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Abstract.
S6K1 is activated by insulin and growth factors via the PI3K and mTOR signalling pathways. S6K1 regulates numerous processes such as protein synthesis, growth, proliferation and longevity and its inhibition has been proposed as a strategy for the treatment of cancer and insulin resistance. Here we describe a novel cell-permeable inhibitor of S6K1, PF-4708671, which specifically inhibits the S6K1 isoform with a Ki of 20 nM and IC_{50} of 160 nM. PF-4708671 prevents the S6K1-mediated phosphorylation of S6 protein in response to IGF1 while having no effect upon the TPA-induced phosphorylation of substrates of the highly related RSK and MSK kinases. PF-4708671 was also found to induce phosphorylation of the T-loop and hydrophobic motif of S6K1, an effect that is dependent upon mTORC1. PF-4708671 is the first S6K1 specific inhibitor to be reported and will be a useful tool for delineating S6K1-specific roles downstream of mTOR.

Introduction.

S6K (p70 ribosomal S6 kinase) is a Ser/Thr kinase belonging to the AGC protein kinase family, which also includes Akt, RSK (p90 ribosomal S6 kinase) and MSK (mitogen-and stress-activated protein kinase) [1]. S6K exists as two isoforms, S6K1 and S6K2 and plays a crucial role in the regulation of protein synthesis and cell growth downstream of the mTOR protein kinase. Indeed, *Drosophila* expressing a mutant of S6K1 and mice lacking S6K1 display a reduced size [2, 3]. The best-characterised substrate of S6K1 is S6 protein, a component of the 40S ribosomal subunit, which can be phosphorylated by both isoforms at five different residues: Ser235, Ser236, Ser240, Ser244 and Ser247 [4]. Fibroblasts and islet pancreatic beta cells derived from knock-in mice in which the five-serine residues are mutated to alanine display a reduced cell size [5]. S6K1 also stimulates translation via the phosphorylation of the RNA helicase promoting factor eIF4B [6] and also eEF2K [7], which is involved in the elongation step of protein synthesis. In addition, recent work indicates that S6K1 phosphorylates the Rictor subunit of mTOR Complex-2 at Thr1135 mediating 14-3-3 binding, although the importance of this phosphorylation is unclear [8-10].

Like many of the kinases in the AGC kinase family, the catalytic activity of S6K1 is stimulated downstream of the PI3K pathway and is dependent upon phosphorylation, in particular at two key sites: the T-loop and the hydrophobic motif. The hydrophobic motif (Thr389 in S6K1) is phosphorylated by mTOR complex 1 (mTORC1), which consists of the mTOR protein kinase bound to Raptor and mLST8 [11, 12]. Phosphorylation of Thr389 allows the binding of Phosphoinositide-dependent kinase 1 (PDK1) via its PIF pocket and subsequently the T-loop (Thr229 in S6K1) is phosphorylated [13].

Many cancer-driving mutations including those in *PTEN*, *PIK3CA*, *Ras*, *TSC1/2* and *LKB1* lead to the activation of the PI3K and mTOR signalling pathways that in turn stimulate S6K1, suggesting S6K1 is a potential target for anticancer drugs. S6K1 activity is also stimulated by nutrients such as amino acids, via mTORC1 [14]. Moreover, S6K1 controls an important feedback loop that inhibits the PI3K-mTOR pathway. This is achieved by S6K1 phosphorylating IRS-1 on multiple Ser residues promoting its degradation [15, 16]. Thus overstimulation of the S6K1 pathway through over-eating switches off the PI3K pathway thereby causing insulin resistance. Consistent with this, mice lacking S6K1 display increased insulin sensitivity and are protected from age and diet-induced obesity [17], indicating S6K1 inhibitors might be effective at counteracting insulin resistance. An interesting recent study also suggests that mice lacking S6K1 have increased longevity [18] and the treatment of mice with rapamycin also enhanced lifespan [19].

To date the cellular effects of S6K1 have largely been inferred using the mTORC1 inhibitor rapamycin. However this is not ideal as mTORC1 also phosphorylates other substrates such as 4EBP1 [20] and the autophagy kinase ULK1 [21-23]. Here we describe the compound PF-4708671, the first specific S6K1 isoform inhibitor to be reported and provide evidence that it can be deployed to inhibit S6K1 activity in cells without inhibiting the activity of the similar AGC kinases such as RSK and MSK. Our findings suggest that PF-4708671 will be a useful tool compound to aid the dissection of signalling processes controlled by S6K1.

Materials and methods.

Materials. Protein G-Sepharose and glutathione-Sepharose were purchased from Amersham Bioscience. ^{32}P γ-ATP was from Perkin-Elmer; IGF1 was from Cell Signaling technology. Tween-20, DMSO, Phorbol-12-Myristate-13-Acetate (TPA) and dimethyl pimelimidate were from Sigma, and CHAPS and rapamycin were from Calbiochem. Akti-1/2, PI-103 and GDC-0941 were synthesised by Dr Natalia Shpiro at the University of Dundee. PF-4708671 was synthesised by Pfizer and Ku-0063794 was synthesised by AstraZeneca. Omnia peptide 6 was purchased from Life Technologies.

Antibodies. The following antibodies were raised in sheep and affinity purified on the appropriate antigen: anti-Raptor (S682B, 3rd bleed; residues 1-20 of human Raptor MESEMLQSPLLGLGEEDEAD, used for immunoblotting and immunoprecipitation), anti-Rictor (S274C, 1st bleed; residues 6-20 of mouse Rictor RGRSLKNLRIRGRND, used for immunoprecipitation and immunoblotting). An antibody that recognizes Rictor phosphorylated at Thr1135 (S998B, 2nd bleed) was raised against residues 1129 - 1141 of human Rictor RRIRTLpTEPSVDL (used for immunoblotting). Anti-Akt1 (S695B, 3rd bleed; residues 466 - 480 of human Akt1 RPHFPQFSYSASGTA, used for immunoblotting), anti-S6K (S417B, 2nd bleed; residues 25 - 44 of human S6K AGVFDIDLDQPEDAGSEDEL, used for immunoblotting and immunoprecipitation). Anti-MSK1 (S804, 2nd bleed; raised against full length MSK1, used for immunoblotting), anti-PRAS40 (S115B, 1st bleed; residues 238 - 256 of human PRAS40 DLPRPRLNTSDFQKLKRKY, used for immunoblotting). An antibody that recognizes PRAS40 phosphorylated at Thr246 (S114B, 2nd bleed) was raised against residues 240 - 251 of human PRAS40 CRPRLNTpSDFQK (used for immunoblotting). Anti-RSK2 (S382B, 1st bleed; residues 712 - 734 of human RNQSPVLEPVGRSTLAQRRGIKK, used for immunoblotting); phospho-RSK Ser227 (#sc-12445-R) and the total mTOR antibody (#sc-1549) were purchased from SantaCruz Biotechnology. The phospho-CREB Ser133 (#05-667) was from Millipore. The phospho-Akt Ser473 (#9271), 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), 4E-BP1 total (#9452), phospho 4E-BP1 Thr37/Thr46 (#9459), phospho 4E-BP1 Ser65 (#9451), phospho-ERK Thr202/Tyr204 ((#9101), total ERK ((#9102), phospho-RSK Thr573 (#9346), phospho-RSK Ser380 (#9341), phospho-GSK3α/β Ser21/9 (#9331), phospho-MSK Thr581 (#9595), CREB (#9197) and phospho-mTOR Ser2448 (#2971) antibodies were purchased from Cell Signaling Technology. For phospho-immunoblotting of the phosphorylated T-loop of S6K we employed the pan-PDK1 site antibody from Cell Signaling Technology (#9379) as previously described [24].The GSK3α/β antibody (44-610) was purchased from Biosource. 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* DH5α using a Qiagen plasmid Mega or Maxi kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. For transient transfections, ten 10-cm-diameter dishes of 293 cells were cultured and each dish was transfected with 5μg of the indicated plasmids using the polyethylenimine method [25].

