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Exploring the specificity of the PI3K family inhibitor LY294002

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The PI3Ks (phosphatidylinositol 3-kinases) regulate cellular signalling 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 the physiological function of PI3K 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 one-dimensional gel electrophoresis and identified by liquid chromatography–tandem MS. The present 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.

Key words: Brd4, chemical proteomic strategy, LY294002, phosphatidylinositol 3-kinase (PI3K), valosin-containing protein (VCP).

INTRODUCTION

PI3Ks (phosphatidylinositol 3-kinases) 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 their critical role in the life, death, growth, proliferation, metabolism and in differentiated cell function has been reviewed recently [1]. The family is made up of 14 enzymes that can be separated into four classes, of which classes I, II and 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, PIP3 [phosphatidylinositol (3,4,5) triphosphate]. 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 (phosphoinositide-dependent kinase 1) and Akt, for example regulate a multitude of targets [5]. Owing 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 p110α [8,9], and loss of or reduced activity of the tumour suppressor PIP3 phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) [10], are reported during tumorigenesis. Many other genes in the PI3K pathway are also tumour 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 elucidate further 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 Wm (Wortmannin) [16,17], a naturally occurring metabolite of *Penicillium funiculosum*, and LY294002 [18], which was derived from the flavonoid quercetin (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/404/bj4040015add.htm). 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 not only mTOR (mammalian target of rapamycin) and DNA-PK (DNA-dependent protein kinase) [19], but also other protein kinases, such as CK2 (casein kinase 2) 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^{2+} signalling [27] and transcription factors such as NF-κB (nuclear factor κB) [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 modelling analysis or *in vitro* activity assays. Previous reports have revealed a new approach to assess drug specificity by direct immobilization

Abbreviations used: AA ATP hydrolysis mutant form of p97/VCP; ALDH, aldehyde dehydrogenase; Brd, bromodomain; CK2, casein kinase 2; DMF, dimethylformamide; EAS, epoxy-activated Sepharose; FCS, fetal calf serum; GSK3, glycogen synthase kinase 3; LC–ESI–MS/MS, liquid chromatography–electrospray–ionization tandem MS; mTOR, mammalian target of rapamycin; PIP3, phosphatidylinositol (3,4,5) triphosphate; PI3K, phosphatidylinositol 3-kinase; PI3KC3, PI3K, class 3; PXDK, pyridoxal kinase; VCP, valosin-containing protein; Wm, Wortmannin; WT, wild-type.

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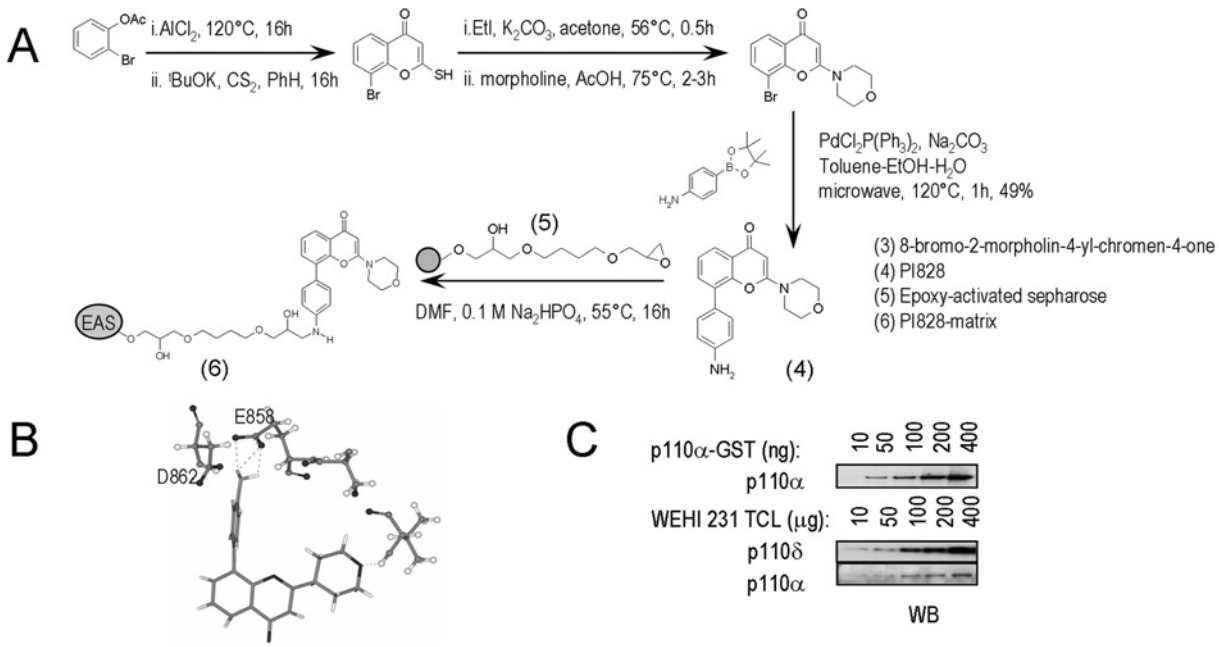


