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Ayaz Najafov, Eeva M Sommer, Jeffrey M Axten, M. Phillip Deyoung, Dario R Alessi. Characterisation of GSK2334470, a novel and highly specific inhibitor of PDK1. *Biochemical Journal*, 2010, 433 (2), pp.357-369. 10.1042/BJ20101732 . hal-00549899

**HAL Id: hal-00549899**

**<https://hal.science/hal-00549899>**

Submitted on 23 Dec 2010

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## **Characterisation of GSK2334470, a novel and highly specific inhibitor of PDK1**

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Keywords: Kinase inhibitor, cancer, PI3K, SGK, RSK, Akt/Akt1 and S6K.  
Running title: Novel small molecule PDK1 inhibitor.

Accepted Manuscript

**Abstract.** Phosphoinositide-dependent protein kinase-1 (PDK1) activates a group of protein kinases belonging to the AGC-kinase family that play important roles in mediating diverse biological processes. Many cancer-driving mutations induce activation of PDK1 targets including Akt, S6K and SGK. Here we describe the small molecule GSK2334470, which inhibits PDK1 with an  $IC_{50}$  of  $\sim 10$  nM, but does not suppress the activity of 93 other protein kinases including 13 AGC-kinases most related to PDK1 at 500-fold higher concentrations. Addition of GSK2334470 to HEK293, U87 or fibroblast cells ablated T-loop residue phosphorylation and activation of SGK isoforms and S6K1 induced by serum or IGF1. GSK2334470 also inhibited T-loop phosphorylation and activation of Akt, but was more efficient at inhibiting Akt in response to stimuli such as serum that activated the PI 3-kinase pathway weakly. GSK2334470 inhibited activation of an Akt1 mutant lacking the PH domain more potently than full length Akt1, suggesting GSK2334470 is more effective at inhibiting PDK1 substrates that are activated in the cytosol rather than at the plasma membrane. Consistent with this, GSK2334470 inhibited Akt activation in knock-in embryonic stem cells, expressing a mutant of PDK1 that is unable to interact with phosphoinositides, more potently than in wild type cells. GSK2334470 also suppressed T-loop phosphorylation and activation of RSK2, another PDK1 target activated by the ERK pathway. However, prolonged treatment of cells with inhibitor was required to observe inhibition of RSK2, indicating that PDK1 substrates possess distinct T-loop dephosphorylation kinetics. Our data define how PDK1 inhibitors affect AGC signalling pathways and suggest that GSK2334470 will be a useful tool for delineating roles of PDK1 in biological processes.

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20101732

## Introduction

3-Phosphoinositide dependent protein kinase-1 (PDK1) plays an important role in growth factor signalling cascades by phosphorylating and activating a group of protein kinases belonging to the AGC-kinase family (cAMP-dependent, cGMP-dependent, and PKC) [1, 2]. These enzymes co-ordinately regulate the cellular machinery controlling protein synthesis, metabolism, survival and proliferation. Kinases activated by PDK1 include isoforms of Akt [3], the p70 ribosomal S6 kinase (S6K1) [4], the serum and glucocorticoid induced protein-kinase (SGK) [5], the p90 ribosomal S6 kinase (RSK) [6], and protein kinase C (PKC) [7]. The significance of the PDK1 pathway in pathological conditions is highlighted by the findings that the majority of human tumours have mutations in genes such as PTEN resulting in over-activation of PDK1 targets that promote proliferation and growth of tumour cells [2]. PDK1 is also frequently overexpressed in a variety of tumours including breast cancer [8, 9]. Reduction of PDK1 expression protects mice from developing tumours resulting from the loss of the PTEN tumour suppressor [10]. These observations indicate that PDK1 inhibitors might have therapeutic utility for the treatment of cancer, a hypothesis that has been difficult to evaluate due to the lack of specific PDK1 inhibitors. Recent work has also suggested that inhibitors of PDK1 might have other benefits such as counteracting resistance of cancer cells to drugs such as tamoxifen [11, 12]. A number of PDK1 inhibitors such as UCN-01 [13, 14], BX-795 [15] and celecoxib derivatives [16] have been described to date, that are poorly specific and/or ineffective at inhibiting PDK1 dependent pathways *in vivo*.

PDK1 activates 23 AGC kinases by phosphorylating a specific Thr or Ser residue located within the T-loop of the kinase domain [1]. Maximal activation also necessitates phosphorylation of a Ser/Thr residue located C-terminal to the catalytic domain, within a region known as the hydrophobic motif. Recent work has established that the mammalian target of rapamycin (mTOR) complex-1 (mTORC1) phosphorylates the hydrophobic motif of S6K1 whilst a distinct mTORC2 complex phosphorylates the hydrophobic motif of Akt and SGK isoforms [17, 18]. In the case of RSK, a second kinase domain, located C-terminal to the AGC catalytic domain is activated by the ERK1/ERK2 pathway, phosphorylates the hydrophobic motif [19].

Agonists induce activation of AGC kinases by diverse mechanisms. In the case of S6K, SGK and RSK isoforms, which are activated in the cytosol, stimuli induce the phosphorylation of hydrophobic motif by activating hydrophobic motif kinases. This phosphorylation promotes interaction, phosphorylation and activation by PDK1 [1, 20]. Activation of Akt occurs at the plasma membrane and necessitates prior activation of the phosphoinositide 3-kinase (PI-3-kinase) and generation of phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P<sub>3</sub>). PtdIns(3,4,5)P<sub>3</sub> binds to the Pleckstrin Homology (PH) domain of Akt not only recruiting it to the cell membrane but also inducing a conformational change that enables PDK1 to phosphorylate the T-loop residue of Akt (Thr308) [21-24]. PDK1 also contains a PH domain that binds with high affinity to PtdIns(3,4,5)P<sub>3</sub>, PtdIns(3,4)P<sub>2</sub> and more weakly to PtdIns(4,5)P<sub>2</sub> [25, 26]. The binding of PDK1 to phosphoinositides does not affect the catalytic activity, but functions to co-localise PDK1 and Akt at the plasma membrane thereby promoting Akt phosphorylation [27].

In this paper we report on the small molecule GSK2334470, that we establish is a highly specific and potent inhibitor of PDK1. We demonstrate that GSK2334470 can be employed in cells to ablate T-loop phosphorylation and activation of SGK, S6K1 and RSK as well also suppressing the activation of Akt. Our data indicate that GSK2334470 will be useful in probing biological processes controlled by PDK1.

### Materials and methods.

**Materials.** GSK2334470 was generated by GlaxoSmithKline [28] and detailed synthesis will be described elsewhere. GlaxoSmithKline will make GSK2334470 available for purchase from Sigma-Aldrich and/or Tocris in the near future. Protein G-Sepharose and glutathione-Sepharose were purchased from Amersham Bioscience.  $^{32}\text{P}$   $\gamma$ -ATP was from Perkin-Elmer. IGF1 was from Cell Signaling technology. DMSO, Phorbol-12-Myristate-13-Acetate (PMA) and Tween-20 were from Sigma. CHAPS was from Calbiochem. PI-103 and GDC-0941 were synthesized by Dr Natalia Shpiro at the MRC Protein Phosphorylation Unit, University of Dundee. Recombinant full length PDK1 was expressed in insect cells [29]. GST-Akt1 and GST- $\Delta$ PH-Akt1 were purified from HEK293 cells treated with 1  $\mu\text{M}$  PI-103 PI 3-kinase inhibitor for 30 min as described previously [22]. Plasmids encoding SGK isoforms were described previously [30, 31]. Littermate wild type PDK1 and homozygous PDK1<sup>K465E/K465E</sup> mouse embryonic stem (ES) cells were cultured as described previously [27].

**Antibodies.** The following antibodies were raised in sheep and affinity purified on the indicated antigen: anti-Akt1 (S695B, 3<sup>rd</sup> bleed; raised against residues 466-480 of human Akt1 RPHFPQFSYSASGTA, used for immunoblotting and immunoprecipitation), anti-S6K (S417B, 2<sup>nd</sup> bleed; raised against residues 25-44 of human S6K AGVFDIDLQPEDAGSEDEL, used for immunoblotting and immunoprecipitation), anti-PRAS40 (S115B, 1<sup>st</sup> bleed; raised against residues 238-256 of human PRAS40 DLPRPRLNTSDFQKLKRY, used for immunoblotting), anti-phospho-PRAS40 Thr246 (S114B, 2<sup>nd</sup> bleed, raised against residues 240-251 of human PRAS40 CRPRLNTpSDFQK, used for immunoblotting), anti-RSK2 (S382B, 1<sup>st</sup> bleed; residues 712-734 of human RNQSPVLEPVGRSTLAQRRGIKK, used for immunoblotting), anti-PDK1 (S682, 3<sup>rd</sup> bleed; raised against residues 544-556 of human PDK1 RQRYQSHPDAAVQ, used for immunoblotting and immunoprecipitation), anti-NDRG1 (S276B 3<sup>rd</sup> bleed; raised against full length human NDRG1, used for immunoblotting) and anti-phospho-NDRG1 T346, T356, T366 (S911B 2<sup>nd</sup> bleed; raised against RSRSHTPSEG, a sequence is common to all the three SGK1 phosphorylation sites on NDGR1, used for immunoblotting). The following commercially-available antibodies were used in this study: phospho-RSK Ser227 (#sc-12445-R) and phospho-SGK1 Ser422 (#sc-16745) were purchased from SantaCruz Biotechnology; phospho-Akt Ser473 (#9271), phospho-Akt Thr308 (#4056), phospho-S6K Thr389 (#9234), phospho S6 ribosomal protein Ser235/Ser236 (#4856), phospho S6 ribosomal protein Ser240/Ser244 (#4838), total S6 ribosomal protein (#2217), phospho-ERK Thr202/Tyr204 (#9101), total ERK (#9102), phospho-RSK Thr573 (#9346), phospho-GSK3 $\alpha/\beta$  Ser21/9 (#9331), phospho-PDK1 Ser241 (#3061) and phospho-NDRG1 Thr346 (#5482) were purchased from Cell Signaling Technology. For immunoblotting of the phosphorylated T-loop of S6K1 we employed the pan-PDK1 site antibody from Cell Signaling Technology (#9379) as previously described [32]. We have also found this pan-PDK1 site antibody efficiently recognises the phosphorylated T-loop of SGK isoforms (Fig 2). The GSK3 $\alpha/\beta$  antibody (44-610) was purchased from Biosource. Anti-GST-HRP conjugate was purchased from Abcam (#ab58626). Secondary antibodies coupled to horseradish peroxidase used for immunoblotting were obtained from Thermo Scientific.

