scale experiments.

#### *Docking studies*

The crystal structure of p110 $\gamma$  [35, 36] was used for homology modeling and inhibitor docking studies, although only the catalytic region of the protein was utilized (residues

725 to 1092). The models were constructed as described previously [37]. Docking was carried out using the application GOLD (Cambridge Crystallographic Data Centre, Cambridge, UK), with GoldScore and point of origin set to C<sub>5</sub> of ATP from which a 10 Å pocket was defined. As a test of accuracy, the docked structure of PI828 was compared to the crystallographically determined structure [35]. The docked and structural coordinates and orientation of PI828 were in very good agreement. A further docking study of LY294009 and PI828 in both VCP (PDB number 1R7R) and ALDH2 (PDB number 1AG8) was carried out. For ALDH2, two pockets were identified, but the NAD- binding pocket proved to be the most probable site of interaction.

## Results

### *Design and Immobilization of PI828; Validation of the Binding Assay*

An analogue of LY294002 was designed following consideration of its binding mode to p110 $\gamma$  as determined by X-ray crystallography [35]. The crystal structure indicated that the morpholine oxygen formed a hydrogen bond with the hinge region Val882, with a further putative interaction existing between the carbonyl oxygen and Lys833. Significantly, the 4-position hydrogen of the exocyclic phenyl substituent, which occupies the ribose binding pocket, was seen to extend out towards solvent. Based on this structural information, we chose to incorporate an amine linker at this position to use as a site of attachment to the resin (Fig.1A; synthesis and immobilization in supplemental data). The precursor to the immobilized ligand, PI828, showed encouraging biochemical inhibition of class I PI3Ks (Table S1). Indeed, PI828 was more potent than LY294002, particularly against p110 $\beta$ . From our modeling study, we postulated that this increase in potency may arise from hydrogen bonding of the PI828 amine protons with Glu858 and Asp862 in the catalytic site (Fig.1B), residues which are unique among all the PI3K class I isoforms (Zvelebil *et al*, submitted manuscript). PI828 was immobilized onto epoxy-activated sepharose (EAS) beads using a procedure similar to that previously developed [28]. Binding of PI3Ks was first examined using recombinant p110 $\alpha$  and then from WEHI231 cells and PI3K catalytic subunits were detected by western blotting (Fig.1C).

Affinity binding conditions were mainly based on previously published work [28] and no binding to blocked EAS without immobilized inhibitor was observed (Fig.2).

*Identification of protein targets of PI828 and Evaluation of Relative Binding Affinity*

We next used the PI828-matrix to perform large-scale pull-down assays in human epithelial cells (HeLa cells) (Table S2) and in the mouse lymphoma B-cell line WEHI231 (Table S3), which expresses high levels of p110 $\delta$  [38]. In the first instance, proteins were eluted in Laemmli sample buffer to ensure complete elution from affinity matrix. In later experiments, specific elution was carried out with LY294002 to evaluate binding profiles. Reproducible protein identification from both cell types was observed although unique proteins were also found which may be related to different protein expression patterns of the two cell types (Fig 2A and Tables S2 and S3). PI3K catalytic subunits were highly represented in the bound fraction, and the most abundant isoform was p110 $\delta$ . The p85 regulatory subunits were also present, which reflects the strong interaction between these two subunits. The class III PI3K, vps34, was also identified together with Beclin-1, a protein known to interact with vps34 [39]. No PI3K-related kinases were identified by mass spectrometry, which may be attributed to their low copy numbers and high molecular weights, although, mTOR was detected by immunoblotting (Fig.2B). The type III phosphatidylinositol-4 kinase, (230 kDa isoform) was also identified, correlating with reports showing its sensitivity to both Wm and LY294002 [40, 41]. The PI828-matrix also bound to an array of proteins with various functions and substrate specificities. These included proteins with a capacity for binding ATP, NAD(P)H, nucleotides and acetylated histones (Fig.3A and 3B and see below).