Figure 1 Synthesis of the LY294002 derivative and evaluation of binding to class I PI3Ks

(**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 to p110β as assessed by Molecular Modelling (using GOLD). Dotted lines represent putative hydrogen bonds. Residues Asp^{862} and Glu^{858} are specific for p110β. (**C**) Efficient binding to the PI828-matrix from recombinant enzyme and total cellular extract. Binding assays with recombinant p110α–GST (glutathione S-transferase) fusion protein or from a total cellular extract of WEHI231 cells. Pull-downs in 10 μl of bead slurry were carried out in high salt with increased concentration of recombinant enzyme or total extract. Levels of p110α and p110δ were evaluated by Western blotting with specific antibodies.

of small molecule inhibitors on to a solid phase and subsequent identification of bound proteins using optimized proteomic methods [28,29]. In the present study, we used an LY294002-derived matrix to isolate and identify its direct molecular targets and to understand further reported off-target effects of this compound.

MATERIALS AND METHODS

Reagents

Tissue culture media and FCS (fetal calf serum) were from Gibco (Invitrogen). Antibodies against p110α and p110δ were a kind gift from Dr Bart Vanhaesebroeck (Ludwig Institute of Cancer Research, London, U.K.). Antibodies against p85 and VCP (valosin-containing protein) were from Santa Cruz Biotechnology. The antibody against ALDH (aldehyde dehydrogenase) was from BD Transduction Laboratories. The antibody against mTOR was from Cell Signaling Technology. LY294002 and LY303511 were from Merck Biosciences. 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, unless otherwise stated.

Synthesis of PI828

The precursor to LY294002 and PI828, 8-bromo-2-morpholin-4-yl-chromen-4-one, was prepared as described previously [30]. To prepare PI828 (Figure 1A) [31], a suspension of 161 mM 8-bromo-2-morpholin-4-yl-chromen-4-one, 177 mM 4-(4,4,5,5-tetramethyldioxaborolan-2-yl)-phenylamine, 484 mM sodium carbonate in toluene/ethanol was flushed with argon. Dichlorobistriphenylphosphine palladium (II) (8 mM) 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 (water saturated with NaCl), separated and dried over $MgSO_4$. The crude product was evaporated on to silica and purified by flash column chromatography [5:95–10:90 % (v/v) methanol/dichloromethane] to give a yellow solid which was further purified by trituration in ether/ethyl acetate (1:1, v/v) to furnish the title compound as a pale yellow solid (177 mg; 49 % recovered yield as a percentage of the theoretical yield).