**General methods.** Tissue culture, immunoblotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. DNA constructs used for transfection were purified from E. coli DH5 $\alpha$  using a Qiagen plasmid Mega or Maxi kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. For transient transfections, ten 10-cm-diameter dishes of HEK-293 cells were cultured and each dish was transfected with 10  $\mu\text{g}$  of the indicated plasmids using the polyethylenimine method [33]. Vesicles containing 100  $\mu\text{M}$  phosphatidylcholine (PC), 100  $\mu\text{M}$  phosphatidylserine and 10  $\mu\text{M}$  PIP<sub>3</sub> (L- $\alpha$ -D-myo-Phosphatidylinositol 3,4,5-triphosphate 3-O-phospho linked, D(+)-sn-1,2-di-O-hexadecanoylglycerol (CellSignals, cat#: 208)) were prepared and activation of Akt in the presence and absence of the PC/PS/PIP<sub>3</sub>

vesicles was undertaken as described previously [34]. Measurement of PDK1 activity employing PDKtide peptide substrate (KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC) was undertaken as described previously [35].

**Buffers.** The following buffers were used: Lysis Buffer CHAPS-LB [40 mM Tris-HCl, pH 7.5, 0.3% Chaps, 120 mM NaCl, 0.27 mM sucrose, 1 mM EDTA, 50 mM NaF, 10 mM  $\beta$ -glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate (added prior to lysis), 1 mM benzamidine (added prior to lysis), 1 mM PMSF (added prior to lysis), 0.1% (v/v) BME (added prior to lysis)], TBS-Tween Buffer [50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.1% (v/v) Tween-20], Kinase Buffer [50 mM Tris-HCl pH 7.5, 0.1 mM EGTA and 0.1% (v/v)  $\beta$ -mercaptoethanol], Wash Buffer [50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1%  $\beta$ -mercaptoethanol, 0.27 m sucrose and 0.03% Brij-35] and Sample Buffer [50 mM Tris-HCl pH 6.8, 6.5% (v/v) Glycerol, 1% (w/v) SDS, and 1% (v/v)  $\beta$ -mercaptoethanol].

**Cell Treatments and Lysis.** Cells were cultured in 10% (v/v) FBS in DMEM (high glucose) and treated with or without different inhibitors as described in the Figure legends. Following treatment, cells were rinsed with 5 ml of ice cold PBS, lysed employing 0.5 ml of the Lysis Buffer, lysates clarified by centrifugation (16,000xg at 4°C for 20 min), supernatants were snap frozen in liquid nitrogen and stored at -80°C until required. Protein concentration was determined using Coomassie Protein Assay Reagent (Thermo Scientific, cat# 1856209).

**Specificity kinase panel.** All assays were performed at The National Centre for Protein Kinase Profiling (<http://www.kinase-screen.mrc.ac.uk/>) as previously described [36]. Briefly, all assays were carried out robotically at room temperature (21°C) and were linear with respect to time and enzyme concentration under the conditions used. Assays were performed for 30 min using Multidrop Micro reagent dispensers (Thermo Electron Corporation, Waltham, MA, U.S.A.) in a 96-well format. The abbreviations for each kinase are defined in legend to Table 1. The concentration of magnesium acetate in the assays was 10 mM and [ $\gamma$ -33P]ATP (~800 cpm/pmol) was used at 5  $\mu$ M for Aurora A, CK2 $\alpha$ , DYRK3, EF2K, ERK1, ERK8, GSK3 $\beta$ , HER4, HIPK2, IGF1R, IKK $\beta$ , IRR, MARK3, MKK1, p38 $\gamma$  MAPK, p38 $\delta$  MAPK, PAK4, PIM2, Akt1 (S473D), PLK1, PKC $\zeta$  and PRK; 20  $\mu$ M for Aurora B, BRSK1, CaMKK $\beta$ , CDK2-CyclinA2, CHK1, CHK2, CK1 $\delta$ , CSK, EPH-B3, ERK2, FGF-R1, GCK, HIPK1, HIPK3, IR, IRAK4, JNK1 $\alpha$ 1, JNK2 $\alpha$ 2, JNK3 $\alpha$ 1, LKB1, MAPKAP-K2, MAPKAP-K3, MARK2, MLK1, MLK3, MSK1, MST2, MST4, NUA1, p38 $\beta$ MAPK, PAK2 (T402E), PAK5, PAK6, PDK1, PIM1, PIM3, PKA, PKC $\alpha$ , PKC $\gamma$ , PRAK, RIPK2, ROCKII, S6K1 (T412E), SGK1 (S422D), SYK, TTK and YES1; 50  $\mu$ M for BRSK2, BTK, CaMK1, DYRK1a, DYRK2, EPH-A2, IKK $\epsilon$ , LCK, MARK4, MELK, MINK1, MNK1, MNK2 $\alpha$ , NEK2A, NEK6, p38 $\alpha$ MAPK, Akt2 (S474D), PKD1, RSK1, RSK2, Src, SRPK1 and TBK1, in order to be at or below the  $K_m$  for ATP for each enzyme [36]. Lipid kinases were assayed as recently described [37].

**Kinase activity assays.** Endogenous Akt, S6K and RSK were immunoprecipitated from 0.1-1 mg of cell lysate for 2 h at 4 °C on a vibrating platform using 3-5  $\mu$ g of the indicated antibodies. For the SGK activity assays 150  $\mu$ g of transfected lysate was incubated with 5  $\mu$ g of glutathione-Sepharose for 3 h at 4 °C. The immunoprecipitates were washed two times with Lysis Buffer containing 0.5 mM NaCl, followed by two washes with Kinase Buffer. Kinase reactions were initiated by a reaction mix to bring the final concentrations of the reaction components to 0.1 mM  $\gamma$ <sup>32</sup>P-ATP (~200 cpm/pmol), 5 mM magnesium acetate, 0.1%  $\beta$ -mercaptoethanol and 30 mM Crosstide peptide (GRPRTSSFAEGKK) as previously described [38]. Reactions were carried out for 20 min at 30 °C on a vibrating platform and stopped by spotting the reactions onto P81 phosphocellulose paper. Cerenkov counting was done after washing the papers in phosphoric acid, rinsing in acetone and air-drying. One unit of activity was defined as that which catalyzed the incorporation of 1 nmol of [<sup>32</sup>P]-phosphate into the substrate over 1hr.

**Purification of GST-Akt1 and GST- $\Delta$ PH-Akt1 from HEK293 cells.** Proteins were batch-purified as described before with slight modifications [34]. Briefly, at 50% confluency,

HEK293 cells in 10 cm dishes were transfected with 10  $\mu$ g of plasmids encoding either GST-Akt or GST- $\Delta$ PH-Akt plasmids using the polyethylenimine method [33]. After 24 h, cells were treated with 1  $\mu$ M PI-103 PI 3-kinase inhibitor to induce dephosphorylation of Akt1 for 30 min and lysed as described above. The pooled supernatants were then incubated with Glutathione-Sepharose (10  $\mu$ l of beads per 10 cm dish) for 1 h at 4 °C. The beads were washed twice with 10 volumes of Lysis Buffer containing 0.5 mM NaCl and 10 times with 10 volumes of Wash Buffer (in order to remove Triton X-100, which interferes with PIP<sub>3</sub> vesicle experiments). Proteins were eluted from the beads by resuspension in an equal amount of Wash Buffer containing 20 mM glutathione (pH 7.5) for 1 h on ice. Supernatants were filtered through a 0.22  $\mu$ m-spin column and aliquots were snap-frozen and stored at -80 °C.

**Immunoblotting.** Total cell lysate (20 $\mu$ g) or immunoprecipitated samples were heated at 95 °C for 5 min in sample buffer, and subjected to 10% polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membranes. Membranes were blocked for 1hr in TBS-Tween buffer containing 5% (w/v) skimmed milk. The membranes were probed with the indicated antibodies in TBS-Tween containing 5% (w/v) skimmed milk or 5% (w/v) BSA for 16 h at 4 °C. Detection was performed using horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence reagent.

## Results

**GSK2334470 is a specific PDK1 inhibitor.** The structure of GSK2334470 is shown in Figure 1A. GSK2334470 inhibited PDK1 from activating full-length Akt1 in the presence of PtdIns(3,4,5)P<sub>3</sub> containing lipid vesicles (Fig 1B) or a mutant of Akt1 lacking the PH domain ( $\Delta$ PH-Akt1) (Fig 1C) with an IC<sub>50</sub> of ~10 nM. GSK2334470 also similarly inhibited PDK1 from phosphorylating the PDKtide peptide substrate (Fig 1D). To evaluate the specificity of GSK2334470, we studied the effect that this compound had on the activity of 95 protein kinases including 13 AGC kinase family members most closely related to PDK1 (Table 1). GSK2334470 was remarkably specific and apart from PDK1, no other kinase tested was significantly inhibited even at a concentration of 1  $\mu$ M (100-fold higher than IC<sub>50</sub> of inhibition of PDK1 in activating full-length Akt).