Competitive binding with an excess of free inhibitor (1 mM LY294002 or PI828) was carried out to validate binding specificities (Fig.2A and 2B). As well as the PI3K isoforms, other previously reported targets of LY294002, such as mTOR, CK2 and GSK3 $\beta$ , showed reduced binding in the presence of excess free inhibitor (Fig.2B). On the other hand, bands corresponding to the family of dehydrogenases showed higher affinity for the PI828-matrix (Fig. 2A and B). Sequential competitive elution experiments with increasing concentrations of LY294002 (Fig.4) demonstrated that the bound proteins had different binding strength for the affinity matrix. The ALDH2 interaction was strong and

10 mM of LY294002 or SDS were necessary to elute it from the PI828-matrix, suggesting high affinity to the beads (Fig.4A and 4B). We next used this affinity platform to assess binding profiles in competing concentrations of PI103 and LY303511 (Fig.4). PI103 is a potent inhibitor of p110 $\alpha$  at low nanomolar concentration, as recently reported [42, 43], and LY303511 is a structurally related analogue of LY294002 which has an amine substitution of its morpholine oxygen group, therefore altering its ability to inhibit PI3Ks [44]. As expected, LY303511 was less efficient at eluting p110 $\delta$  than LY294002 or PI103. PI103 was unable to efficiently elute mTOR in our competition assay although it can inhibit its activity [42] (Fig.4). Both, CK2 and GSK3 $\beta$  were eluted at low concentrations of LY303511 and PI103 suggesting a high affinity for these inhibitors.

#### *Evaluation of putative targets of LY294002*

Some of the putative targets were further evaluated to address the functional impact of LY294002 on their activity. Casein kinase 2 (CK2), a previously reported target of LY294002 [20], showed robust binding to the PI828-beads with complete depletion of CK2 from samples achieved under the conditions used (data not shown). Notably, both catalytic and regulatory subunits of CK2 were recovered. The binding was also shown to be selective (Fig.2B and 4B) and CK2 was catalytically inhibited by LY294002 (Table 1). LY294002 was also able to reduce the kinase activity of both isoforms of the serine/threonine kinase Glycogen synthase kinase 3 (GSK3  $\alpha$  and  $\beta$ ) (Table S4). Another PI3K un-related target was the p97/Valosin-containing protein (VCP), a member of the type II AAA ATPase family. The presence of p97/VCP and other chaperones with ATPase activity in the PI828 pull-down assay is interesting since it suggests a novel class of targets of LY294002. To confirm our observation, we compared binding of recombinant wild type p97/VCP (WT), and a mutant defective in ATP binding (K251A; K524A, AA) to the PI828-matrix (Fig.5A) [33]. This validated the direct interaction of VCP with the PI828-matrix, but also showed that these critical lysines are not required for binding to PI828, since similar binding was observed for the WT and mutant VCP. We also characterized the binding by competing with an excess of free compound or ATP showing that high concentration of free inhibitor (2 mM) plus ATP (10 mM) were required to reduce VCP binding (Fig.5A). Homology modeling based on the crystal

structure of ATP-bound VCP allowed both LY294002 and PI828 with its linker to be docked into the ATP binding site (Fig.5B). In the LY294002-bound form, one putative hydrogen bond was observed with Ile380 but also between the linker and Arg256. Furthermore, the linker was exposed to the solvent channel, providing enough space for the bulky matrix not to impede binding. Thus, it appears that VCP may be a direct target of LY294002 in cells, which may perturb its chaperone functions.

## Discussion

The use of the chemical proteomic platform developed here has revealed a new subset of possible targets for LY294002 whose inhibition could affect various cellular processes particularly metabolism, transcription or protein trafficking and dynamics (Fig.3). The major non-lipid kinases identified in this study have been previously reported (CK2, mTOR, GSK3 $\beta$ ), which emphasizes the thoroughness of this assay. It is likely that the PI3K-independent cellular effects observed with LY294002 are in fact due to inhibition of one or more of these three kinases. The serine/threonine kinase CK2 plays a putative role in proliferation and other cellular processes through activation of transcription factors and other cell signaling proteins and its overexpression in cancer has been reported [45]. One possible mechanism is that inhibition of CK2 by LY294002 could affect phospholipase C and calcium signaling [27]. Additionally, GSK3 is involved in the regulation of glycogen metabolism through the inactivation of glycogen synthase, but it also has an important role in regulating many signaling pathways [46]. This kinase is a known substrate of Akt [47] and is therefore regulated downstream of the class I PI3Ks. It will therefore be important to note the direct effect of LY294002 and other PI3K-specific inhibitors on this enzyme in future studies. Other targets had also previously been reported to be directly inhibited by LY294002, such as the cAMP phosphodiesterase 2 (PDE2) (Tables 2 and 3 Supplemental data) [48]. However, the kinase Pim-1 was not identified in our assay and its binding to the PI828-matrix needs to be assessed by immunoblotting. Absence of Pim-1 could however reflect the proposed binding mode of LY294002 to this kinase where the orientation of LY294002 is distinct from p110 $\gamma$  [21]. Under this conformation, it is possible that the bulky matrix disrupts the binding to our