Synthesis of PF-4708671. In the first stage 5-ethyl-4-(piperazin-1-yl)pyrimidine was generated as follows: In a 40 ml vial, 5-ethyl-pyrimidin-4-ol (524 mg, 4.22 mmol) and PyBOP (2940 mg, 5.49 mmol) were dissolved in dry DMF (5 ml). DBU (1 ml, 6.5 mmol) was added and the mixture was stirred at RT for 1 h. tert-butyl piperazine-

1-carboxylate (1570 mg, 8.44 mmol) was added and the mixture continued stirring at RT for 48 h. The reaction mixture was diluted with H_2O (150 ml). The solution was extracted with EtOAc (2 x 100 ml). The organic layer was washed with Brine (100 mL) and dried over Na_2SO_4. The organics were concentrated *in vacuo* to afford a light yellow oil. The crude oil was purified on SiO_2 using EtOAc/Heptane (10%-100% gradient). 1H NMR (400 MHz, *DMSO-d6*) d ppm 8.54 (s, 1 H), 8.28 (s, 1 H), 3.41 - 3.47 (m, 4 H), 3.31 - 3.36 (m, 4 H), 2.60 (q, *J*=7.39 Hz, 2 H), 1.42 (s, 9H), 1.16 - 1.22 (m, 3 H). The oil (724 mg) was then dissolved in 20 % TFA/DCM solution (10 ml). The solution was stirred at RT for 2.5 h. Solvent was removed in vacuo. The residue was dissolved in 1N NaOH and extracted with dichloromethane and concentrated to leave the product as a colourless oil (264 mg).

In the second stage PF-4708671 (2-((4-(5-ethylpyrimidin-4-yl)piperazin-1-yl)methyl)-5-(trifluoromethyl)-1H-benzo[d]imidazole) was generated as follows: To a solution of 5-ethyl-4-(piperazin-1-yl)pyrimidine (130 mg, 0.31 mmol) and 2-(chloromethyl)-5-(trifluoromethyl)-1H-benzo[d]imidazole (73 mg, 0.31 mmol) in DMF (2 ml) was added di-isopropyl-ethylamine (5 eq) and the reaction heated at 80 ºC for 18 h. Reaction was purified on SiO_2 (10% (v/v) MeOH/EtOAc) and isolated as a tan solid, 38 mg. 1H NMR (400 MHz, DMSO-d6) d ppm 1.18 (t, J=7.43 Hz, 3 H) 2.55 - 2.64 (m, 6 H) 3.39 - 3.44 (m, 4 H) 3.85 (s, 2 H) 7.49 (s, 1 H) 7.70 (s, 1 H)7.86 (s, 1 H) 8.25 (s, 1 H) 8.52 (s, 1 H) 12.81 (s, 1 H); LRMS calc. 390.148, found (M+1) 391.2.

PF-4708671 will be available to purchase from Sigma-Aldrich as well as Tocris.

Buffers. The following buffers were used: Tris lysis buffer [50mM Tris-HCl (pH 7.5), 1mM EGTA, 1mM EDTA, 0.3% (w/v) CHAPS, 1mM sodium orthovanadate, 10mM sodium-ß-glycerophosphate, 50mM sodium fluoride, 5mM sodium pyrophosphate, 0.27M sucrose, 0.15M NaCl, 0.1 % (v/v) 2-mercaptoethanol, 1mM benzamidine and 0.1mM phenylmethylsulphonylfluoride], Buffer A [50mM Tris-HCl (pH 7.5), 0.1mM EGTA and 0.1% (v/v) 2-mercaptoethanol], Hepes lysis buffer [40mM Hepes (pH 7.5), 120mM NaCl, 1mM EDTA, 0.3% (w/v) CHAPS, 10mM sodium pyrophosphate, 10mM sodium-ß-glycerophosphate, 50mM sodium fluoride, 0.5mM sodium orthovanadate, 1mM benzamidine and 0.1mM phenylmethylsulphonylfluoride], Hepes kinase buffer [25mM Hepes (pH 7.5), 50mM KCl], TBS-Tween Buffer [50mM Tris/HCl pH 7.5, 0.15M NaCl and 0.1% (v/v) Tween-20] and Sample Buffer [50mM Tris/HCl pH 6.8, 6.5% (v/v) Glycerol, 1% (w/v) SDS, and 1% (v/v) 2-mercaptoethanol]. Omnia Assay buffer [20mM Tris-HCl (pH 7.5), 15mM $MgCl_2$, 0.1mM EDTA, 50mM NaCl, 1mM DTT, 200µM ATP, and 5µM Omnia peptide 6.

Cell lysis. 293 cells were cultured and treated as described in the figure legends. Following treatment, cells were rinsed once with ice cold PBS and then lysed using Tris lysis buffer. Whole-cell lysates were centrifuged (18, 000 x g at 4 °C for 20 min), supernatants were removed and stored at -80 °C until required.

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 [26]. 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 the legend to Table 1. The concentration of magnesium acetate in the assays was 10mM and [γ-33P]ATP (~800 cpm/pmol) was used at 5µM for CK2α, DYRK3, EF2K, ERK1, ERK8, GSK3ß, HIPK2, IGF1R, IRR, MARK3, MKK1, p38γ MAPK, p38δ MAPK, PAK4, PIM2, Akt1, PLK1, PKCζ and PRK2; 20µM for CaMKKß, CDK2/cyclin A, CHK1, CHK2, CK1δ, CSK, EPH-B3, FGF-R1, IR, JNK1α1, JNK2α2, MAPKAP-K2, MST2,

MST4, p38ß MAPK, PKA, PAK5, PAK6, PDK1, PIM1, PIM3, PKCα, ROCKII, PRAK, SGK1, SYK, VEGFR and YES1; 50μM for AMPK, BRSK2, BTK, CaMK1, DYRK1a, DYRK2, EPH-A2, ERK2, IKKε, LCK, MELK, NEK2A, NEK6, p38α, PhKγ1, Akt2, PKD1, SRPK1 and TBK1, or 100μM for MSK1, RSK1, RSK2, S6K1 and S6K2 in order to be at or below the Km for ATP for each enzyme [26].