**GSK2334470 suppresses SGK isoform T-loop phosphorylation and activity.** To determine whether GSK2334470 could inhibit PDK1 activity in cells, we evaluated its impact on phosphorylation and activation of SGK isoforms induced by IGF1. We first monitored the effect that increasing concentrations of GSK2334470 had on the activity of endogenous SGK isoforms induced by IGF1-stimulation, by analysing phosphorylation of the physiological SGK-specific substrate termed N-myc downstream-regulated gene-1 (NDRG1) [39]. GSK2334470 induced significant dose dependent inhibition of endogenous NDRG1 with over 50% reduction in phosphorylation observed at doses of 0.1-0.3  $\mu$ M (Fig 2A). As endogenous levels of SGK isoforms in HEK293 cells are too low to study their phosphorylation state and activity [18], we overexpressed SGK1 (Fig 2B), SGK2 (Fig 2C) or SGK3 (Fig 2D) in HEK293 cells and analysed the impact of GSK2334470 on IGF1-induced phosphorylation of the T-loop (PDK1 site) as well as intrinsic kinase activity. Stimulation of serum starved HEK293 cells with IGF1 in the absence of GSK2334470 induced marked T-loop phosphorylation of each isoform of SGK that was accompanied by increased kinase activity measured after immunoprecipitation (Fig 2). GSK2334470 induced a significant dose-dependent inhibition of the T-loop phosphorylation of each SGK isoform. Significant inhibition of T-loop phosphorylation was observed at low concentrations of 30 nM and was almost abolished at ~0.1  $\mu$ M inhibitor (Fig 2). GSK2334470 similarly inhibited SGK isoform activity and at ~0.1  $\mu$ M, kinase activity was reduced to below levels observed in non-stimulated cells. Consistent with GSK2334470 inhibiting SGK isoform activity in cells, the drug suppressed phosphorylation of NDRG1 at similar doses to which it inhibited T-loop phosphorylation and kinase activity (Fig 2).

**GSK2334470 suppresses S6K1 phosphorylation and activity.** We investigated the effect of adding increasing amounts of GSK2334470 on endogenous S6K1 activity as well as T-loop and hydrophobic motif phosphorylation in HEK293 cells cultured in the presence of serum (Fig 3A). Under these conditions, 1  $\mu$ M GSK2334470 ablated S6K1 activity and phosphorylation of the T-loop (Thr229). Consistent with previous work showing that inhibition of S6K1 T-loop phosphorylation inhibits phosphorylation of the hydrophobic motif [40-42], GSK2334470 inhibited hydrophobic motif phosphorylation of S6K1 to a similar extent as T-loop phosphorylation (Fig 3A). GSK2334470 also inhibited phosphorylation of the ribosomal S6 protein, an S6K substrate [4] (Fig 3A). The ability of GSK2334470 to suppress S6K1 activity and phosphorylation was rapid, with near maximal inhibition seen within 10 min and sustained for at least 2 h, the longest time point examined (Fig 3B). GSK2334470 also suppressed S6K1 activity and phosphorylation induced by IGF1 stimulation of serum starved HEK293 cells, although 3-fold higher concentrations were required to fully inhibit S6K1, compared to cells cultured in serum (compare Fig 3A with Fig 3C). This is likely to be explained by the significantly higher degree of activation of the PI 3-kinase pathway and hence higher S6K1 activity induced by IGF1 compared to serum. Similar results have been observed for other signal transduction inhibitors such as Ku-0063794 [43] and PF-4708671 [37] where ~3-fold higher dose of these drugs are required to inhibit signalling responses in IGF1-treated HEK293 cells compared to serum.



**GSK2334470 partially suppresses Akt1 phosphorylation and activity.** We next studied the effect of adding increasing doses of GSK2334470 on the activity, T-loop (Thr308) as well as hydrophobic motif phosphorylation (Ser473) of Akt1 in HEK293 cells cultured in the presence of serum (Fig 4A). Similar to S6K1, GSK2334470 inhibited Thr308 phosphorylation as well as kinase activity. Although, GSK2334470 did not suppress Akt1 activity to the same extent as treatment of cells with the PI 3-kinase inhibitor PI-103, 1 and 3  $\mu\text{M}$  GSK2334470 markedly inhibited the phosphorylation of several Akt substrates (Foxo, GSK3 and PRAS40). GSK2334470 did not significantly inhibit Ser473 hydrophobic motif phosphorylation of Akt1. GSK2334470 also induced near maximal inhibition of Akt1 activity and phosphorylation within 5 min and Akt substrate phosphorylation (Foxo, GSK3 and PRAS40) was inhibited at slightly latter time point (10 min), as might be expected (Fig 4B).

When cells are stimulated with IGF1, Akt1 is activated to ~20-fold higher levels than in cells cultured in serum (~18 mU/mg compared to 0.8 mU/mg) (Fig 4). Following IGF1 stimulation, GSK2334470 even when deployed at high concentrations of 3  $\mu\text{M}$  did not significantly inhibit Akt activation or phosphorylation of Thr308 or Ser473 (Fig 4C).

To study whether the ability of Akt to be activated by PDK1 at the plasma membrane might account for the reduced sensitivity to GSK2334470, we compared the effects that GSK2334470 had on over-expressed full length Akt or a mutant of Akt lacking the PH domain ( $\Delta\text{PH-Akt1}$ ) in serum (Fig 5A) and IGF1-stimulated HEK293 cells (Fig 5B). This revealed that GSK2334470 suppressed T-loop phosphorylation of  $\Delta\text{PH-Akt1}$  with similar potency to that observed for SGK isoforms (Fig 2) and S6K1 (Fig 3). However, in the case of full-length overexpressed Akt1, GSK2334470 was much less efficient at inducing T-loop dephosphorylation compared to  $\Delta\text{PH-Akt1}$  (Fig 5).

We also investigated the ability of GSK2334470 to inhibit Akt phosphorylation in previously described homozygous PDK1<sup>K465E/K465E</sup> knock-in embryonic stem (ES) cells expressing a mutant of PDK1 incapable of binding phosphoinositides [27] (Fig 5C). These studies revealed that GSK2334470 inhibited phosphorylation of Thr308 and Akt substrates (PRAS40 and GSK3) more potently in PDK1<sup>K465E/K465E</sup> knock-in cells compared to control littermate PDK1<sup>+/+</sup> ES cells (Fig 5C). We observed that 0.3  $\mu\text{M}$  GSK2334470 significantly inhibited phosphorylation of Akt or PRAS40/GSK3 in PDK1<sup>K465E/K465E</sup> knock-in but not wild type ES cells (Fig 5C).

**Investigation of the effects of GSK2334470 U87 cells and fibroblasts.** We investigated the ability of GSK2334470 to inhibit Akt (Fig 6A), S6K1 (Fig 6B) as well as SGK1 (Fig 6C) activation in U87 glioblastoma cells that lack expression of PTEN. Consistent with the idea that loss of PTEN would result in a reasonably potent activation of the Akt pathway, we found that high dose of 3  $\mu\text{M}$  GSK2334470 only partially suppressed Thr308 phosphorylation or Akt activation ~ 3-fold. GSK2334470 almost reduced S6K1 activity to the basal levels observed in cells treated with 1  $\mu\text{M}$  PI-103 PI 3-kinase inhibitor (Fig 6B). In contrast, 1  $\mu\text{M}$  GSK2334470 effectively suppressed SGK1 activity as judged by the inhibition of NDRG1 phosphorylation (Fig 6C).

In mouse embryonic fibroblasts cultured in serum, in which PI 3-kinase pathway activation would be expected to be moderately activated, 1  $\mu\text{M}$  GSK2334470 suppressed Akt Thr308 phosphorylation and activity to the same extent as 1  $\mu\text{M}$  PI-103 (Fig 7A). In mouse embryonic fibroblasts (Fig 7A), we observed that in contrast to HEK293 cells (Fig 4) and U87 cells (Fig 6A), GSK2334470 inhibited phosphorylation of Akt at its hydrophobic motif to a similar extent as T-loop phosphorylation. Moreover, 1  $\mu\text{M}$  GSK2334470 also potently suppressed activation of S6K1 (Fig 7B) as well as SGK1 (Fig 7C).

**GSK2334470 inhibits RSK2 activity.** To define whether GSK2334470 inhibited RSK2, we incubated HEK293 cells cultured in serum with 3  $\mu\text{M}$  drug for up to 24 h and evaluated how this affected activity of endogenous RSK2 (Fig 8A). This revealed that incubation of cells with 3  $\mu\text{M}$  GSK2334470 for 4 h induced ~50 % inhibition of RSK2 activity that was accompanied by a partial dephosphorylation of the PDK1 T-loop residue (Ser227). After 8 h

and 24 h, RSK2 activity and T-loop phosphorylation was suppressed by over 90%. As expected, GSK2334470 did not affect the phosphorylation of RSK2 at Thr573 (Fig 8A), which is phosphorylated independently of PDK1 by the ERK1/ERK2. We also incubated HEK293 cells for 8h with increasing concentrations of GSK2334470 and observed that 0.1  $\mu$ M GSK2334470 induced ~50% inhibition of RSK2 activity which was almost completely suppressed at 1  $\mu$ M GSK2334470 (Fig 8B). Similar observations were made in U87 cells (Fig 8C) as well as mouse embryonic fibroblasts (Fig 8D), where prolonged 8 to 24h incubation with 3  $\mu$ M GSK2334470 was required to substantially inhibit RSK2 activity and induce T-loop dephosphorylation.

## Discussion.

In this study we have characterised GSK2334470, a novel small molecule cell-permeable PDK1 inhibitor that does not significantly inhibit the activity of 93 other protein kinases tested including 13 AGC kinases most closely related to PDK1. GSK2334470 is therefore much more specific than other reported PDK1 inhibitors including the staurosporine analogue UCN-01 [13] or BX-795 [15] that inhibits several other kinases more potently than PDK1 [44, 45]. At concentrations of 0.1-1.0  $\mu\text{M}$ , GSK2334470 suppressed to basal levels the T-loop phosphorylation and activation of cytosolic PDK1 substrates SGK and S6K1 that do bind  $\text{PtdIns}(3,4,5)\text{P}_3$ . GSK2334470 also inhibited phosphorylation of NDRG1 and the S6 protein, physiological substrates for SGK1 [39] and S6K1 [4], respectively. GSK2334470 also effectively suppressed RSK2 T-loop phosphorylation and activity in all three cell lines studied (Fig 8). Our data indicate that the turnover of RSK2 T-loop phosphorylation site in serum cultured cells is relatively slow, as about 8 h is required to induce substantial dephosphorylation of this residue in contrast to 10-30 min for other PDK1 substrates studied (Akt, S6K1 and SGK isoforms). Similar results have been obtained in embryonic stem cells expressing a gatekeeper mutant of PDK1 that is sensitive to the NM-PP1 inhibitor, where 24h was required to inactivate RSK2, whereas other AGC kinases were inactivated within 1 h [46]. Overall, these studies reveal that PDK1 substrates display significant differences in kinetics of T-loop dephosphorylation.