immobilized compound. We were also able to validate the direct binding of VCP to PI828-matrix *in vitro* and docking studies revealed a putative binding mode to the ATP binding pocket. It will be interesting to further evaluate the effect of LY294002 on the activity of this ATPase, since it seems to play a decisive role in the control of the ubiquitin-degradation machinery and protein trafficking [49]. Metabolite kinases were also found to be putative targets of LY294002, including fructosamine 3-kinase, ketosamine 3-kinase, galactokinase, phosphofructokinase and pyridoxal kinase (PXDK). The latter is involved in the metabolism of vitamin B6 and was exclusively found in the HeLa pull-down experiments, which may reflect the reported low expression of PXDK in lymphomas [50]. Interestingly, this protein was found to bind roscovitine, an inhibitor of cyclin-dependant kinases [51]. The potential effect of LY294002 on PXDK activity requires further assessment. The most abundant protein bound to the PI828-matrix was aldehyde dehydrogenase 2 (ALDH2), one of five dehydrogenases identified. Previous chemical proteomic approaches also reported significant binding of dehydrogenase enzymes to inhibitor matrices [28, 29]. For example, Knockaert *et al* showed binding of paullone cyclin-dependant kinase inhibitors to malate dehydrogenase with reduction of enzymatic activity. We carried out homology modeling based on the crystal structure of ALDH bound to NADH, and in the most probable conformation, LY294002 was docked in the NAD-binding pocket, suggesting direct competition with substrate binding (data not shown). A putative hydrogen bond between the backbone of the enzyme and the linker region of PI828 could explain the strong interaction observed in the competition experiments. Another group of identified proteins contained bromodomains (Tables S2 and S3), suggesting that LY294002 (or PI828) may directly target these domains. Bromodomain-containing proteins are a class of proteins capable of interacting with chromatin through acetylated histones [52] and which can act as transcriptional activators [53, 54]. The major Brd protein present in our pull-down was Brd4. This represents an interesting target since it may correlate with a reported inhibitory effect of LY294002 on the regulation of transcription [24].

The goal of this work was the identification of novel targets of LY294002 to further understand its reported off-target effects. Our strategy has enabled the identification of novel targets un-related to PI3Ks whose inhibition could contribute to

the cellular effects observed upon treatment with LY294002. This approach represents a valid tool to establish a global interaction map of compounds of interest. It will be interesting to further evaluate the effect of LY294002 on the activity of some of these proteins, which will provide useful information not only in the context of LY294002, but also for the validation of novel generations of more potent and specific inhibitors. Furthermore, this approach has the potential to be used as a robust high-throughput platform to validate the specificity of new PI3K inhibitors and novel therapeutic agents.

### Acknowledgements

The authors would like to thank Henrik Daub and Klaus Godl for important advice and support in the immobilization of PI828. We are grateful of Yihong Ye for the VCP constructs and Bart Vanhaesebroeck for p110 antibodies. We would also like to thank Barbara Geering, Jaime Millán Martinez and Pedro Cutillas for constructive discussions. This work was funded by the Ludwig Institute for Cancer Research (SG, MW, MZ, JT) and Piramed Ltd (SS, TH, NS).

### References

- 1 Engelman, J. A., Luo, J. and Cantley, L. C. (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat.Rev Genet.* **7**, 606-619
- 2 Carpenter, C. L. and Cantley, L. C. (1996) Phosphoinositide kinases. *Curr.Opin.Cell Biol.* **8**, 153-158
- 3 Vanhaesebroeck, B. and Waterfield, M. D. (1999) Signaling by distinct classes of phosphoinositide 3-kinases. *Exp.Cell Res.* **253**, 239-254
- 4 Pawson, T. and Nash, P. (2003) Assembly of cell regulatory systems through protein interaction domains. *Science* **300**, 445-452
- 5 Rameh, L. E. and Cantley, L. C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol.Chem.* **274**, 8347- 8350
- 6 Drees, B. E. M. G. B., Rommel, C. and Prestwich, G. D. (2004) Therapeutic potential of phosphoinositide 3-kinase inhibitors. *Expert Opin.Ther.Patents* **14**, 703-732
- 7 Rogers, S. J., Box, C., Harrington, K. J., Nutting, C., Rhys-Evans, P. and Eccles, S. A. (2005) The phosphoinositide 3-kinase signalling pathway as a therapeutic target in squamous cell carcinoma of the head and neck. *Expert.Opin.Ther.Targets.* **9**, 769-790