Lipid kinase panel.
Sphingosine Kinase 1 (SPHK1) was assayed as follows: SPHK1 (diluted in 50mM Tris/HCl pH 7.5, 150mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 1mM DTT) was assayed against Sphingosine in a final volume of 50 μl containing 50mM Tris/HCl pH 7.5, 150mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 10μM Sphingosine, 10μM ATP, 1mM DTT and incubated for 30 min at room temperature.
Sphingosine Kinase 2 (SPHK2) was assayed as follows: SPHK2 (diluted in 50mM Tris/HCl pH 7.5, 200mM KCl, 5mM $MgCl_2$, 1mM EGTA, 1mM DTT) was assayed against Sphingosine in a final volume of 50 μl containing 50mM Tris/HCl pH 7.5, 200mM KCl, 5mM $MgCl_2$, 1mM EGTA, 10μM Sphingosine, 1μM ATP, 1mM DTT and incubated for 30 min at room temperature.
Choline Kinase was assayed as follows: Choline Kinase (diluted in 25mM glycine-NaOH pH 8.5, 67mM KCl, 5mM $MgCl_2$) was assayed against Choline in a final volume of 50μl containing 25mM glycine-NaOH ph 8.5, 67mM KCl, 5mM $MgCl_2$, 1mM Choline, 1μM ATP, 1mM DTT and incubated for 30 min at room temperature. These three assays were stopped by addition of 50μl Kinase Glo Plus Reagent, incubated for 10 min at room temperature and read for 1 sec/well.
Class 1 PI3Kα was assayed as follows: PI3Kα (diluted in 50mM Hepes pH 7.5, 150mM NaCl, 0.02% Na Cholate, 1mM DTT) was assayed against PtdIns(4,5)P_2 diC8 in a final volume of 50μl containing 37mM Hepes pH 7.5, 111mM NaCl, 0.02% Na Cholate, 5mM DTT, 5mM $MgCl_2$, 1mM ATP, 2μM PtdIns(4,5)P_2 and incubated for 70 min at room temperature. Assays were stopped by addition of a 5.5μl solution of 50mM EDTA and 0.02% (w/v) sodium Cholate. 25μl of the resultant mixture was transferred to Lumitrac 200 plate. Detection mix (41mM Hepes pH 7.5, 123mM NaCl, 1.7μg GST-GRP1, 0.16μM PtdIns(3,4,5)P_3 Biotin, 1.6μg Streptavidin Allophycocyanin, 0.96μg/ml Eu Chelate labelled antibody) was added to give a final volume of 50μl and incubated for 20 min at room temperature before reading.
Class 1 PI3Kß was assayed as follows: PI3Kß (diluted in 50mM Hepes pH 7.5, 150mM NaCl, 0.02% Na Cholate, 1mM DTT) was assayed against PtdIns(4,5)P_2 diC8 in a final volume of 50μl containing 37mM Hepes pH 7.5, 111mM NaCl, 0.02% Na Cholate, 5mM DTT, 5mM $MgCl_2$, 1mM ATP, 2μM PtdIns(4,5)P_2 and incubated for 70 min at room temperature. Assays were stopped by addition of 5.5μl 50mM EDTA and 25μl transferred to Lumitrac 200 plate. Detection mix (41mM Hepes pH 7.5, 123mM NaCl, 1.7μg GST-GRP1, 0.16μM PtdIns(3,4,5)P_3 Biotin, 1.6μg Streptavidin Allophycocyanin, 0.96μg/ml Eu Chelate labelled antibody) was added to give a final volume of 50μl and incubated for 20 min at room temperature before reading.
Class 2 PI3KC2ß was assayed as follows: PI3KC2ß (diluted in 20mM Tris/HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM DTT, 0.5mM EGTA, 0.02% CHAPS) was assayed against phosphoinositide substrate in a final volume of 50μl containing 19mM Tris/HCl pH 7.5, 143mM NaCl, 0.96mM EDTA, 0.96mM DTT, 0.48mM EGTA, 0.02% CHAPS, 20μM phosphatidylinositol, 0.2mM ATP, 2mM $MgCl_2$ and incubated for 30 min at room temperature. Assays were stopped by addition of 5.5μl 50mM EDTA, 2% CHAPS and 25μl transferred to Lumitrac 200 plate. Detection mix (18.6mM Tris/HCl pH 7.5, 140mM NaCl, 0.9mM DTT, 0.02% CHAPS, 1.4μg SGK PX, 0.06μM PtdIns(3)P Biotin, 1.6μg Streptavidin Allophycocyanin, 0.64μg/ml Eu Chelate labelled antibody) was added to give a final volume of 50ml and incubated for 30 min at room temperature before reading.
Class 3 VPS34 was assayed as follows: VPS34 (diluted in 20mM Tris/HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM DTT, 0.5mM EGTA, 0.02% CHAPS) was assayed against phosphoinositide substrate in a final volume of 50μl containing 19mM Tris/HCl pH 7.5, 143mM NaCl, 0.96mM EDTA, 0.96mM DTT, 0.48mM EGTA,

0.02% CHAPS, 20μM phosphatidylinositol, 0.2mM ATP, 2mM $MnCl_2$ and incubated for 60 min at room temperature. Assays were stopped by addition of 5.5μl 50mM EDTA, 2% CHAPS and 25μl transferred to Lumitrac 200 plate. Detection mix (18.6mM Tris/HCl pH 7.5, 140mM NaCl, 0.9mM DTT, 0.02% CHAPS, 1.4μg SGK PX, 0.06μM PtdIns(3)P Biotin, 1.6μg Streptavidin Allophycocyanin, 0.64μg/ml Eu Chelate labelled antibody) was added to give a final volume of 50ml and incubated for 30 min at room temperature before reading.

mTORC1 activity assays. 293 cells were lysed in Hepes lysis buffer and 3mg of lysate was pre-cleared by incubation with 5μl of Protein G-Sepharose conjugated to pre-immune IgG. The lysates were then incubated with 5μl of Protein G-Sepharose covalently conjugated to either 5μg anti-Raptor antibody, or 5μg pre-immune IgG for 1.5 hrs at 4ºC on a vibrating platform. The immunoprecipitates were washed four times with Hepes lysis buffer, followed by two washes with Hepes kinase buffer. For maximal mTORC1 activity, the lysis buffer for the initial two wash steps contained 0.5M NaCl. Kinase reactions were initiated by adding 0.1mM ATP and 10mM Mg in the presence or absence of PF-4708671 and inactive GST-S6K1 (0.5μg). Reactions were carried out for 30min at 30ºC on a vibrating platform and stopped by the addition of SDS sample buffer. Reactions were filtered through a 0.22 μm Spin-X filter and samples subjected to electrophoresis and immunoblot analysis.

Purification of GST-S6K1 and GST-S6K2 from 293 cells
24hrs post-transfection, 293 cells, which had been transfected with GST-S6K1 or GST-S6K2, were serum starved for 16hrs. Cells were treated with 50ng/ml IGF1 for 40min (in order to obtain active GST-S6K1 and GST-S6K2) or with 0.1μM rapamycin for 30min (to obtain inactive GST-S6K1) and harvested in Tris-CHAPS lysis buffer. 3mg lysate was affinity purified on 10μl glutathione-Sepharose for 1hr at 4°C on a rotating wheel. The resulting precipitates were washed twice with Tris-CHAPS lysis buffer, twice with buffer A and twice with buffer A containing 0.27M sucrose. GST-tagged proteins were eluted from the resin by resuspension in an equal amount of buffer A containing 0.27M sucrose and 10mM glutathione (pH7.5-8) for 1hr on ice. Supernatants were filtered through a 0.22μm-spin column and aliquots were snap-frozen and stored at -80°C.

Protein kinase activity assays.
For selectivity IC_{50} assays purified active GST-S6K1, GST-S6K2, His-MSK1 [residues 2-802], His-RSK1 [residues 1-735] and His-RSK2 [residues 2-740] (0.5 units/ml) were assayed for 30min at 30°C in a 50μl assay mixture in buffer A containing either 30μM Crosstide (GRPRTSSFAEG, for S6K1, S6K2 and MSK1) or 30μM Long S6 (KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK, for RSK1 and RSK2), 10mM magnesium acetate and 100μM [γ-^{32}P] ATP. Reactions were terminated and incorporation of [γ-^{32}P]-phosphate into the peptide substrate was determined by applying the reaction mixture onto P81 phosphocellulose paper and scintillation counting after washing the papers in phosphoric acid. One unit of activity was defined as that which catalysed the incorporation of 1 nmol of [^{32}P]-phosphate into the substrate. To determine the Ki for PF-4708671 full-length recombinant S6K1 was added to a final concentration of 5 nM to Omnia Assay buffer containing variable concentrations of compound. The reaction was run for 60 min at 30°C in a 50μl assay volume. The fluorescence of the peptide was monitored at an excitation wavelength of 360 nm and an emission wavelength of 485 nm. The rate of the reaction at each compound concentration was normalized to the DMSO control rate and this normalized rate versus concentration was fit to the Morrison tight-binding equation for a competitive inhibitor to provide the true Ki [27]. In order to assay S6K activity in 293 cell lysates, cells were lysed in Tris lysis buffer. 0.5mg of lysate was incubated with 5 μg of S6K antibody conjugated to Protein G-Sepharose for 1hr at 4 ºC on a vibrating platform. Immunoprecipitates were washed twice with lysis buffer, twice with buffer A and kinase activity was assayed exactly as before using the Crosstide peptide.