Production of immobilized PI828

EAS (epoxy-activated Sepharose 6B) beads (1 g; GE Healthcare) were incubated overnight at 55 °C with 2 vol. of 20 mM PI828 in 50 % (v/v) DMF (dimethylformamide)/0.1 M sodium phosphate buffer (pH 6.8), with constant shaking in the dark. The resin was then washed in 2 vol. of 50 % (v/v) DMF/0.1 M Na_2CO_3 and incubated for 16 h at 40 °C in the dark with 2 vol. of 1 M ethanolamine. Further washes were performed as follows: 3 × 50 % DMF/0.1M Na_2CO_3; 1 × 0.1 M $NaHCO_3$/0.5 M NaCl; 1 × 0.1 M sodium acetate (pH 4.0)/0.1 M NaCl; 1× H_2O; 1 × 20 % (v/v) ethanol. The lilac-coloured resin was stored in 20 % (v/v) ethanol 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 (24 °C) and was terminated by addition of PBS. IC_{50} values were subsequently determined using

a sigmoidal dose–response curve fit (variable slope). CK2 and GSK3β (glycogen synthase kinase 3β) inhibition was established by kinase selectivity screening. Inhibitor (10 μM; PI828 and LY294002) was tested against the Upstate panel of kinases in 10 μM ATP (Upstate Kinase Profiler^{TM} version 7.0, www.upstate.com).

Cell lysis and affinity purification experiments

HeLa cells (2.5×10^9 cells; Cilbiotech, Belgium) were lysed in 30ml of lysis buffer A [20 mM Hepes, pH 7.5, 150 mM NaCl, 0.25 % Triton X-100, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol supplemented with protease and phosphatase inhibitors (100 μg/ml AEBSF, 17 μg/ml aprotinin, 5 μM BpVphen, 5 μM fenvalerate, 1 μg/ml leupeptin, 1 μM okadaic acid, 1 μg/ml pepstatin and 2 mM sodium orthovanadate)]. WEHI231 cells were grown in suspension in RPMI 1640 medium supplemented with 10 % (v/v) FCS, 2 mM L-glutamine, 50 μM 2-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 containing 10 mM ATP and 20 mM $MgCl_2$. Affinity complexes were washed extensively in lysis buffer A adjusted to 1M NaCl and then in lysis buffer A. Complete elution was achieved in 2× Laemmli sample buffer [50 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.1 % Bromophenol Blue and 2 % (v/v) 2-mercaptoethanol] with boiling. Specific elutions were also carried out with free inhibitor (LY294002, PI828, PI103 or LY303511) in lysis buffer A containing 10 mM ATP and 20 mM $MgCl_2$.

Protein separation and MS

Eluted proteins were separated by one-dimensional gel electrophoresis and one-tenth of the sample was kept for Western blotting. 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 described previously [32]. Each sample (half of the total peptide mixture) was analysed by LC–ESI–MS/MS (liquid chromatography–electrospray ionization–tandem MS) using an Ultimate nano HPLC (Dionex Ltd) with a PepMap C18 75 μm inner diameter column (Dionex) at a flow rate of 300 nl/min, linked on-line to a QTof-I mass spectrometer (Waters). Processed mass spectra (MassLynx, Waters) were searched against the updated IPI (international protein index) human (HeLa) or mouse (WEHI 231) databases using Mascot version 2.0.02 (Matrix Science). Identifications were accepted when a minimum of two individual peptide masses matched a protein (mass error of $\pm$ 100 milli-mass units, 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 WT (wild-type) and AA (ATP hydrolysis mutant) p97/VCP constructs were a kind gift from Dr Y. Ye (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health, Bethesda, MD, U.S.A.) [33]. Full-length mouse His-tagged p97/VCP was cloned into a pQE9 vector (Qiagen) [33,34] and expressed in *Escherichia coli* following induction with 0.5 mM IPTG (isopropyl β-D-thiogalactoside). Recombinant proteins were purified as described previously [34]. WT and AA VCP (2 μg each) were incubated with 10 μl of bead slurry and pull-downs were carried out as described for the large-scale experiments.

Docking studies

The crystal structure of p110γ [35,36] was used for homology modelling and inhibitor docking studies, although only the catalytic region of the protein was utilized (residues 725–1092). 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_5 of ATP from which a 10 Å (1 Å = 0.1 nm) pocket was defined. As a test of accuracy, the docked structure of PI828 was compared with the crystallographically determined structure [35]. The docked and structural co-ordinates and orientation of PI828 were in very good agreement. A further docking study of LY294002 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.