- 8 Kang, S., Bader, A. G. and Vogt, P. K. (2005) Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc.Natl.Acad.Sci.U.S.A* **102**, 802-807
- 9 Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S. M., Riggins, G. J., Willson, J. K., Markowitz, S., Kinzler, K. W., Vogelstein, B. and Velculescu, V. E. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science* **304**, 554
- 10 Cully, M., You, H., Levine, A. J. and Mak, T. W. (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat.Rev Cancer* **6**, 184-192
- 11 Hennessy, B. T., Smith, D. L., Ram, P. T., Lu, Y. and Mills, G. B. (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat.Rev Drug Discov.* **4**, 988-1004
- 12 Stephens, L., Williams, R. and Hawkins, P. (2005) Phosphoinositide 3-kinases as drug targets in cancer. *Curr.Opin.Pharmacol.* **5**, 357-365
- 13 Brachmann, S. M., Yballe, C. M., Innocenti, M., Deane, J. A., Fruman, D. A., Thomas, S. M. and Cantley, L. C. (2005) Role of phosphoinositide 3-kinase regulatory isoforms in development and actin rearrangement. *Mol.Cell Biol.* **25**, 2593-2606
- 14 Okkenhaug, K., Bilancio, A., Farjot, G., Priddle, H., Sancho, S., Peskett, E., Pearce, W., Meek, S. E., Salpekar, A., Waterfield, M. D., Smith, A. J. and Vanhaesebroeck, B. (2002) Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* **297**, 1031-1034
- 15 Foukas, L. C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskett, E., Sancho, S., Smith, A. J., Withers, D. J. and Vanhaesebroeck, B. (2006) Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation. *Nature* **441**, 366-370
- 16 Arcaro, A. and Wymann, M. P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296 (Pt 2)**, 297-301
- 17 Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y. and Matsuda, Y. (1993) Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J Biol.Chem.* **268**, 25846-25856
- 18 Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J.Biol.Chem.* **269**, 5241-5248
- 19 Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., Jr. and Abraham, R. T. (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J* **15**, 5256-5267
- 20 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem.J.* **351**, 95-105

- 21 Jacobs, M. D., Black, J., Futer, O., Swenson, L., Hare, B., Fleming, M. and Saxena, K. (2005) PIM-1 ligand-bound structures reveal the mechanism of serine/threonine kinase inhibition by LY294002. *J.Biol.Chem.*
- 22 Abdul-Ghani, R., Serra, V., Gyorffy, B., Jurchott, K., Solf, A., Dietel, M. and Schafer, R. (2006) The PI3K inhibitor LY294002 blocks drug export from resistant colon carcinoma cells overexpressing MRP1. *Oncogene* **25**, 1743-1752
- 23 Choi, E. K., Park, H. J., Ma, J. S., Lee, H. C., Kang, H. C., Kim, B. G. and Kang, I. C. (2004) LY294002 inhibits monocyte chemoattractant protein-1 expression through a phosphatidylinositol 3-kinase-independent mechanism. *FEBS Lett.* **559**, 141-144
- 24 Kim, Y. H., Choi, K. H., Park, J. W. and Kwon, T. K. (2005) LY294002 inhibits LPS-induced NO production through a inhibition of NF-kappaB activation: independent mechanism of phosphatidylinositol 3-kinase. *Immunol.Lett.* **99**, 45-50
- 25 Poh, T. W. and Pervaiz, S. (2005) LY294002 and LY303511 sensitize tumor cells to drug-induced apoptosis via intracellular hydrogen peroxide production independent of the phosphoinositide 3-kinase-Akt pathway. *Cancer Res.* **65**, 6264-6274
- 26 Yamaguchi, K., Lee, S. H., Kim, J. S., Wimalasena, J., Kitajima, S. and Baek, S. J. (2006) Activating transcription factor 3 and early growth response 1 are the novel targets of LY294002 in a phosphatidylinositol 3-kinase-independent pathway. *Cancer Res.* **66**, 2376-2384
- 27 Tolloczko, B., Turkewitsch, P., Al Chalabi, M. and Martin, J. G. (2004) LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] affects calcium signaling in airway smooth muscle cells independently of phosphoinositide 3-kinase inhibition. *J Pharmacol.Exp.Ther.* **311**, 787-793
- 28 Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M. and Daub, H. (2003) An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc.Natl.Acad.Sci.U.S.A* **100**, 15434-15439
- 29 Knockaert, M., Wieking, K., Schmitt, S., Leost, M., Grant, K. M., Mottram, J. C., Kunick, C. and Meijer, L. (2002) Intracellular Targets of Paullones. Identification following affinity purification on immobilized inhibitor. *J Biol.Chem.* **277**, 25493-25501
- 30 Abbott, B. and Thompson, P. (2003) Synthetic Studies of the Phosphatidylinositol 3-Kinase Inhibitor LY294002 and Related Analogues. *Aust.J.Chem.* **56**, 1099-1106
- 31 Abbott, B. M. and Thompson, P. E. (2004) PDE2 inhibition by the PI3 kinase inhibitor LY294002 and analogues. *Bioorg Med Chem Lett* **14**, 2847-2851
- 32 Gharbi, S., Gaffney, P., Yang, A., Zvelebil, M. J., Cramer, R., Waterfield, M. D. and Timms, J. F. (2002) Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. *Mol.Cell Proteomics* **1**, 91-98
- 33 Ye, Y., Meyer, H. H. and Rapoport, T. A. (2003) Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of