A key observation that may be relevant to development of other PDK1 inhibitors, is that GSK2334470 inhibits Akt1 activation less efficiently than S6K1 and SGK isoforms. Under conditions of low PI 3-kinase pathway activity (serum stimulation), GSK2334470 effectively inhibited Akt1 T-loop phosphorylation and activity as well as phosphorylation of Akt substrates (GSK3, Foxo and PRAS40) (Fig 4A and Fig 7A). However, in response to IGF1, that induces strong activation of the PI 3-kinase pathway, GSK2334470 was ineffective at inhibiting T-loop phosphorylation of endogenous (Fig 4C) or overexpressed full length Akt (Fig 5). GSK2334470 also did not completely suppress Akt activation in glioblastoma U87 cells that lack PTEN, which would be expected to result in an intermediate activation of Akt pathway (Fig 6A).

Previous biochemical analysis revealed that activation of full length Akt by PDK1 undertaken in the presence of  $\text{PtdIns}(3,4,5)\text{P}_3$  containing lipid vesicles was remarkably efficient, requiring 100-1000-fold lower levels of PDK1 compared to substrates not possessing a PH domain [22, 47]. The unusually high rate at which PDK1 can activate full length Akt1 in the presence of lipid vesicles containing  $\text{PtdIns}(3,4,5)\text{P}_3$ , is likely to be a result of both PDK1 and Akt possessing  $\text{PtdIns}(3,4,5)\text{P}_3$ -binding PH domains enabling the co-localisation these enzymes on a two dimensional membrane surface, thereby hugely enhancing the probability of interaction of PDK1 with Akt. As activation of Akt at the plasma membrane proceeds so efficiently, it is possible that only a very small fraction of endogenous PDK1 is actually required to activate Akt. In agreement with this notion, no inhibition of Akt activation was observed in embryonic stem cells or mice expressing 5 to 10-fold lower than normal expression of PDK1 [48, 49]. Thus the inability of GSK2334470 to suppress Akt activation under conditions of high pathway activation, could be explained by a small residual pool of non-inhibited PDK1 still capable of activating Akt at the membrane in inhibitor treated cells. This would also explain why  $\Delta\text{PH}$ -Akt, which is localised in the cytosol was inhibited more potently than full length Akt by GSK2334470 (Fig 5A and B). Moreover, the finding that GSK2334470 inhibited Akt phosphorylation in PDK1<sup>K465E/K465E</sup> ES cells, in which PDK1 can no longer interact with phosphoinositides, more potently than in wild type cells, is also consistent with the idea that it is harder to inhibit Akt activation by PDK1 associated with  $\text{PtdIns}(3,4,5)\text{P}_3$  on the membrane. It is also possible that PDK1 associated with the plasma membrane may not be as effectively exposed to GSK2334470 as cytosolic PDK1. However, at least in vitro, GSK2334470 effectively inhibited activation of Akt in the presence of  $\text{PtdIns}(3,4,5)\text{P}_3$ -containing lipid vesicles (Fig 1B). Another possibility is that in vivo, the pool of membrane-localised PDK1 is complexed to other protein(s) that influence kinase domain structure in a manner that renders it less potently inhibited by GSK2334470.

The finding that GSK2334470 more efficiently suppresses Akt activity under conditions of weaker PI 3-kinase pathway stimulation has implications for the use of this drug. Our data implies that different concentrations of PDK1 inhibitors may be required to inhibit Akt activity in cells dependent on the level of PI3-kinase activation. If the aim is to inhibit the activity of RSK, then long (up to 8h) exposure of cells with GSK2334470 is required to achieve substantial inhibition of this enzyme. Effects of GSK2334470 that are observed over shorter periods of time are unlikely to be due to inhibition of RSK isoforms. Our data would suggest that other PDK1 inhibitors being developed would also suppress activation of non-PtdIns(3,4,5)P<sub>3</sub> binding cytosolic targets SGK or S6K more efficiently than Akt, especially in cells treated with agonists that induce a large activation of PI 3-kinase. If only a low percentage of cellular PDK1 is required to maximally activate Akt at the plasma membrane, this may indicate that it will be challenging to develop a drug that effectively suppresses Akt, especially in response to stimuli or mutations that induce large activation of the PI 3-kinase pathway.

Our data suggest that GSK2334470 does not inhibit mTORC2, as this compound did not suppress hydrophobic motif phosphorylation endogenous Akt1 (Fig 4) or overexpressed Akt1 (Fig 5) or ΔPH-Akt1 (Fig 5). In contrast, GSK2334470 inhibited the hydrophobic motif phosphorylation of endogenous Akt in mouse embryonic fibroblasts cells exposed to serum to a similar extent as Thr308 phosphorylation (Fig 4). Structural analysis of Akt2, has established that T-loop phosphorylation and hydrophobic-motif phosphorylation co-operate to stabilise the structure of the Akt kinase domain [50, 51]. These studies indicate that inhibiting Thr308 phosphorylation by treatment with GSK2334470 would lead to a less stable Akt1 conformation, in which Ser473 would not interact with the kinase domain and would thus be exposed and accessible to becoming dephosphorylated by protein phosphatase(s). This may explain why GSK2334470 could lead to the loss of Akt Ser473 phosphorylation. However, further work is required to determine why GSK2334470 affects Ser473 phosphorylation in mouse embryonic fibroblasts, but not HEK293 or U87 cells.

Although Akt is considered to be one of the key enzymes driving the growth and proliferation of cancer cells, there is increasing evidence that Akt-independent pathways, perhaps requiring SGK isoforms may play a crucial role in driving expansion of a number of tumours [52]. Moreover, recent work carried out in *C. elegans* has disputed the widely believed opinion that Akt is the key mediator of signalling downstream of mTOR and at least in this species, SGK rather than Akt is the key mediator of growth, fat metabolism, reproduction and life-span [53, 54]. Akt has many other vital functions in cells and therefore an anticancer drug that potently inhibited Akt and other PDK1 substrates may have significant side effects. It will be very interesting to evaluate whether a compound like GSK2334470 that inhibits S6K and SGK isoforms more potently than Akt, would be effective at suppressing growth of various cancers and whether it would be better-tolerated than other PI 3-kinase pathway inhibitors being developed and/or evaluated in clinical trials. It would also be of interest to assess the effects of combining low doses of PDK1 and mTOR kinase inhibitors as it might be envisaged that inhibiting both key upstream activators would be more effective at suppressing actions of AGC kinases in cancer than employing PDK1 or mTOR inhibitors individually. Despite the complications of GSK2334470 not completely inhibiting Akt activity under conditions of high PI 3-kinase pathway activation, this compound will be a very useful research tool to probe signalling responses downstream of PDK1 and represents a useful addition to our armoury of effective signal transduction inhibitors to dissect biological roles of protein kinases.

**Acknowledgements.**

We are grateful to Juan M. García-Martínez, Stephan Wullschleger, Laura Pearce, Nick Leslie, Alexander Gray and Ian Batty for valuable discussions. We thank the staff at the National Centre for Protein Kinase Profiling ([www.kinase-screen.mrc.ac.uk](http://www.kinase-screen.mrc.ac.uk)) for undertaking the kinase specificity screening, the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) for DNA sequencing and the protein production and antibody purification teams [Division of Signal Transduction Therapy (DSTT), University of Dundee] co-ordinated by Hilary McLauchlan and James Hastie for expression and purification of antibodies. We thank the Medical Research Council, and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck-Serono and Pfizer) for financial support.

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## Figure and Table Legends

**Table 1 Effect of GSK2334470 upon the activity of 95 protein kinases.** Results are presented as a percentage of kinase activity in control incubations, in which GSK2334470 was omitted. Protein kinases were assayed as described in Materials and methods and the results are an average of three separate reactions  $\pm$  S.D. † indicates AGC kinase family members. Abbreviations not defined in main text: AMPK, AMP-activated protein kinase; BRSK, brain-specific kinase; BTK, Bruton's tyrosine kinase; CaMK, calmodulin-dependent kinase; CaMKK, CaMK kinase; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; CK, casein kinase; CLK, CDC-like kinase; CSK, C-terminal Src kinase; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; EF2K, elongation-factor-2 kinase; EPH, ephrin; ERK, extracellular signal-regulated kinase; FGF-R, fibroblast growth factor receptor; GCK, germinal centre kinase; GSK, glycogen synthase kinase; HIPK, homeodomain-interacting protein kinase; IGF1R, IGF1 receptor; IKK, inhibitory  $\alpha$ B kinase; IR, insulin receptor; IRAK, Interleukin-1 Receptor-Associated Kinase; IRR, insulin-related receptor; JNK, c-Jun N-terminal kinase; Lck, lymphocyte cell-specific protein tyrosine kinase; LKB1, Ser/Thr Kinase 11; MAPK, Mitogen-activated protein kinase; MAPKAP-K, MAPK-activated protein kinase; MARK, microtubule-affinity-regulating kinase; MELK, maternal embryonic leucine-zipper kinase; MKK, MAPK kinase; MLCK, smooth muscle myosin light-chain kinase; MLK, mixed lineage kinase; MNK, MAPK-integrating protein kinase; MSK, mitogen- and stress-activated protein kinase; MST, mammalian homologue Ste20-like kinase; NEK, NIMA (never in mitosis in *Aspergillus nidulans*)-related kinase; NUAK, SnF1-like Kinase; PAK, p21-activated protein kinase; PDK, Phosphoinositide-dependent kinase; PHK, phosphorylase kinase; PIM, provirus integration site for Moloney murine leukaemia virus; PKA, cAMP dependent protein kinase; PKC, Protein kinase C; PKD, protein kinase D; PLK, polo-like kinase; PRAK, p38-regulated activated kinase; PRK, protein kinase C-related kinase; ROCK, Rho-dependent protein kinase; RSK, Ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; SRPK, serine arginine protein kinase; SYK, spleen tyrosine kinase; TBK1, TANK-binding kinase 1; VEGFR, vascular endothelial growth factor receptor; YES1, Yamaguchi sarcoma viral oncogene homologue 1.