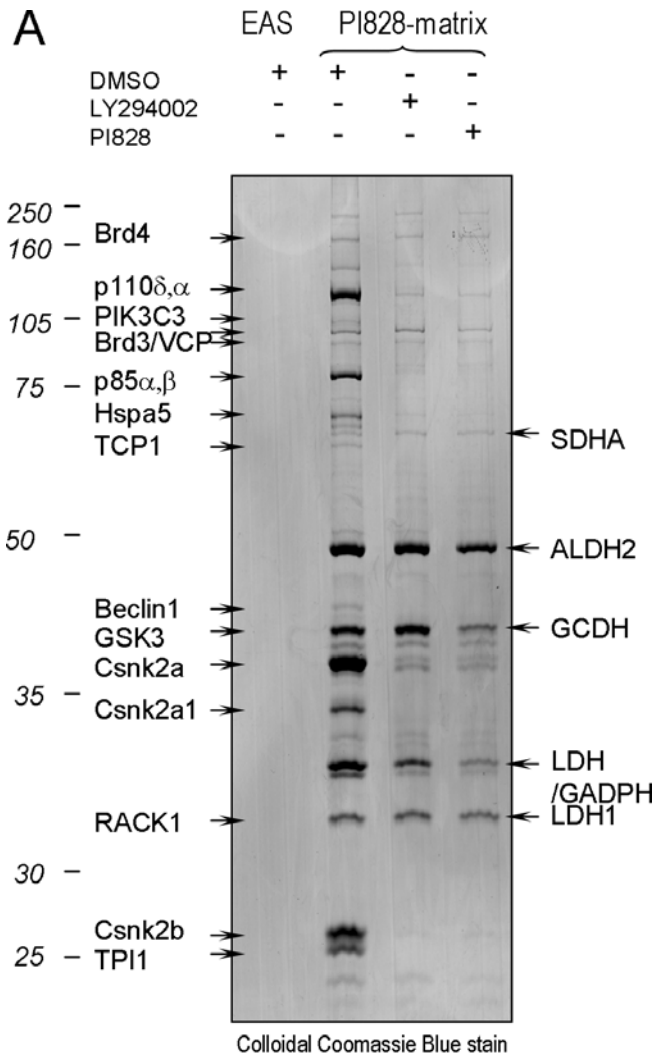
RESULTS

Design and immobilization of PI828; validation of the binding assay

An analogue of LY294002 was designed following consideration of its binding mode to p110γ, as determined by X-ray crystallography [35]. The crystal structure indicated that the morpholine oxygen formed a hydrogen bond with the hinge region residue Val^{882}, with a further putative interaction existing between the carbonyl oxygen and Lys^{833}. Significantly, the 4-position hydrogen of the exocyclic phenyl substituent, which occupies the ribose-binding pocket, was seen to extend out towards the solvent. On the basis of this structural information, we chose to incorporate an amine linker at this position to use as a site of attachment to the resin (Figure 1A; see also the synthesis and immobilization section of Supplemental material at http://www.BiochemJ.org/bj/404/bj4040015add.htm). The precursor to the immobilized ligand, PI828, showed encouraging biochemical inhibition of class I PI3Ks (see Supplementary Table S1 at http://www.BiochemJ.org/bj/404/bj4040015add.htm). Indeed, PI828 was more potent than LY294002, particularly against p110β. From our modelling study, we postulated that this increase in potency may arise from hydrogen-bonding of the PI828 amine protons with Glu^{858} and Asp^{862} in the catalytic site (Figure 1B), residues which are unique among all the PI3K class I isoforms (M. J. Zvelebil, S. J. Shuttleworth and M. D. Waterfield, unpublished work). PI828 was immobilized on to EAS beads using a procedure similar to a method developed previously [28]. Binding of PI3Ks was first examined using recombinant p110α and then from WEHI231 cells, and PI3K catalytic subunits were detected by Western blotting (Figure 1C). Affinity binding conditions were mainly based on previously published work [28] and no binding to blocked EAS without immobilized inhibitor was observed (Figure 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; see Supplementary Table S2 at http://www.BiochemJ.org/bj/404/bj4040015add.htm) and in the mouse lymphoma B-cell line WEHI231 (see Supplementary Table S3 at http://www.BiochemJ.org/bj/404/bj4040015add.htm), which expresses high levels of p110δ [38]. In the first instance, proteins were eluted by Laemmli sample