- nonubiquitinated polypeptide segments and polyubiquitin chains. *J Cell Biol.* **162**, 71-84
- 34 Meyer, H. H., Shorter, J. G., Seemann, J., Pappin, D. and Warren, G. (2000) A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J* **19**, 2181-2192
- 35 Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P. and Williams, R. L. (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Mol.Cell* **6**, 909-919
- 36 Walker, E. H., Perisic, O., Ried, C., Stephens, L. and Williams, R. L. (1999) Structural insights into phosphoinositide 3-kinase catalysis and signalling. *Nature* **402**, 313-320
- 37 Pirola, L., Zvelebil, M. J., Bulgarelli-Leva, G., Van Obberghen, E., Waterfield, M. D. and Wymann, M. P. (2001) Activation loop sequences confer substrate specificity to phosphoinositide 3-kinase alpha (PI3Kalpha). Functions of lipid kinase-deficient PI3Kalpha in signaling. *J Biol.Chem.* **276**, 21544-21554
- 38 Bilancio, A., Okkenhaug, K., Camps, M., Emery, J. L., Ruckle, T., Rommel, C. and Vanhaesebroeck, B. (2006) Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood* **107**, 642-650
- 39 Kihara, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2001) Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep.* **2**, 330-335
- 40 Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J. and Balla, T. (1996) Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry* **35**, 3587-3594
- 41 Meyers, R. and Cantley, L. C. (1997) Cloning and characterization of a wortmannin-sensitive human phosphatidylinositol 4-kinase. *J Biol.Chem* **272**, 4384-4390
- 42 Fan, Q. W., Knight, Z. A., Goldenberg, D. D., Yu, W., Mostov, K. E., Stokoe, D., Shokat, K. M. and Weiss, W. A. (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* **9**, 341-349
- 43 Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W. A., Williams, R. L. and Shokat, K. M. (2006) A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* **125**, 733-747
- 44 Ding, J., Vlahos, C. J., Liu, R., Brown, R. F. and Badwey, J. A. (1995) Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. *J Biol.Chem* **270**, 11684-11691
- 45 Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D. and Seldin, D. C. (2001) Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**, 3247-3257
- 46 Doble, B. W. and Woodgett, J. R. (2003) GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci.* **116**, 1175-1186