**Table 2. Effect of GSK2334470 upon activity of 15 lipid kinases.** Results are tabulated as a percentage of lipid kinase activity in control incubations, in which GSK2334470 was omitted. Lipid kinases were assayed as described previously [37], and the results are an average of three separate reactions  $\pm$  S.D. Abbreviations: PI3K, Phosphatidylinositol 3-kinase; PIK4CA, Phosphatidylinositol 4-kinase catalytic alpha subunit; PIK4CB, Phosphatidylinositol 4-kinase catalytic beta subunit; PIP5K2A, Phosphatidylinositol 5-phosphate 4-kinase type II alpha; VPS34, phosphoinositide-3-kinase, class 3; SPHK, sphingosine kinase; CHK, choline kinase; DGK, diacylglycerol kinase.

**Figure 1. GSK2334470 inhibits PDK1 *in vitro*.** (A) Structure of GSK2334470. (B & C) Effect of GSK2334470 on PDK1 activity assayed studying activation of either full length Akt1 (B) (assayed in the presence of 10  $\mu$ M PtdIns(3,4,5)P<sub>3</sub> in lipid vesicles containing 0.1 mM phosphatidylcholine and 0.1 mM phosphatidylserine [34]) or  $\Delta$ PH-Akt1 (C). (D) Effect of GSK2334470 on ability of PDK1 to phosphorylate the PDKtide peptide [35] was analysed. Results are plotted as percentage of the maximal activity (no inhibitor). The dotted line indicated 50% inhibition level. The data is average of triplicate reactions.

**Figure 2. Effect of GSK2334470 on SGK activity in HEK293 cells.** (A) HEK293 cells were serum starved overnight and treated with the indicated concentrations of GSK2334470 for 30 min and stimulated with 50 ng/ml IGF1 for 30 min. Cells were lysed and lysates immunoblotted with the indicated antibodies. (B-D) HEK293 cells were transfected with constructs encoding for GST- $\Delta$ N-SGK1 (lacks residues 1 to 60) (B), GST-SGK2 (C) or GST-SGK3 (D). 24 h post transfections cells were deprived of serum overnight and treated with the indicated concentration of GSK2334470 prior to stimulation with IGF1 as in (A). SGK isoforms were affinity purified on glutathione-Sepharose and their catalytic activities were assayed using Crosstide as a substrate peptide. Each bar represents the mean specific activity

± S.D from two different samples, with each sample assayed in duplicate. Affinity purified SGK1 was also subjected to immunoblotting with an anti-GST antibody (SGK-Total). Cell lysates were also analysed by immunoblotting with the other indicated antibodies. Similar results were obtained in two separate experiments.

**Figure 3. Effect of GSK2334470 on S6K1 activity in HEK293 cells.** (A) HEK293 cells cultured in medium containing 10% (v/v) fetal bovine serum, were treated with the indicated concentrations of GSK2334470 for 30 min. Cells were lysed, endogenous S6K1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity ± S.D. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. (B) As in (A), except cells were treated with 3 μM GSK2334470 for indicated time points. (C) As in (A), except cells were serum-starved overnight, treated with the indicated concentrations of GSK2334470 for 30 min prior to stimulation with 50 ng/ml of IGF1 for 30 min minutes. Similar results were obtained in three separate experiments.

**Figure 4. Effect of GSK2334470 on Akt activity in HEK293 cells.** HEK293 cells cultured in medium containing 10% (v/v) fetal bovine serum, were treated with the indicated concentrations of GSK2334470 for 30 min. Cells were lysed, endogenous Akt1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity ± S.D. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. (B) As in (A), except cells were treated with 3 μM GSK2334470 for indicated time points. (C) As in (A), except cells were serum-starved overnight, treated with the indicated concentrations of GSK2334470 for 30 min prior to stimulation with 50 ng/ml of IGF1 for 30 min minutes. Similar results were obtained in three separate experiments.

**Figure 5. Association of Akt with PtdIns(3,4,5)P<sub>3</sub> suppresses sensitivity to GSK2334470.** (A) HEK293 cells were transfected with constructs expressing either full length GST-Akt1 or GST-ΔPH-Akt. Cells were cultured in the presence of medium containing 10% (v/v) fetal bovine serum and 48 h post transfection, cells were treated with the indicated concentrations of GSK2334470 for 30 min. Akt form were affinity purified on glutathione-Sepharose and their catalytic activities were assayed using Crosstide as a substrate peptide. Each bar represents the mean specific activity ± S.D. from 3 separate samples. Purified Akt forms were also subjected to immunoblot analysis with the indicated antibodies. (B) As in (A), except cells serum-starved for 16 h prior to treatment with GSK2334470 and stimulated with 50 ng/ml of IGF1 for 30 min. Similar results were obtained in three separate experiments. (C) Wild type PDK1<sup>+/+</sup> and homozygous knockin PDK1<sup>K465E/K465E</sup> [27], were deprived of serum for 4 h, treated with the indicated concentrations of GSK2334470 for 30 min prior to stimulation with 50 ng/ml of IGF1 for 30 min minutes. Cells were lysed, endogenous Akt1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity ± S.D. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies.

**Figure 6. Effect of GSK2334470 on Akt and S6K activity in U87 cells.** U87 cells cultured in medium containing 10% (v/v) fetal bovine serum, were treated with the indicated concentrations of GSK2334470 for 30 min. Cells were lysed, endogenous Akt1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity ± S.D. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. (B) As in (A), except for endogenous S6K1 was immunoprecipitated and assayed. (C) As in (A) except that lysates were immunoblotted with the total and phospho-NDRG1 antibody as a readout of endogenous SGK activity. Similar results were obtained in three separate experiments.

**Figure 7. Effect of GSK2334470 on Akt and S6K activity in mouse embryonic fibroblasts.** Mouse embryonic fibroblasts cultured in medium containing 10% (v/v) fetal bovine serum, were treated with the indicated concentrations of GSK2334470 for 30 min. Cells were lysed, endogenous Akt1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  S.D. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. **(B)** As in **(A)**, except for endogenous S6K. Similar results were obtained in three separate experiments.

**Figure 8. Effect of GSK2334470 on RSK2 activity in serum-cultured HEK293 cells.** **(A)** HEK293 cells cultured in medium containing 10% (v/v) fetal bovine serum, were treated with 3  $\mu$ M GSK2334470 for indicated time points. Cells were lysed, endogenous RSK2 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Each bar represents the mean specific activity  $\pm$  S.D from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. Similar results were obtained in two separate experiments. **(B)** as in **(A)** except that cells were treated with the indicated concentrations of GSK2334470 for 8h before lysis. **(C & D)** as in **(A)** except that U87 cells **(C)** or mouse embryonic fibroblasts **(D)** were employed.