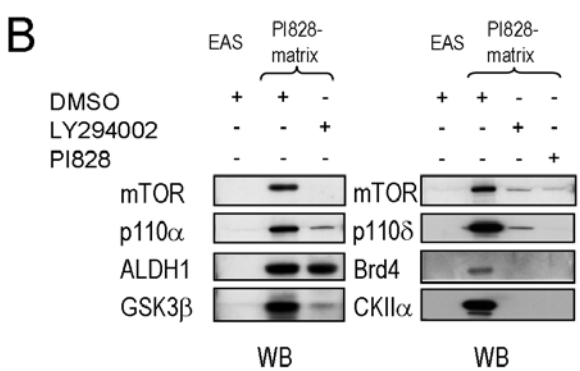
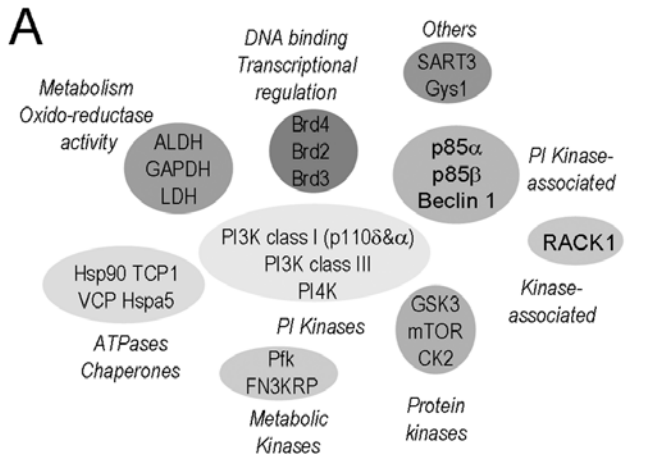


Figure 2 Identification of cellular targets of LY294002

Total cellular extracts (15 mg) from WEHI231 cells grown in 10% (v/v) FCS were incubated in batches 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 by 2× Laemmli sample buffer, then separated on a one-dimensional PAGE and proteins were detected by colloidal Coomassie Blue staining. The entire lanes were dissected and proteins identified by LC–ESI–MS/MS. Identified proteins are reported on the gel image. (**B**) In parallel, samples were analysed by Western blotting with specific antibodies against p110α, p110δ, mTOR, ALDH1, Brd4, GSK3β and CK2α.



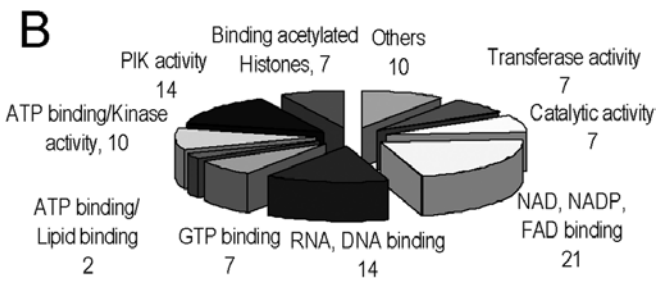


Figure 3 LY294002-interacting proteins and their cellular functions

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

buffer to ensure complete elution from the 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 the different protein expression patterns of the two cell types (Figure 2A and Supplementary Tables S2 and S3). PI3K catalytic subunits were highly represented in the bound fraction, and the most abundant isoform was p110δ. The p85 regulatory subunits were also present, which reflects the strong interaction between these two subunits. PI3KC3 (PI3K, class 3, also known as Vps34), was also identified together with Beclin-1, a protein known to interact with PI3KC3 [39]. No PI3K-related kinases were identified by MS, which may be attributed to their low copy numbers and high molecular masses, although mTOR was detected by immunoblotting (Figure 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 (Figure 3 and see below).