Immunoblotting. Total cell lysate (20µg) or immunoprecipitated samples were heated at 70°C for 5 min in sample buffer, and subjected to 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 16hrs at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

Results.

PF-4708671 is a specific S6K1 inhibitor. PF-4708671, a piperazinyl-pyrimidine analogue was synthesised as described in the Materials and Methods (Fig1A). PF-4708671 inhibited the activity of full length S6K1 in vitro with a Ki of 20 nM and S6K1 isolated from IGF1-stimulated 293 cells with an IC50 of 0.16μM (Fig 1B) and only inhibited very weakly the closely related S6K2 isoform (IC50 65μM) (Fig 1C). We next studied the effect of PF-4708671 on the activity of RSK1, RSK2 and MSK1 that are related to S6K1. We found that PF-4708671 inhibited RSK1 (IC50 of 4.7μM) and RSK2 (IC50 of 9.2μM) over 20-fold less potently than S6K1. PF-4708671 inhibited MSK1 (IC50 of 0.95μM) four fold more weakly than S6K1. To further evaluate the specificity of PF-4708671, we studied the effect of PF-4708671 upon the activity of 77 protein kinases including 13 AGC kinase family members that were assayed at ATP concentrations approximate to their K_m constant for ATP (Table 1). At 10μM PF-4708671, which nearly ablated S6K1, only MSK1 was inhibited by more than 75%. In addition PF-4708671 did not inhibit 10 lipid kinases tested (Table 2).

PF-4708671 suppresses S6K1 activity. We tested the effect of adding increasing amounts of PF-4708671 on the IGF1-induced phosphorylation of the ribosomal S6 protein, a well-characterised substrate of S6K1. We monitored the phosphorylation of two sets of residues that are phosphorylated by S6K1, namely Ser235/Ser236 as well as Ser240/Ser244 [28]. Stimulation of serum starved 293 cells with IGF1 induced marked phosphorylation of these two sets of sites on S6 protein, which as expected was inhibited by the dual PI3K/mTOR inhibitor PI-103 (1μM) as well as with the mTORC1 specific inhibitor rapamycin (0.1μM). Phosphorylation of S6 protein was inhibited by PF-4708671 in a dose dependent manner so that it was significantly reduced by 3μM and almost abolished by 10μM inhibitor (Fig 2A). We also observed that PF-4708671 suppressed the phosphorylation of two other S6K1 substrates: mTOR at Ser2448 [29] and Rictor at Thr1135 [8-10]. However, PF-4708671 did not suppress the phosphorylation of S6K1 at its activating Thr389 (mTORC1 site) or Thr229 (PDK1 site) residues, indicating that the drug is not inhibiting upstream components of the PI-3K pathway. Consistent with this, PF-4708671 did not affect the phosphorylation or electrophoretic mobility of 4EBP1, an mTORC1 substrate. In addition PF-4708671 did not inhibit the IGF1-induced phosphorylation of Akt at Thr308 or Ser473, nor phosphorylation of the Akt specific substrate PRAS40, suggesting that it was specifically inhibiting S6K1 signalling.

PF-4708671 does not suppress phosphorylation of RSK and MSK substrates under conditions in which it inhibits S6K1 activity. As PF-4708671 also inhibited RSK and MSK isoforms in vitro, albeit at lower potency than S6K1, we evaluated whether PF-4708671 inhibited these kinases in cells. Previous work has established that treatment of 293 cells with phorbol esters leads to the activation of RSK, MSK as well as S6K1 via the ERK signalling pathway [30, 31]. Under these conditions RSK phosphorylates GSK3α/β at Ser21/Ser9 [32], MSK phosphorylates CREB at Ser133 [30] and S6K1 phosphorylates S6 protein [4]. Consistent with PF-4708671 inhibiting S6K1 activity we observed that TPA-induced phosphorylation of S6 protein was suppressed by PF-4708671 in a dose dependent manner, similar to that seen in IGF1-stimulated 293 cells (Fig 2B). However, even at 10μM PF-4708671, which completely ablates S6 protein phosphorylation, no inhibition of GSK3α/β (mediated by RSK) or CREB phosphorylation (mediated by MSK) was observed. This indicates that PF-4708671 is not markedly inhibiting RSK or MSK isoforms in vivo.

PF-4708671 promotes rapid phosphorylation of S6K1 at Thr229 and Thr389. We observed that the addition of PF-4708671 to 293 cells cultured in the absence (Fig 3A) or presence of serum (Fig 3B) led to a significant dose dependent enhancement of S6K1 phosphorylation at both Thr229 and Thr389. This was accompanied by an increase in S6K1 activity (measured after immunoprecipitation and extensive washing to remove the inhibitor). Maximal stimulation of S6K1 phosphorylation and activity

plateaued at 3-10μM PF-4708671 and was not further increased by 30μM PF-4708671. However, when compared to IGF1stimulation, PF-4708671-induced activation of S6K1 was ~5-fold less in the presence of serum and 10-fold less in the absence of serum (Fig 3A and 3B). Stimulation of S6K1 phosphorylation at Thr389 and Thr229 was rapid, with a significant increase observed after 1 min. In contrast, PF-4708671 did not stimulate the phosphorylation of Akt at Thr308 or Ser473, nor affect the phosphorylation status of PRAS40 (Fig 3C and 3D).

Ability of PF-4708671 to stimulate S6K1 phosphorylation is dependent upon mTORC1. We next decided to investigate whether PF-4708671-induced S6K1 phosphorylation was dependent upon mTORC1. We observed that PF-4708671 only induced significant phosphorylation of S6K1 in the presence of amino acids, conditions under which mTORC1 activity is maximal (Fig 4A). We also noticed in these experiments that overnight serum starvation of 293 cells resulted in a marked reduction in levels of S6K1 protein (Fig 4A). We have also made similar observations in mouse embryonic fibroblast cells (LP data not shown), and it would be interesting to further explore the mechanism by which S6K1 levels reduce following serum starvation. In addition the ability of PF-4708671 to stimulate phosphorylation of S6K1 at Thr389 and Thr229 was suppressed by inhibitors targeting components of the PI3K pathway. The dual PI3K and mTOR inhibitor PI-103 (1μM), the PI3K specific inhibitor GDC-0941 (1μM), the Akt inhibitor Akti-1/2 (10μM) [33] and the mTOR kinase inhibitor Ku-0063794 (1μM) [34] all suppressed phosphorylation of S6K1 induced by PF-4708671 (Fig 4A). Moreover, rapamycin (0.1μM), which potently inhibits mTORC1 (but not mTORC2), prevented PF-4708671-induced phosphorylation of S6K1.