Table 1

Kinase	Percentage of activity remaining			Kinase	Percentage of activity remaining			Kinase	Percentage of activity remaining		
	0.01µM GSK-2334470	0.1µM GSK-2334470	1µM GSK-2334470		0.01µM GSK-2334470	0.1µM GSK-2334470	1µM GSK-2334470		0.01µM GSK-2334470	0.1µM GSK-2334470	1µM GSK-2334470
<b>MKK1</b>	86±17	101±12	84±24	<b>SmMLCK</b>	116±6	113±4	92±8	<b>EF2K</b>	108±2	100±3	97±13
<b>ERK1</b>	93±5	106±12	94±15	<b>PHK</b>	106±8	108±3	103±8	<b>HIPK1</b>	102±3	105±2	102±7
<b>ERK2</b>	117±8	111±10	99±4	<b>CHK1</b>	111±11	106±5	104±5	<b>HIPK2</b>	119±1	124±17	113±2
<b>JNK1</b>	92±3	108±5	99±6	<b>CHK2</b>	129±25	121±5	88±11	<b>HIPK3</b>	111±2	105±7	106±10
<b>JNK2</b>	117±6	126±19	120±17	<b>GSK3b</b>	108±4	111±6	109±9	<b>PAK2</b>	105±8	119±2	105±16
<b>JNK3</b>	106±7	106±8	100±2	<b>CDK2-Cyclin A</b>	104±3	101±11	105±10	<b>PAK4</b>	108±1	117±16	106±4
<b>p38a MAPK</b>	105±5	109±8	103±3	<b>PLK1</b>	95±5	113±2	97±12	<b>PAK5</b>	97±2	101±11	94±17
<b>p38b MAPK</b>	113±6	129±18	109±17	<b>Aurora A</b>	112±7	115±4	92±9	<b>PAK6</b>	108±7	120±17	124±11
<b>p38g MAPK</b>	109±6	110±5	106±3	<b>Aurora B</b>	105±6	106±2	83±1	<b>MST2</b>	116±4	128±11	112±3
<b>p38d MAPK</b>	106±2	113±12	108±5	<b>LKB1</b>	104±8	124±8	100±5	<b>MST4</b>	112±7	108±14	119±5
<b>ERK8</b>	106±12	117±7	107±1	<b>AMPK</b>	107±2	111±7	87±1	<b>GCK</b>	109±3	116±14	101±5
<b>RSK1</b>	123±12	113±3	94±9	<b>MARK2</b>	113±1	114±0	109±10	<b>MINK1</b>	121±4	110±8	105±4
<b>RSK2</b>	96±20	97±6	79±9	<b>MARK3</b>	113±5	109±7	114±15	<b>MLK1</b>	110±1	111±1	107±15
<b>PDK1</b>	62±8	12±5	2±0	<b>MARK4</b>	106±7	114±6	102±11	<b>MLK3</b>	100±5	110±2	87±7
<b>PKBa</b>	179±71	117±22	97±6	<b>BRSK1</b>	110±17	95±6	57±1	<b>IRAK4</b>	109±0	112±5	106±1
<b>PKBb</b>	125±11	118±14	100±8	<b>BRSK2</b>	114±2	110±0	62±3	<b>RIPK2</b>	110±1	114±15	107±21
<b>SGK1</b>	96±1	96±4	37±4	<b>MELK</b>	115±12	77±1	23±0	<b>TTK</b>	90±1	98±10	82±10
<b>S6K1</b>	111±4	106±16	100±8	<b>NUAK1</b>	120±19	105±3	47±7	<b>Src</b>	101±5	105±1	97±4
<b>PKA</b>	103±13	111±15	98±5	<b>CK1</b>	100±1	108±6	98±11	<b>Lck</b>	112±18	107±3	114±19
<b>ROCK 2</b>	96±4	92±4	70±0	<b>CK2</b>	103±0	104±12	94±3	<b>CSK</b>	103±7	104±5	100±12
<b>PRK2</b>	90±10	102±12	82±1	<b>DYRK1A</b>	102±2	107±9	103±3	<b>YES1</b>	122±1	119±14	101±8
<b>PKCa</b>	104±4	105±13	94±3	<b>DYRK2</b>	100±3	108±5	102±11	<b>IGF-1R</b>	126±35	115±5	107±13
<b>PKCz</b>	113±0	105±10	72±11	<b>DYRK3</b>	100±19	103±5	93±8	<b>IR</b>	101±8	103±4	102±5
<b>PKD1</b>	99±5	116±0	104±11	<b>NEK2a</b>	118±15	118±11	106±12	<b>IRR</b>	111±4	111±8	109±1
<b>MSK1</b>	102±0	100±2	94±3	<b>NEK6</b>	98±6	101±6	97±7	<b>HER4</b>	106±10	107±5	106±9
<b>MNK1</b>	106±14	108±5	111±9	<b>IKKb</b>	105±3	112±7	98±5	<b>FGF-R1</b>	119±3	119±1	110±1
<b>MNK2</b>	91±2	100±28	106±1	<b>IKKe</b>	114±9	97±2	74±9	<b>VEG-FR</b>	98±6	108±1	89±6
<b>MAPKAP-K2</b>	104±2	111±13	101±1	<b>TBK1</b>	110±6	115±11	109±16	<b>EPH A2</b>	110±4	118±12	114±15
<b>MAPKAP-K3</b>	112±18	112±11	100±16	<b>PIM1</b>	99±1	100±3	98±3	<b>EPH-B3</b>	106±11	107±8	91±0
<b>PRAK</b>	81±2	92±7	76±3	<b>PIM2</b>	97±7	100±28	97±20	<b>SYK</b>	109±14	105±4	103±10
<b>CAMKKb</b>	112±9	123±11	127±11	<b>PIM3</b>	105±4	112±14	97±5	<b>BTK</b>	126±9	121±15	110±2
<b>CAMK1</b>	108±3	112±3	117±17	<b>SRPK1</b>	106±5	120±9	106±8				

Table 2

Kinase	Percentage of activity remaining		
	0.1µM GSK2334470	1µM GSK2334470	10µM GSK2334470
PI3K a	106±2	105±4	104±13
PI3K b	102±6	108±9	97±1
PI3K d	119±8	104±25	73±4
PI3K g	98±3	83±1	70±0
VPS34 + VPS15	96±0	102±19	89±5
PIP5K2A	102±2	97±3	86±8
SPHK1	106±10	102±2	94±2
SPHK2	103±2	100±1	91±1
CHK a	102±3	94±8	101±1
DGK b	99±0	100±0	93±8
PIK4CA	100±2	94±1	103±1
PIK4CB	99±2	89±1	97±1
CHK b	113±5	95±5	87±4
DGK g	99±6	100±3	101±5
DGK z	110±19	86±8	101±11

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Figure 1

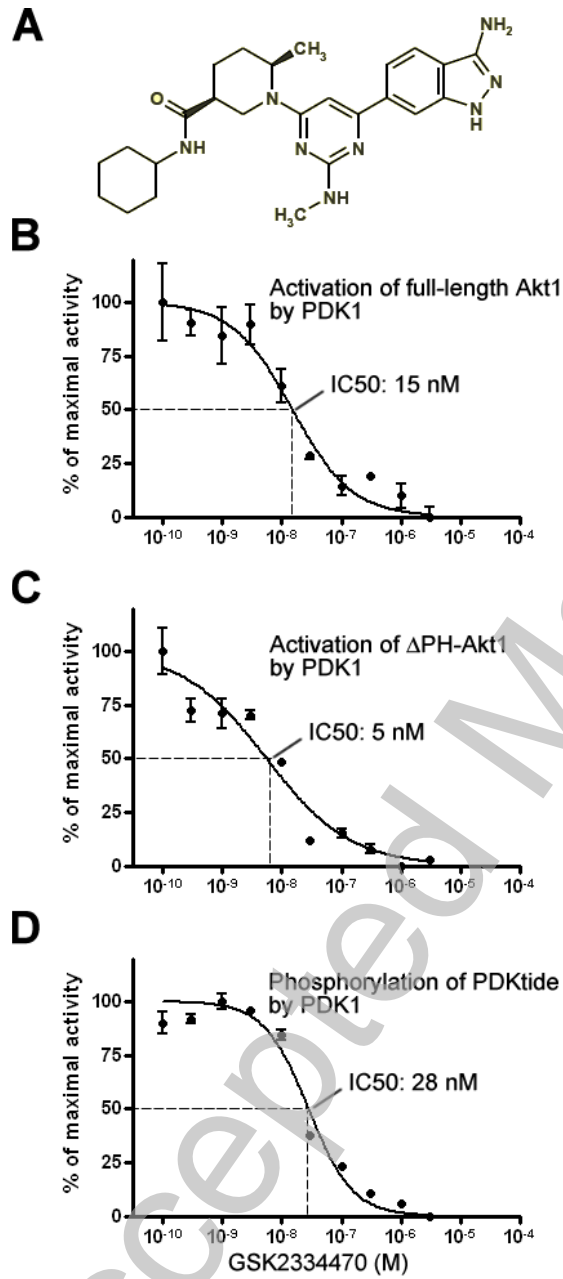
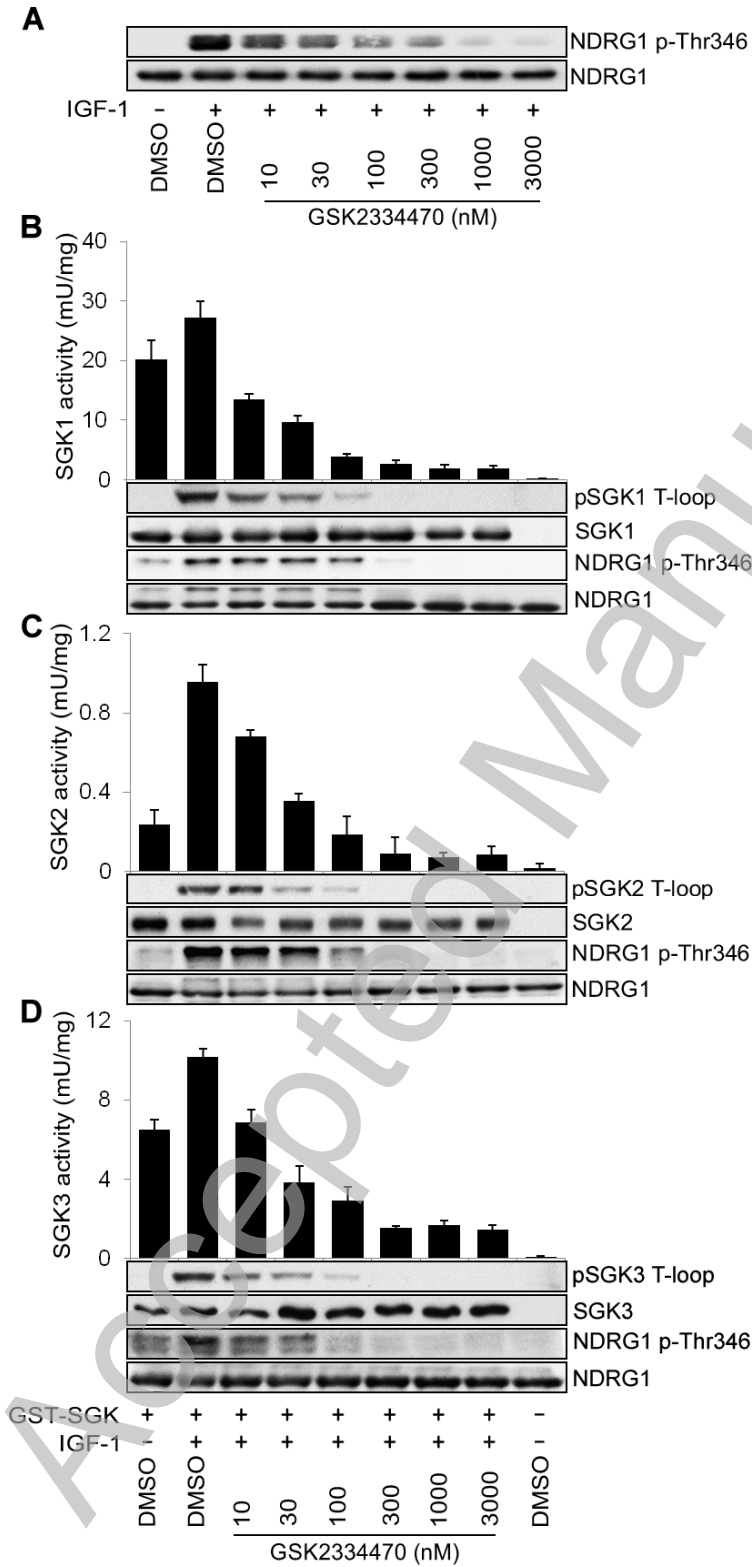