Competitive binding with an excess of free inhibitor (1 mM LY294002 or PI828) was carried out to validate binding specificities (Figure 2). As well as the PI3K isoforms, other previously reported targets of LY294002, such as mTOR, CK2 and GSK3β, showed reduced binding in the presence of excess free inhibitor (Figure 2B). On the other hand, bands corresponding to the family of dehydrogenases showed higher affinity for the PI828-matrix (Figure 2). Sequential competitive elution experiments with

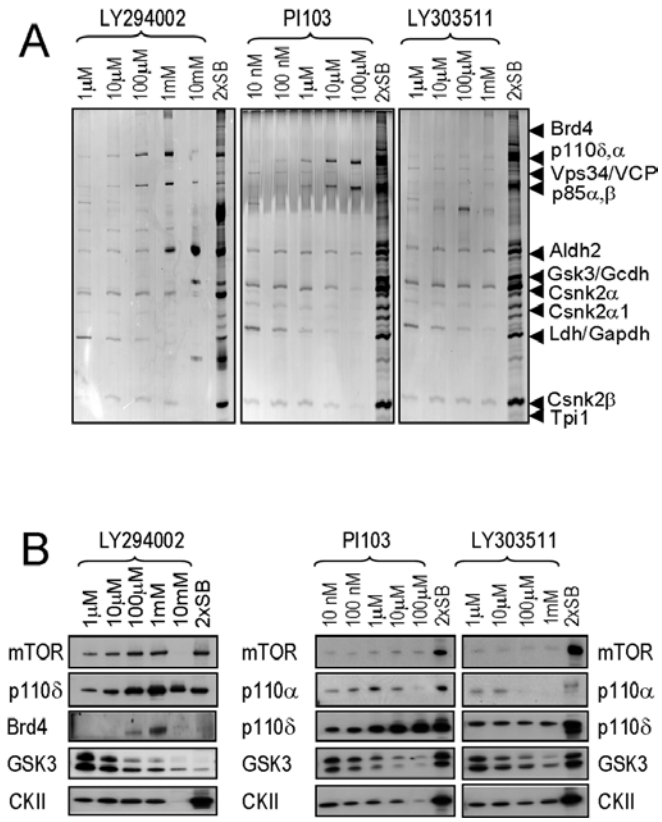


Figure 4 Evaluation of relative binding affinities by competitive elution by LY294002, PI103 and LY303511

Bound material in low or high salt was eluted by increased concentration of inhibitor in 10 mM ATP and 20 mM $MgCl_2$. Owing 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 by 2× Laemmli sample buffer. Each eluted fraction was separated by one dimensional-PAGE. The elution pattern was observed by colloidal Coomassie Blue staining (**A**) and in parallel by Western blotting with specific antibodies (**B**).

increasing concentrations of LY294002 (Figure 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 (Figure 4). We next used this affinity platform to assess binding profiles in competing concentrations of PI103 and LY303511 (Figure 4). PI103 is a potent inhibitor of p110α at low nanomolar concentration, as reported recently [42,43], and LY303515 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 p110δ than LY294002 or PI103. PI103 was unable to efficiently elute mTOR in our competition assay, although it can inhibit its activity (Figure 4) [42]. Both CK2 and GSK3β 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. 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 (results not shown).

Table 1 LY294002 is a potent inhibitor of CK2

In vitro lipid kinase assays were carried out with both compounds (LY294002 and PI828) for CK2. Inhibitor concentrations for 50 % inhibition (IC_{50}) were established with recombinant CK2. h, human.

		Mean IC_{50} (μM)				
Corporate ID	Compound	p110α	CK2(h)	CK2α2(h)	cSRC(h)	Yes(h)
PI000828	PI828	0.173	0.149	1.127	> 10000	> 10000
PI001395	LY294002	0.186	0.098	3.869	> 10000	> 10000