PF-4708671 does not affect activity of mTORC1. In order to determine the effect that PF-4708671 had on mTORC1 activity we immunoprecipitated the mTORC1 complex from 293 cells treated in the presence or absence of PF-4708671. The Raptor immunoprecipitates were washed in the presence or absence of 0.5M NaCl and activity assayed in the absence of PF-4708671 by monitoring phosphorylation of recombinant S6K1 at Thr389. As reported previously [35] washing mTORC1 immunoprecipitates with 0.5M NaCl markedly enhanced mTORC1 activity, presumably due to the removal of the inhibitory PRAS40 subunit (Fig 4C). The activity of mTORC1 measured with or without a 0.5M NaCl wash was not affected by incubating cells with concentrations of PF-4708671 up to 10μM. This observation suggests that PF-4708671 is not directly activating mTORC1. We also observed that when in vitro mTORC1 kinase assays were carried out in the presence of PF-4708671, there was no effect upon the phosphorylation of S6K1 by mTORC1 in vitro (Supplementary figure 1).

Discussion.

In this study we have described a novel cell-permeable S6K1 inhibitor, which suppresses the phosphorylation of the S6K1 substrates S6, Rictor (Thr1135) and mTOR (Ser2448). In vitro specificity analysis also suggests that PF-4708671 does not significantly inhibit the activity of the closely related S6K2 isoform (Fig 1C) or a number of other AGC kinases (Akt1, Akt2, PKA, PKCα, PKCε, PRK2, ROCK2, RSK1, RSK2, SGK1) in vitro. From a panel of 77 protein kinases and 10 lipid kinases tested only MSK1 was significantly inhibited albeit 4-fold less potently than S6K1. Our cell based studies support the conclusion that PF-4708671 is a specific inhibitor, as it does not inhibit the phosphorylation of Akt, RSK and MSK1 substrates.

We observed that treating cells with PF-4708671, although suppressing phosphorylation of established S6K1 substrates, also induced rapid phosphorylation of both the T-loop and hydrophobic motif of S6K1. This was observed under conditions of serum starvation or in the presence of serum but not seen when cells were treated with IGF1 or TPA, agonists that induce maximal phosphorylation of S6K1. It is possible that PF-4708671-induced phosphorylation is a result of inhibiting the negative feedback loop in which S6K1 phosphorylates IRS-1, promoting its degradation and suppressing PI3K pathway activity [15, 16]. However, it is not clear whether this mechanism would be sufficiently rapid to account for the PF-4708671-induced phosphorylation of S6K1, as this was observed after just 1 min. Moreover, we were unable to detect phosphorylation of IRS-1 at Ser302, nor any change in the protein level of IRS-1 following PF-4708671 treatment (LRP, unpublished observations). We also observed that PF-4708671 did not promote phosphorylation of Akt at Thr308 or Ser473 or phosphorylation of the PRAS40 Akt substrate. While inhibitors of PI3K and Akt suppressed the ability of PF-4708671 to stimulate phosphorylation of S6K1, these drugs inactivate mTORC1, which is likely to account for this observation. Consistent with this, the ability of PF-4708671 to promote S6K1 phosphorylation is ablated with other treatments that inactivate mTORC1 including amino acid starvation and treatment with rapamycin. It is possible that binding of PF-4708671 to S6K1 promotes its phosphorylation by mTORC1 in a manner similar to Akt inhibitors such as A-443654 which stimulate phosphorylation of Akt at Ser473 and Thr308 [36]. Moreover, another PKC Inhibitor termed Bim1 which is a relatively non-selective PKC inhibitor also promotes marked hyper-phosphorylation of PKC isoforms Our observations indicate that this is not the case as PF-4708671 did not affect the phosphorylation of S6K1 at Thr389 by immunoprecipitated mTORC1 in vitro (Fig 4C and Supplementary Fig 1). Further work is required, but our results hint that there is an additional feedback pathway by which S6K1 might regulate its own phosphorylation by mTORC1. Obviously this may be complex to unravel, as PF-4708671 does not enhance activity of immunoprecipitated mTORC1 or promote phosphorylation of the 4E-BP1 mTORC1 substrate.

Until now no S6K1 inhibitors have been available and inhibitors of the PI3K pathway such as PI-103 and GDC-0941 and rapamycin have been employed to infer roles of S6K1. This is not ideal as PI3K pathway inhibitors also prevent the activity of other signalling components (including that of Akt). While rapamycin specifically targets mTORC1, S6K1 is not the only substrate of this mTOR complex, which also phosphorylates 4EBP1, important for Cap-dependent translation. Moreover, it is likely that other mTORC1 substrates exist. Comparison of the effects of rapamycin and PF-4708671 will help to dissect the relative contribution of S6K1 to physiological processes controlled by mTORC1 such as growth, translation, proliferation, longevity and insulin resistance.

One other benefit of an S6K1-specific inhibitor is that it would help to distinguish between the cellular roles of S6K1 and S6K2. Both isoforms share a high degree of similarity although S6K1 is found predominantly in the cytosol, whereas S6K2 seems to be restricted to the nucleus [37]. While S6K1$^{-/-}$ mice are significantly smaller [3], the birth weight of S6K2$^{-/-}$ mice is similar to that of wild-type mice [38]. It is often

very difficult to determine the specific roles of isoforms of kinases. Thus far only SKAR [39] and Rictor [9] have been shown to be specific S6K1 substrates. No specific substrates of S6K2 have yet been described. Hence PF-4708671 could help to elucidate isoform-specific functions of S6K1 and S6K2. For example treatment of S6K2$^{-/-}$ KO MEFS with PF-4706871 would allow the identification of proteins that are specifically phosphorylated by S6K1.

Previous studies characterising S6K1 knockout mice revealed significant residual phosphorylation of ribosomal S6 protein, which suggested that S6K2 may also phosphorylate ribosomal S6 protein [3, 38]. However our data demonstrate that concentrations of PF-4708671 that do not inhibit S6K2, ablate phosphorylation of ribosomal S6 protein at Ser235, Ser236, Ser240 and Ser244 within 30 min. This suggests that S6K1 is the predominant kinase that phosphorylates ribosomal S6 protein at least in 293 cells that were employed in this study. It is likely that in the S6K1 knockout mice, compensatory pathways have evolved to enable ribosomal S6 protein to become phosphorylated. Indeed mRNA levels of S6K2 were markedly elevated in all tissues of S6K1 knockout mice [3].

Taken together with the recent advances in the discovery of novel specific kinase inhibitors such as Akti-1/2 [33] or MK-2206 [40] (Akt inhibitors) and BI-D1870 (RSK inhibitor) [31], the identification of PF-4708671 should help to yield further information on the specific cellular roles of AGC kinases. In addition the development of this S6K1 inhibitor, together with the recently solved structure of the inactive conformation of S6K1 [41] could act as a basis upon which to develop improved S6K1 kinase inhibitors, which might one day contribute to the treatment of human diseases including cancer and insulin-resistance.

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Figure Legends

Table 1 Effect of PF-4708671 upon the activity of 77 protein kinases

Results are presented as a percentage of kinase activity in control incubations, in which PF-4708671 was omitted. Protein kinases were assayed as described in Materials and methods and the results are an average of three separate reactions ± S.D. † indicates AGC kinase family members, * indicates inhibition of >2-fold. 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; CSK, C-terminal Src kinase; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; EF2K, elongation-factor-2 kinase; EPH, ephrin; FGF-R, fibroblast growth factor receptor; GSK, glycogen synthase kinase; HIPK, homeodomain-interacting protein kinase; IGF1R, IGF1 receptor; IKK, inhibitory κB kinase; IR, insulin receptor; IRR, insulin-related receptor; JNK, c-Jun N-terminal kinase; Lck, lymphocyte cell-specific protein tyrosine kinase; 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; 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; 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 PF-4708671 upon the activity of 10 lipid kinases

Results are presented as a percentage of lipid kinase activity in control incubations, in which PF-4708671 was omitted. Lipid kinases were assayed as described in Materials and methods and the results are an average of three separate reactions ± S.D. Abbreviations not defined in main text: 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.