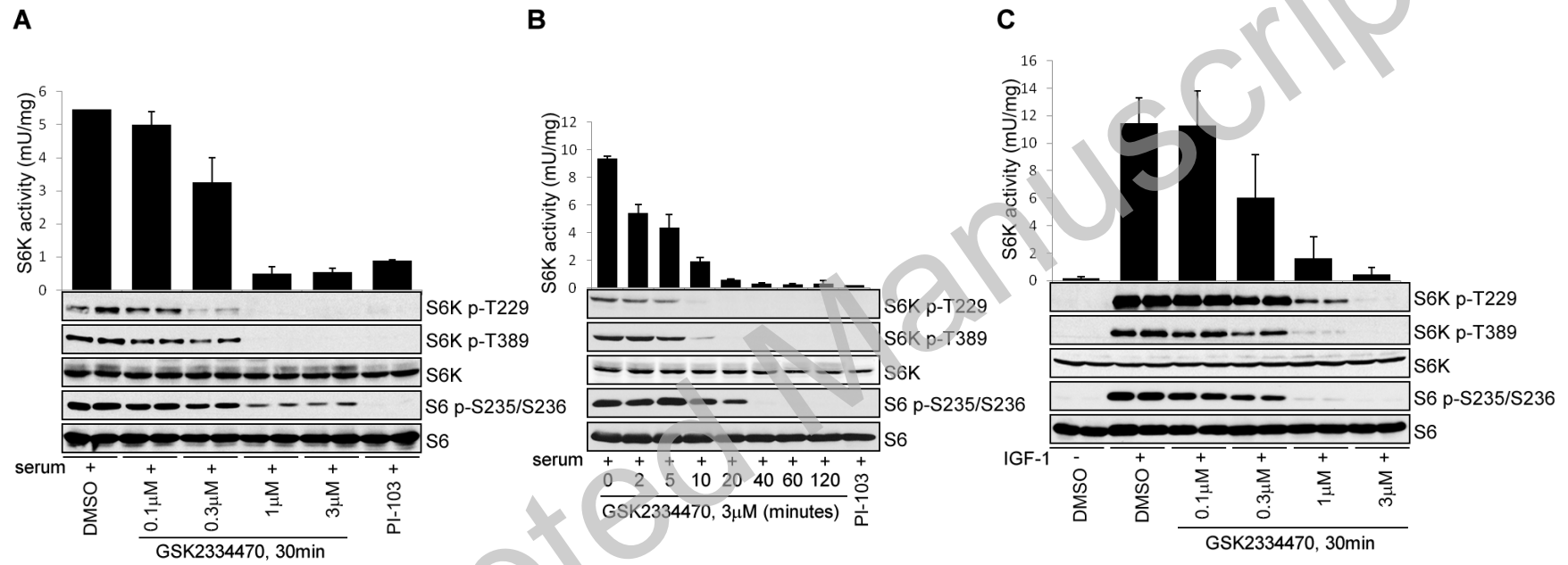
Figure 2



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Figure 3



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Figure 4

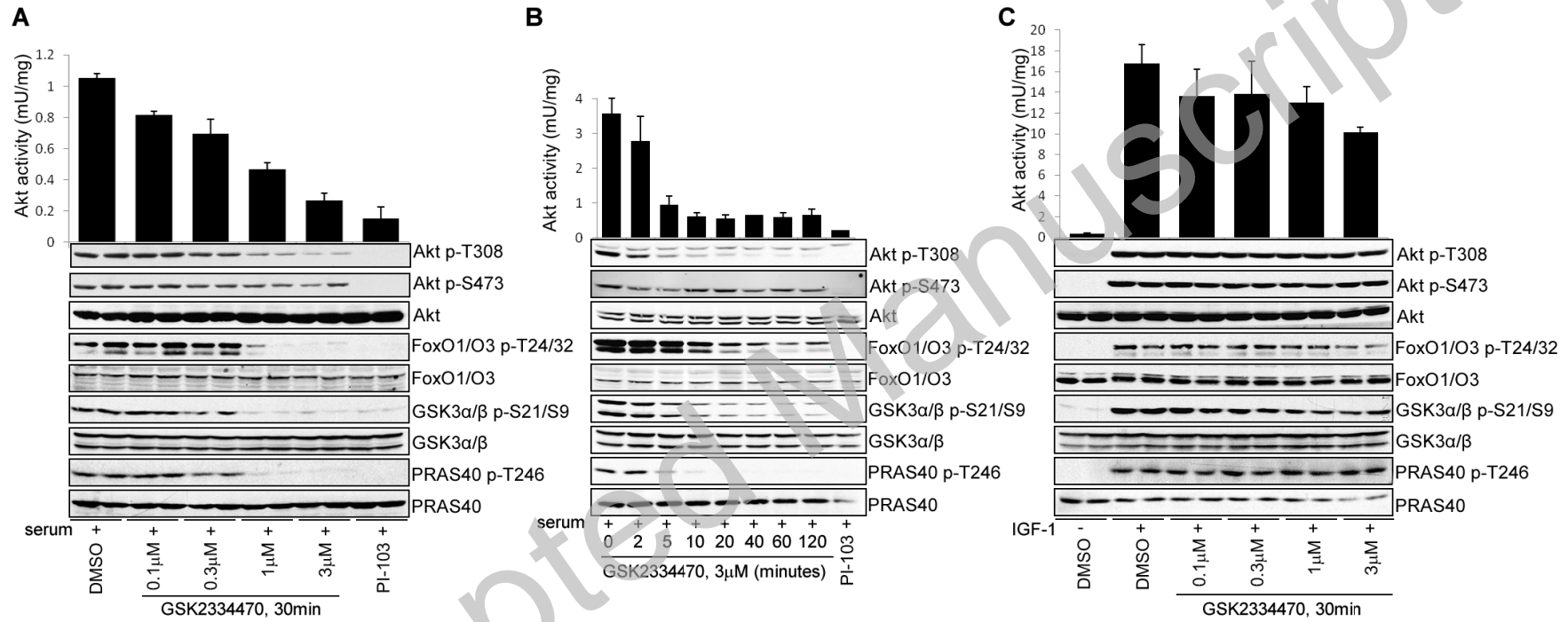
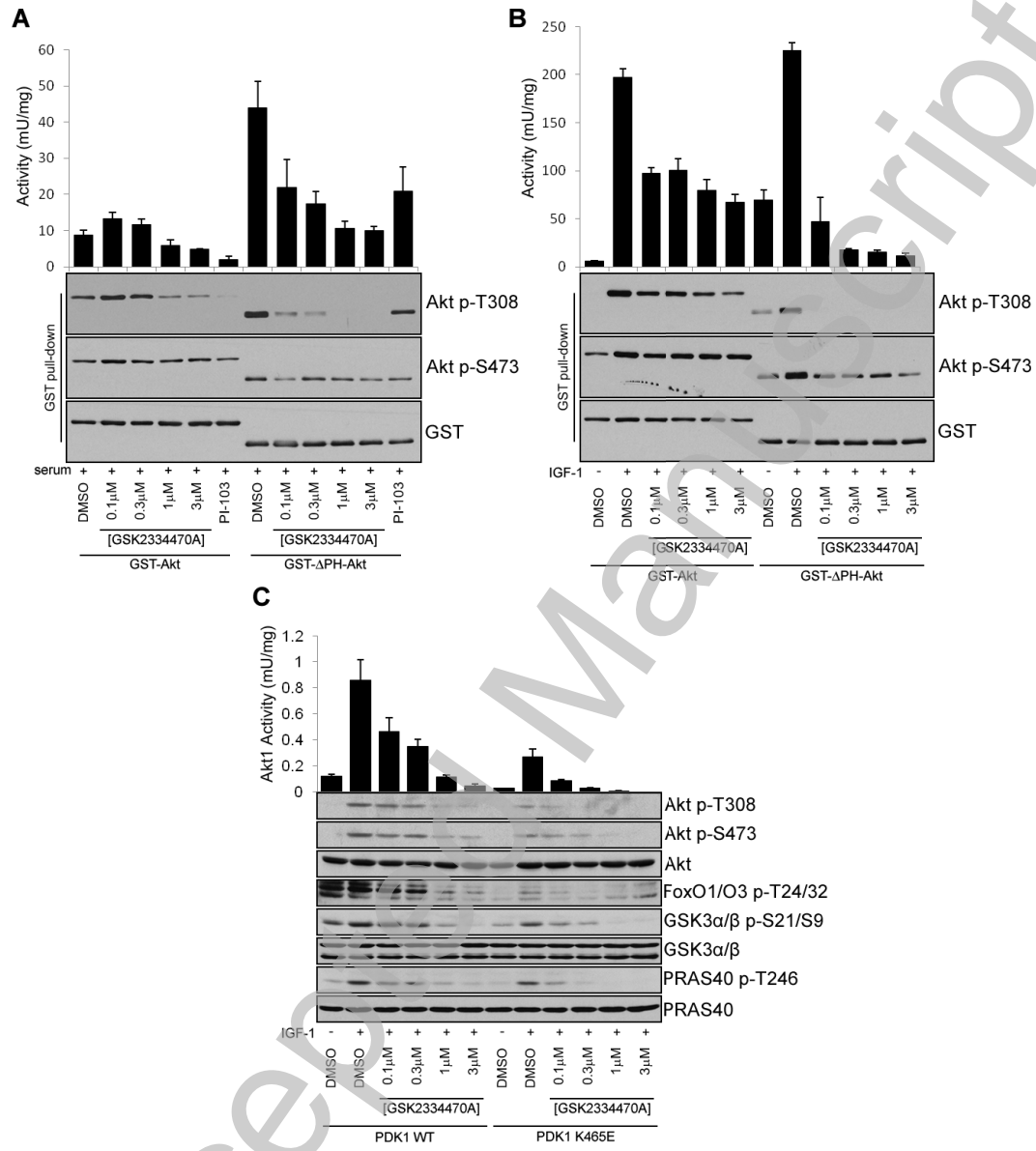
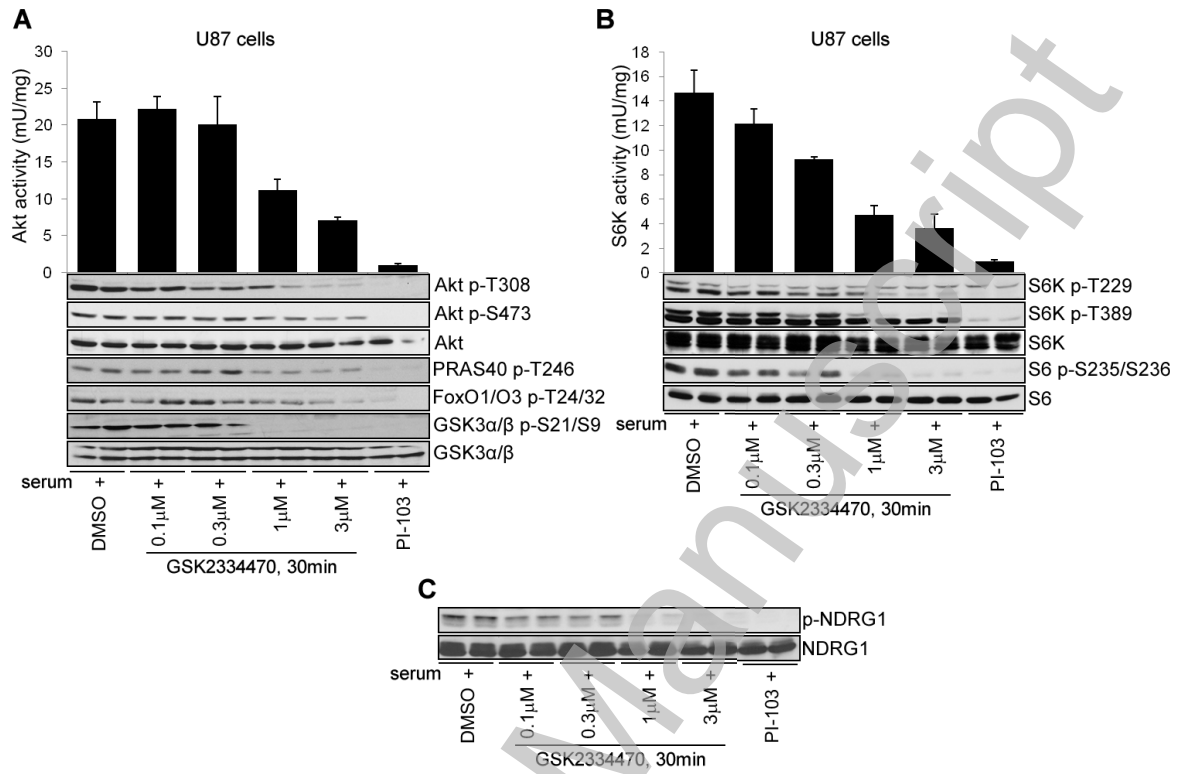