Notably, both catalytic and regulatory subunits of CK2 were recovered. The binding was also shown to be selective (Figures 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 kinases GSK3α and β (see Supplementary Table S4 at http://www.BiochemJ.org/bj/404/bj4040015add.htm). Another PI3K unrelated target was the p97/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 WT p97/VCP, and a mutant defective in ATP binding (K251A, K524A) to the PI828-matrix (Figure 5A) [33]. This validated the direct interaction of VCP with the PI828-matrix, but also showed that these critical lysine residues 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 (Figure 5A). Homology modelling 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 (Figure 5B). In the LY294002-bound form, one putative hydrogen bond was observed with Ile^{380} and also between the linker and Arg^{256}. 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 in the present study 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 (Figure 3). The major non-lipid kinases identified in the present study have been reported previously (CK2, mTOR, GSK3β) [20,21], which emphasizes the thoroughness of this assay. It is likely that the PI3K-independent cellular effects observed with LY294002 are in fact due to the 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 signalling 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 signalling [27]. Additionally, GSK3 is involved in the regulation of glycogen metabolism through the inactivation of glycogen synthase and it also has an important role in regulating many signalling 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

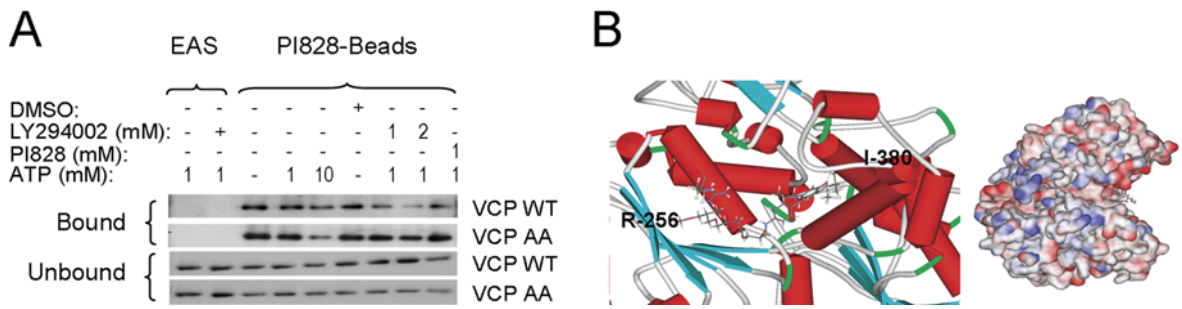


Figure 5 AAA ATPase p97/VCP is able to bind PI828-matrix directly

(**A**) An *in vitro* binding experiment was carried out with recombinant WT or AA mutant VCP. Binding was carried out in various concentrations of inhibitor and ATP and bound proteins were eluted by 2× Laemmli sample buffer. Proteins were visualized by Western blotting with anti-VCP antibodies. (**B**) PI828 was docked in the ATP-binding pocket of VCP.

important to note the direct effect of LY294002 and other PI3K-specific inhibitors on this enzyme in future studies. Other targets have also been reported previously to be directly inhibited by LY294002, such as the cAMP PDE2 (phosphodiesterase 2; see Supplementary Tables S2 and S3) [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 p110γ [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 the PI828-matrix *in vitro* and docking studies revealed a putative binding mode to the ATP-binding pocket. It will be interesting to evaluate further 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 PXDK (pyridoxal kinase). 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-dependent kinases [51]. The potential effect of LY294002 on PXDK activity requires further assessment. The most abundant protein bound to the PI828-matrix was 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. [29] showed binding of paullone cyclin-dependent kinase inhibitors to malate dehydrogenase with a reduction of enzymatic activity. We carried out homology modelling 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 (results 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 Brds (bromodomains; see Supplementary Tables S2 and S3), suggesting that LY294002 (or PI828) may directly target these domains. Brd-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 understand further its reported off-target effects. Our strategy has enabled the identification of novel targets unrelated 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 evaluate further 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.

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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. et al. (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. and Waterfield, M. D. et al. (2002) Impaired B and T cell antigen receptor signaling in p110δ PI3-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 p110α 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**, 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, Jr, J. C. 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. **280**, 13728–13734
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-κB 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 α (PI3Kα): functions of lipid kinase-deficient PI3Kα 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 p110δ isoform of PI3K in B-cell antigen and IL-4 receptor signaling: comparative analysis of genetic and pharmacologic interference with p110δ 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. et al. (2006) A pharmacological map of the PI3-K family defines a role for p110α 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. et al. (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

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