- 47 Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789
- 48 Abbott, B. M. and Thompson, P. E. (2004) PDE2 inhibition by the PI3 kinase inhibitor LY294002 and analogues. *Bioorg.Med.Chem Lett.* **14**, 2847-2851
- 49 Halawani, D. and Latterich, M. (2006) p97: The cell's molecular purgatory? *Mol.Cell* **22**, 713-717
- 50 Kang, J. H., Hong, M. L., Kim, D. W., Park, J., Kang, T. C., Won, M. H., Baek, N. I., Moon, B. J., Choi, S. Y. and Kwon, O. S. (2004) Genomic organization, tissue distribution and deletion mutation of human pyridoxine 5'-phosphate oxidase. *Eur J Biochem* **271**, 2452-2461
- 51 Bach, S., Knockaert, M., Reinhardt, J., Lozach, O., Schmitt, S., Baratte, B., Koken, M., Coburn, S. P., Tang, L., Jiang, T., Liang, D. C., Galons, H., Dierick, J. F., Pinna, L. A., Meggio, F., Totzke, F., Schachtele, C., Lerman, A. S., Carnero, A., Wan, Y., Gray, N. and Meijer, L. (2005) Roscovitine targets, protein kinases and pyridoxal kinase. *J Biol.Chem* **280**, 31208-31219
- 52 Dey, A., Chitsaz, F., Abbasi, A., Misteli, T. and Ozato, K. (2003) The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc.Natl.Acad.Sci.U.S.A* **100**, 8758-8763
- 53 Farina, A., Hattori, M., Qin, J., Nakatani, Y., Minato, N. and Ozato, K. (2004) Bromodomain protein Brd4 binds to GTPase-activating SPA-1, modulating its activity and subcellular localization. *Mol.Cell Biol.* **24**, 9059-9069
- 54 Sinha, A., Faller, D. V. and Denis, G. V. (2005) Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A1. *Biochem.J.* **387**, 257-269

## Figure Legends

**Table 1: LY294002 is a potent inhibitor of CK2.** In-vitro lipid kinase assays were carried out with both compounds (LY294002 and PI828) for casein kinase 2 (CK2). Inhibitor concentrations for 50% inhibition ( $IC_{50}$ ) were established with recombinant CK2.

**Figure 1: Synthesis of the LY294002 derivative and evaluation of binding to class I PI3Ks.** (A) Synthesis of PI828 (4) and the immobilized derivative, PI828-matrix (5) starting from the 8-Bromochromenone derivative (3). (B) Putative binding mode of PI828 in p110 $\beta$  as assessed by Molecular Modelling (using GOLD). Green-dotted lines represent putative hydrogen bonds. Residues D862 and E858 are specific for p110 $\beta$ . (C) Efficient binding to the PI828-matrix from recombinant enzyme and total cellular extract. Binding assays with recombinant p110 $\alpha$ -GST fusion protein or from a total cellular extract of WEHI 231 cells. Pull-downs in 10  $\mu$ l of beads slurry were carried out in high

salt with increased concentration of recombinant enzyme or total extract. Levels of p110 $\alpha$  and p110 $\delta$  were evaluated by western blotting with specific antibodies.

**Figure 2: Identification of cellular targets of LY294002.** A total cellular extract from WEHI231 cells grown in 10% FCS (15 mg) was incubated in batch with the PI828-matrix or the control saturated EAS devoid of the compound. Specific binding was validated by competition assay with 1 mM of LY294002 or PI828. (A) Bound material was eluted in 2x Laemmli sample buffer separated on a 1D-PAGE and proteins were detected by colloidal coomassie staining. The entire lanes were dissected and proteins identified by LC-MS/MS. Identified proteins are reported on the gel image (B) In parallel, samples were analyzed by western blotting with specific antibodies to p110 $\alpha$ , p110 $\delta$ , mTOR, ALDH1, Brd4, GSK3 $\beta$  and CK2 $\alpha$ .

**Figure 3: (A) LY294002-interacting proteins and their cellular functions. (B) Identified proteins share different substrate specificity**