Figure 5



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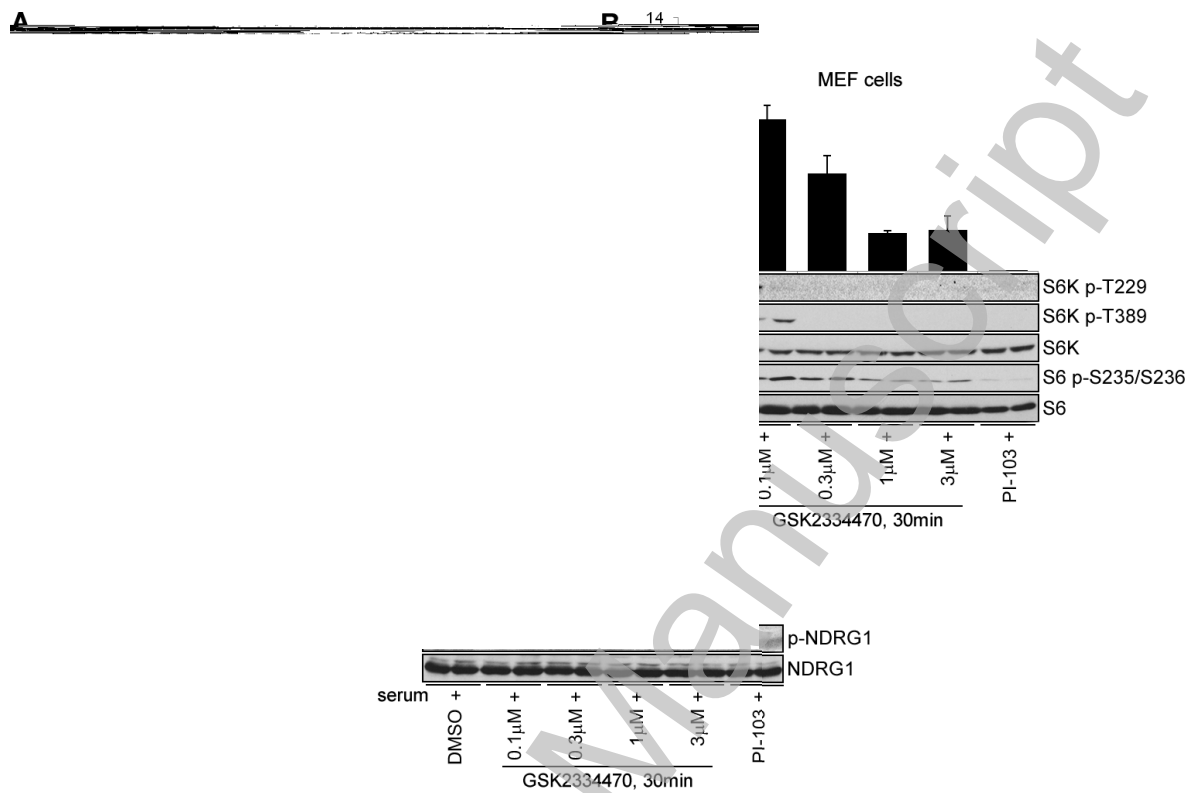
Figure 6



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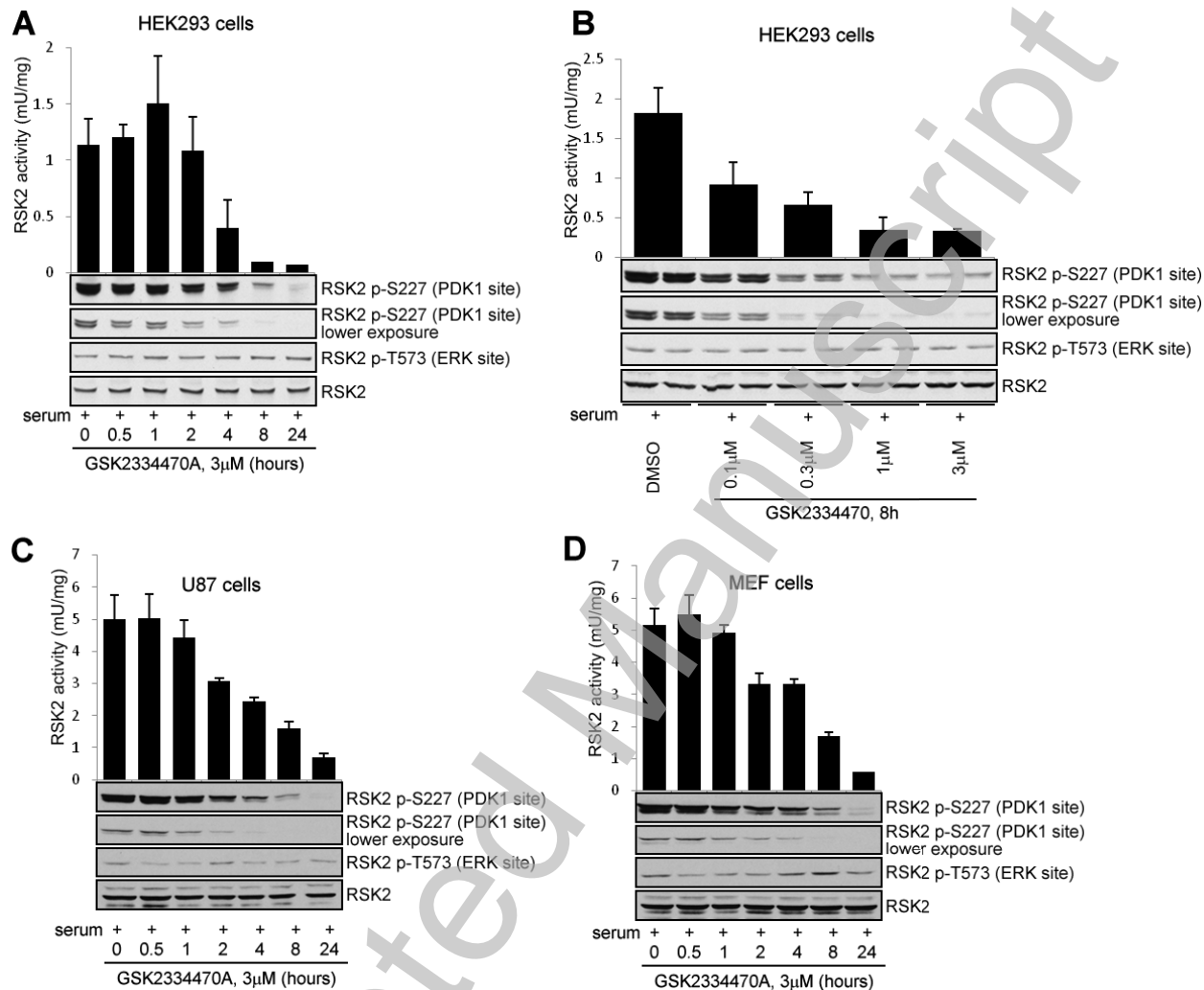
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Figure 7



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Figure 8



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