Figure 1 PF-4708671 inhibits S6K1 in vitro

(**A**) Structure of the kinase inhibitor, PF-4708671 (2-((4-(5-ethylpyrimidin-4-yl)piperazin-1-yl)methyl)-5-(trifluoromethyl)-1H-benzo[d]imidazole). (**B**) Active GST-S6K1 (isolated from IGF1-stimulated 293 cells), GST-S6K2 (isolated from IGF1-stimulated 293 cells) (**C**), His-MSK1 (baculovirus expressed) (**D**), His-RSK1 (baculovirus expressed) (**E**) or His-RSK2 (baculovirus expressed) (**F**) were assayed in the presence or absence of increasing concentrations of PF-4706871 with 10mM Mg and 100μM ATP. Results are presented as percentage of kinase activity relative to the control measured in the presence of DMSO. Results are the average of at least duplicate reactions where similar results were obtained in at least one other experiment. Dashed line indicates concentration at which 50% of S6K1 activity is inhibited.

Figure 2 PF-4708671 inhibits S6K1 activity in vivo

293 cells that had been deprived of serum for 16hrs were treated with the indicated concentrations of PF-4708671 for 30min prior to stimulation with either 50ng/ml IGF1 (**A**) or 400ng/ml TPA (**B**) for 30min. Cells were lysed and lysates

immunoblotted with the indicated antibodies as described in the materials and methods section. For detection of Rictor phosphorylated at T1135, Rictor was immunoprecipitated from 3mg lysate and immunoblotting carried out using Rictor and pT1135 antibodies. Similar results were obtained in three separate experiments.

Figure 3 PF-4708671 induces phosphorylation of S6K1
(**A**) 293 cells that had been serum starved for 16hrs were treated with the indicated concentrations of PF-4708671 for 30min. Cells were lysed and S6K1 immunoprecipitated and catalytic activity assessed employing the Crosstide substrate. Ear bar represents the mean specific activity ± S.E.M. from 3 separate samples. Cell lysates were also analysed by immunoblotting using the indicated antibodies. (**B**) As in (**A**) except cells were cultured in the presence of 10% foetal bovine serum. (**C**) As in (**A**) except cells were treated with 10μM PF-4708671 for the indicated times. (**D**) As in (**C**) except cells were maintained in the presence of 10% foetal bovine serum. Results are representative of at least two separate experiments.

Figure 4 PF-4708671-induced phosphorylation of S6K1 is dependent upon mTORC1
(**A**) 293 cells were serum starved for 16hrs before incubation for 1hr in amino acid-free EBSS (Earle's Balanced Salt Solution) medium containing 10% dialysed serum. Cells were then incubated in the absence or presence of the indicated concentrations of PF-4708671 for 30min prior to re-addition of physiological levels of amino acids for an additional 30min. Cell lysates were analysed by immunoblotting with the indicated antibodies. (**B**) 293 cells cultured in the presence of 10% foetal bovine serum were pre-treated with the indicated concentrations of PI-103, GDC-0941, Akti-1/2, Rapamycin or Ku-0063794 for 30min prior to treatment with 10μM PF-4708671 for 30min. Cell lysates were subjected to immunoblotting analysis with the antibodies shown. (**C**) 293 cells were treated with either DMSO or the indicated amounts of PF-4708671 for 30min before lysis. Lysates were then subjected to immunoprecipitation with preimmune IgG (IgG) or Raptor (R) antibodies and immunoprecipitates washed with lysis buffer with or without 0.5M NaCl. In vitro kinase assays were carried out using dephosphorylated GST-S6K1 as the substrate at 30°C for 30min. Kinase reactions and cell lysates were subjected to immunoblotting using the indicated antibodies. Results are representative of at least two separate experiments.

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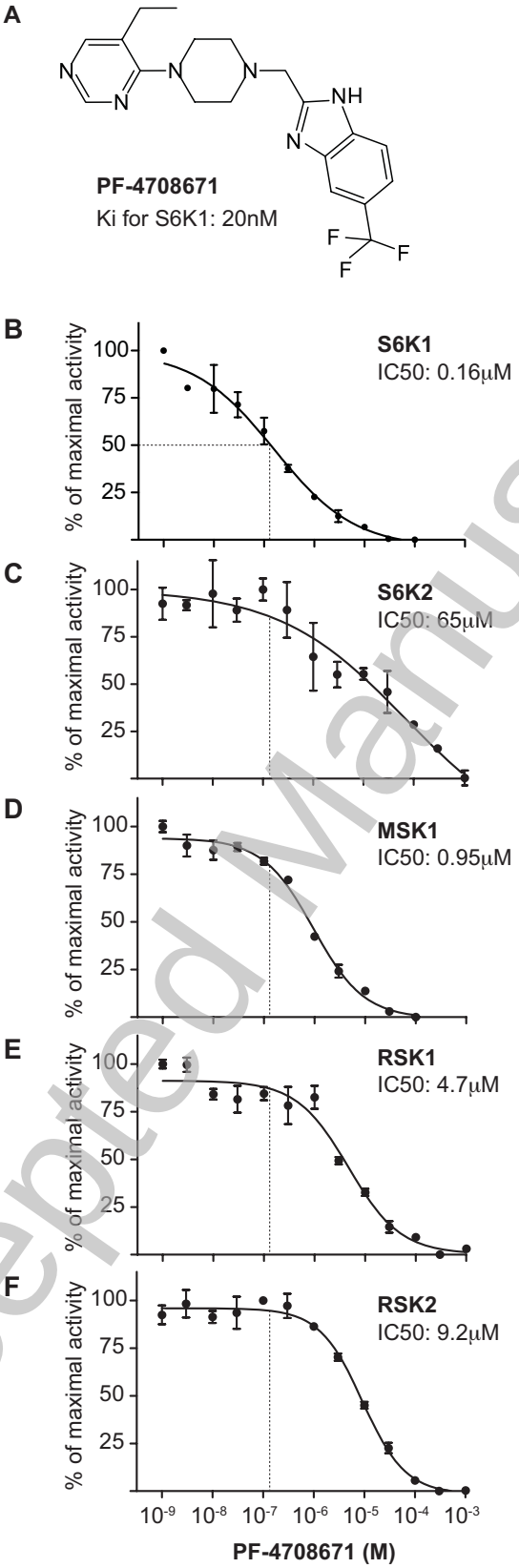