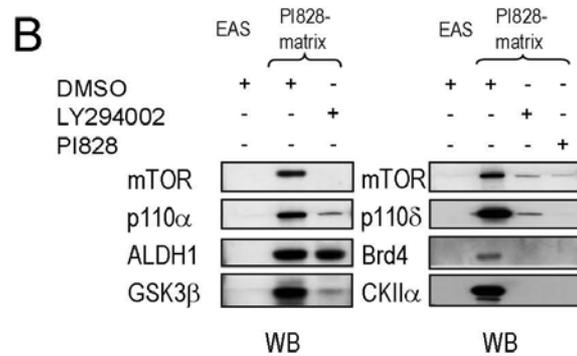
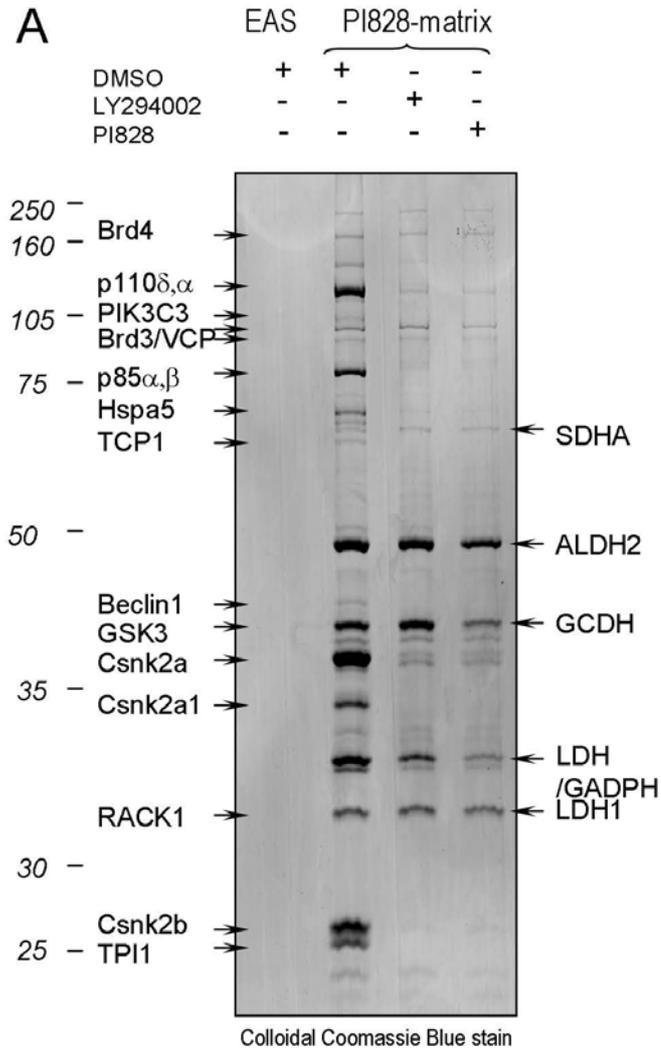
**Figure 4: Evaluation of relative binding affinities by competitive elution with LY294002, PI103 and LY303511.** Bound material in low or high salt was eluted with increased concentration of inhibitor in 10 mM ATP and 20 mM MgCl<sub>2</sub>. Due to its higher potency for the class I PI3Ks, PI103 was used at lower concentration than LY294002 and LY303511. A final elution step was carried out in 2x Laemmli sample buffer. Each eluted fractions were separated by 1D-PAGE. The elution pattern was observed by colloidal coomassie blue staining (A) and in parallel by western blotting with specific antibodies (B).

**Figure 5: The AAA ATPases p97/VCP is able to bind directly PI828-matrix.** (A) In-vitro binding experiment was carried out with recombinant VCP (WT: wild type; AA: mutant defective in ATP hydrolysis). Binding was carried out in various concentrations of inhibitor and ATP and bound proteins were eluted in 2x Laemmli sample buffer. Proteins were visualized by western blotting with anti-VCP antibodies. (B) PI828 was docked in the ATP binding pocket of VCP.

**Table 1: LY294002 is a potent inhibitor of CK2.** In-vitro lipid kinase assays were carried out with both compounds (LY294002 and PI828) for casein kinase 2 (CK2). Inhibitor concentrations for 50% inhibition ( $IC_{50}$ ) were established with recombinant CK2.

Corporate ID	Compound	Mean $IC_{50}$ ( $\mu$ M)				
		p110 $\alpha$	CK2(h)	CK2 $\alpha$ 2(h)	cSRC(h)	Yes(h)
PI000828	PI828	0.173	0.149	1.127	>10,000	>10,000
PI001395	LY294002	0.186	0.098	3.869	>10,000	>10,000