FIGURE 1

Kinase	Percentage of activity remaining			Kinase	Percentage of activity remaining		
	0.1μM PF-4708671	1μM PF-4708671	10μM PF-4708671		0.1μM PF-4708671	1μM PF-4708671	10μM PF-4708671
AMPK	100±0	88±3	46±1*	MKK1	117±8	104±2	96±1
Aurora B	89±5	87±3	49±9*	MLCK	95±9	79±3	87±16
BRSK2	88±4	84±6	83±2	MNK1	101±1	91±3	79±2
BTK	83±4	87±7	92±8	MNK2α	92±2	93±10	79±11
CAMK1	106±9	94±22	78±12	MSK1[=]	80±6	42±2*	14±0*
CAMKKβ	99±12	98±0	80±1	MST2	95±3	85±5	77±3
CDK2/Cyclin A	102±21	101±10	88±8	MST4	87±4	77±5	46±3*
CHK1	90±17	83±16	83±5	NEK2A	95±3	94±3	91±3
CHK2	101±3	73±17	72±6	NEK6	88±4	75±2	80±11
CK1δ	96±7	93±3	71±2	PAK4	93±7	88±3	88±7
CK2α	96±10	92±2	83±12	PAK5	100±23	88±11	87±0
CSK	94±10	93±6	98±6	PAK6	99±3	83±9	86±4
DYRK1A	99±4	94±6	85±3	PDK1[=]	90±6	86±16	95±2
DYRK2	97±17	104±3	101±2	PhKγ1	100±7	96±10	104±7
DYRK3	106±2	92±6	83±11	PIM1	88±2	90±4	78±6
EF2K	90±1	73±2	78±4	PIM2	90±6	90±7	100±12
EPH-A2	95±12	86±5	89±15	PIM3	93±2	88±8	83±2
EPH-B3	132±2	139±6	101±5	PKA[=]	84±0	83±1	34±10*
ERK1	93±16	85±9	83±28	Akt1[=]	87±2	69±5	52±5
ERK2	93±8	85±1	86±6	Akt2[=]	74±7	73±2	60±2
ERK8	85±1	83±16	71±9	PKCα[=]	119±10	106±9	62±0
FGFR1	91±1	92±2	63±1	PKCζ[=]	78±8	75±19	70±16
GSK3β	87±5	96±8	92±2	PKD1	94±5	83±5	67±3
HIPK2	108±8	86±16	88±2	PLK1	98±6	87±1	88±4
IGF1R	72±6	95±19	82±9	PRAK	114±10	97±10	87±6
IKKβ	195±131	230±167	192±131	PRK2[=]	95±1	86±7	84±3
IKKε	103±6	99±1	96±5	ROCKII[=]	98±2	91±12	58±10
IR	108±5	95±5	96±8	RSK1[=]	85±0	83±4	33±2*
IRR	88±8	96±14	77±16	RSK2[=]	100±3	86±8	45±7*
JNK1α1	111±34	107±6	95±0	S6K1[=]	58±8	23±2*	7±2*
JNK2α2	92±3	93±2	98±5	S6K2[=]	100±8	65±5	55±4
Lck	91±7	92±10	88±6	SGK1[=]	106±3	74±7	65±6
p38α MAPK	86±3	79±12	86±5	Src	103±10	99±31	92±4
p38β MAPK	88±7	89±10	97±11	SRPK1	108±11	106±15	101±16
p38γ MAPK	107±10	102±0	103±4	SYK	121±14	100±12	91±4
p38δ MAPK	89±10	82±0	91±3	TBK1	100±10	89±0	93±5
MAPKAP-K2	88±22	105±5	78±13	VEGFR	97±10	83±3	37±2*
MARK3	94±8	83±4	82±0	YES1	98±1	84±1	76±7
MELK	94±5	101±11	87±1				

Table 1 Effect of PF-4708671 upon the activity of 77 protein kinases

Kinase	Percentage of activity remaining	
	1μM PF-4708671	10μM PF-4708671
PI3Kα	107±6	117±6
PI3Kβ	103±6	115±7
PI3Kγ	101±6	102±7
PIK4CA	106±5	102±12
PIK4CB	103±2	99±11
PIP5K2A	101±5	100±2
SPHK1	99±5	103±6
SPHK2	100±13	98±4
CHKA	98±3	94±4
DGKβ	98±2	99±1

Table 2 Effect of PF-4708671 upon the activity of 10 lipid kinases

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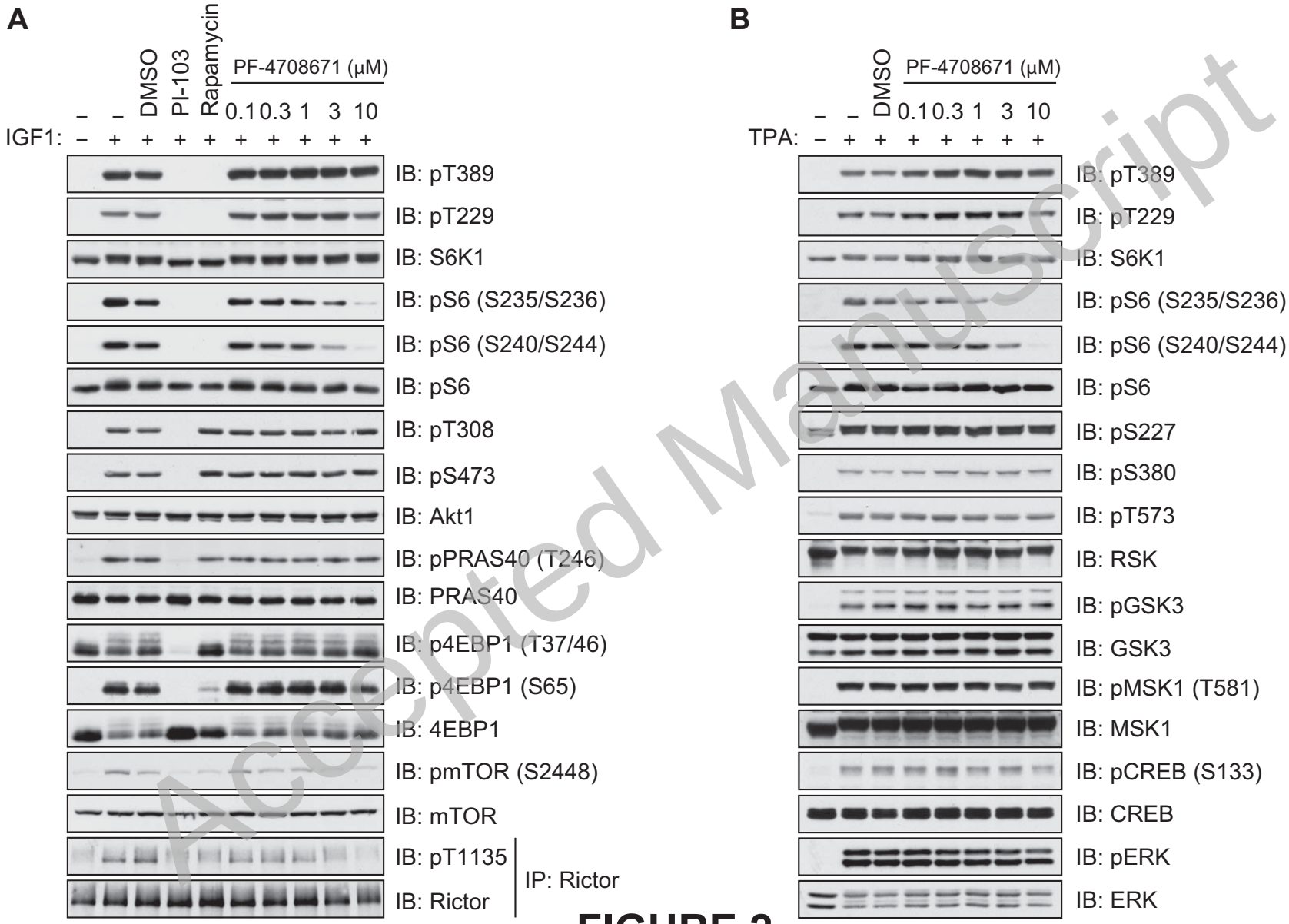

FIGURE 2

Biochemical Journal Immediate Publication. Published on 12 Aug 2010 as manuscript BJ20101024

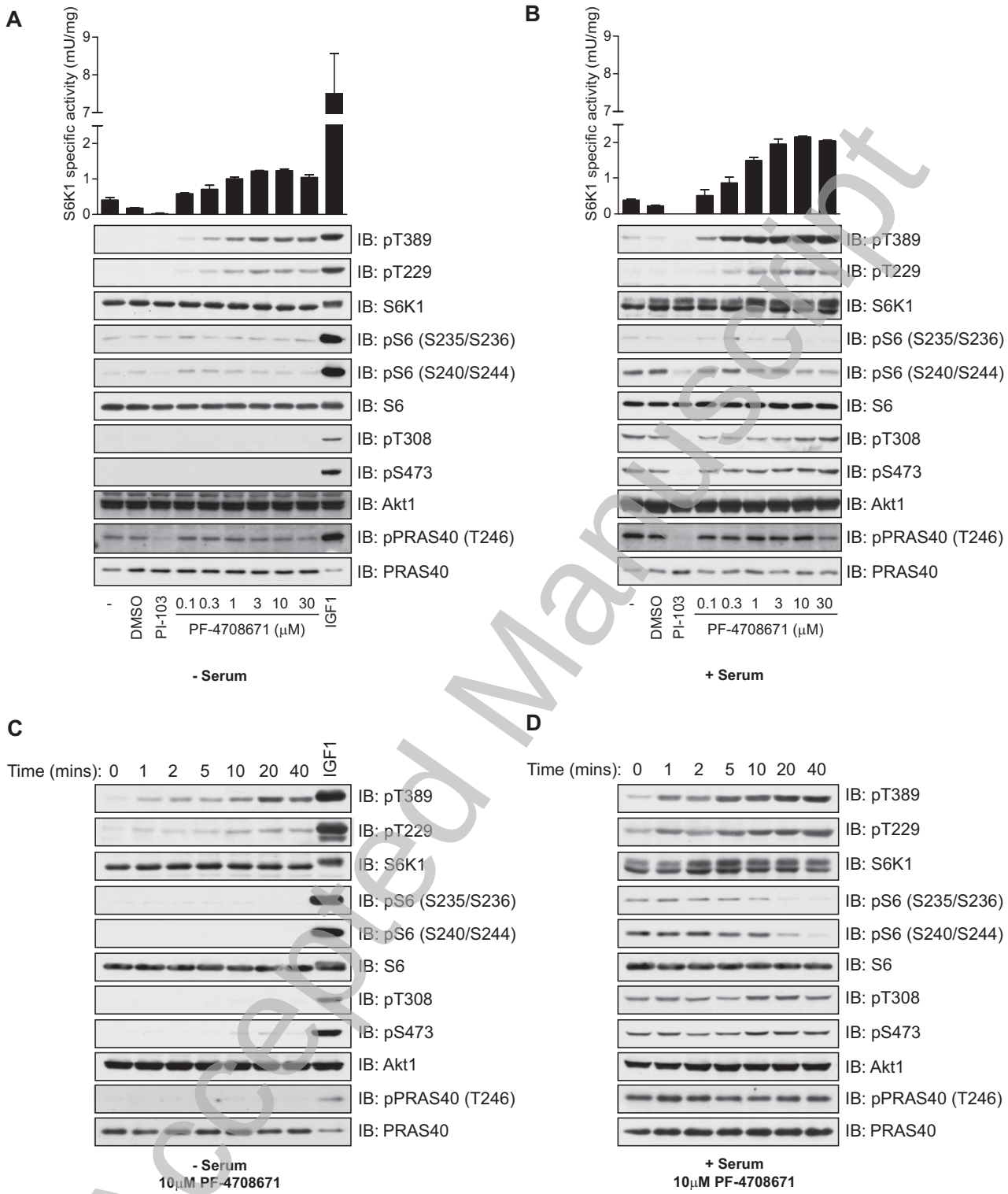

FIGURE 3

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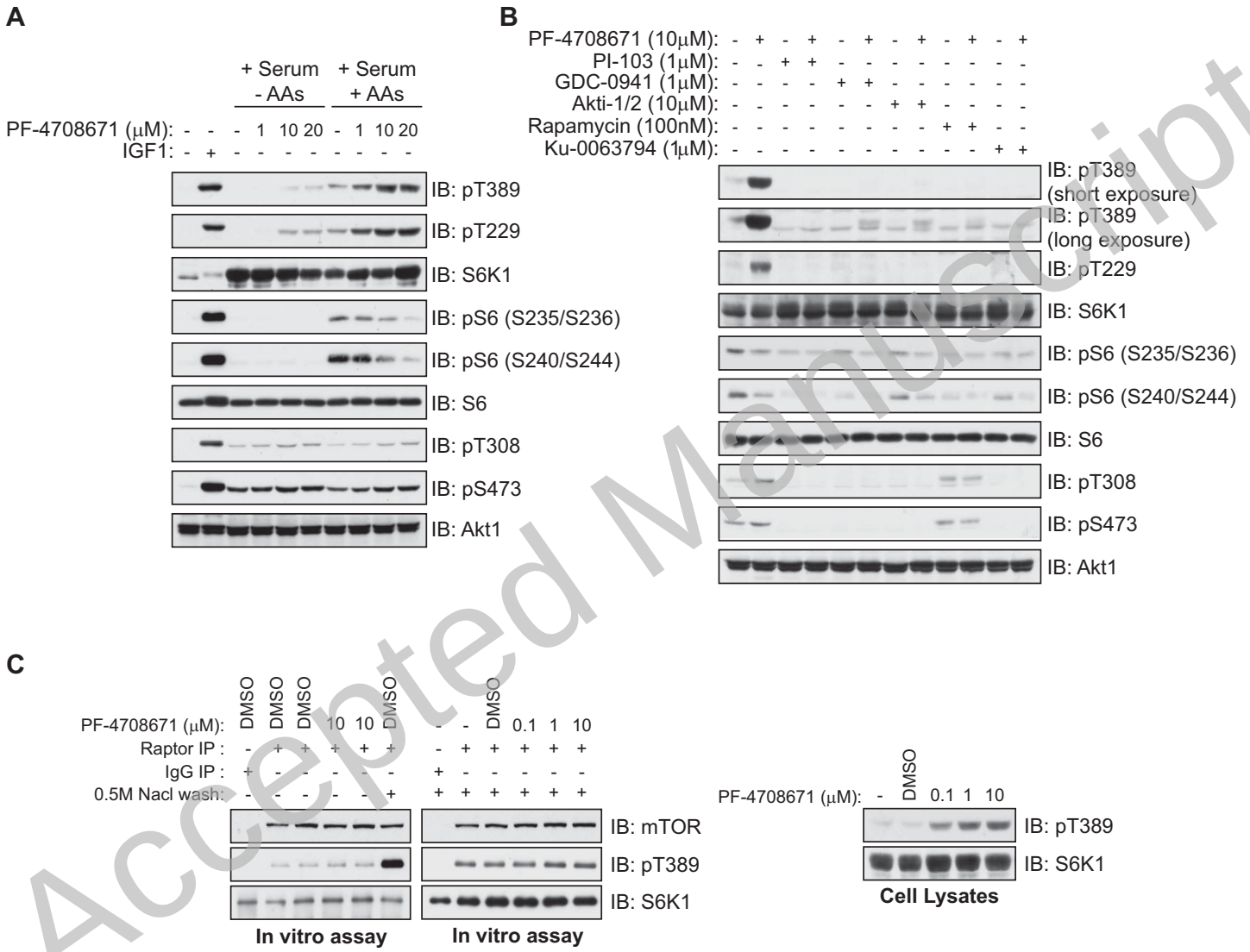

FIGURE 4