**Fig.2**

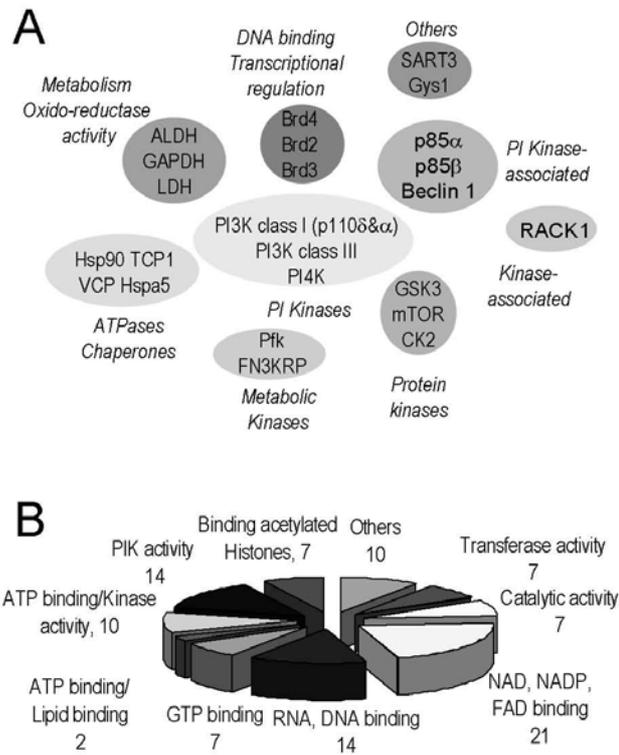


Fig.3

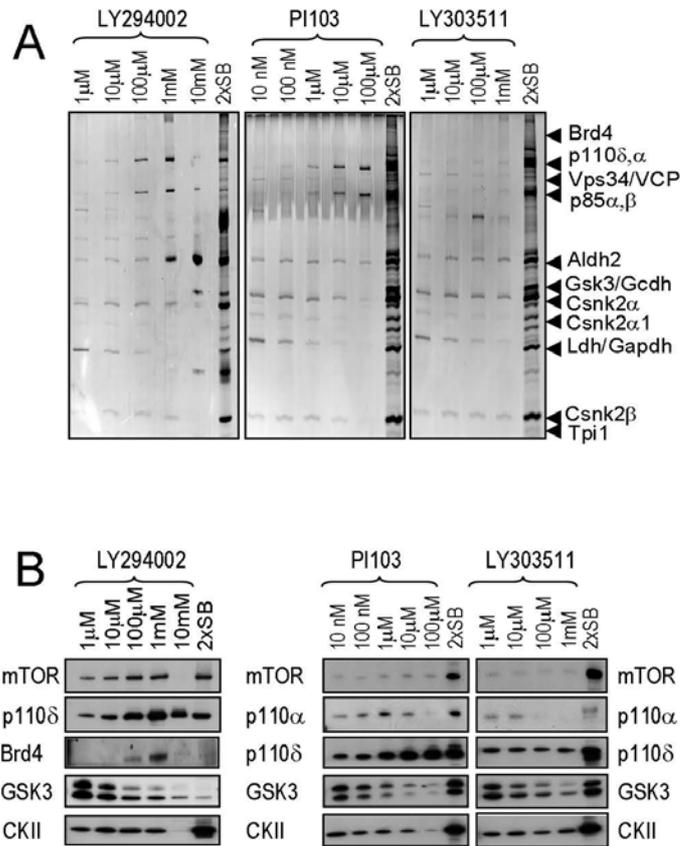


Fig.4

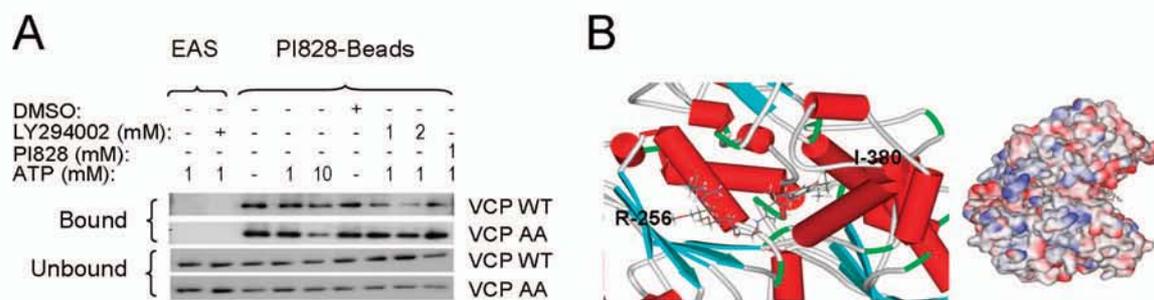


Fig.5