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BI-D1870 is a specific inhibitor of the p90 Ribosomal S6 Kinase (RSK) isoforms in vitro and in vivo

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Running Title: Identification of a p90RSK inhibitor.

Abbreviations: GST, glutathione-S-transferase; MKK1, mitogen activated protein kinase kinase-1; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; MSK1, mitogen and stress activated protein kinase-1; S6K, p70 ribosomal S6 kinase; RSK, p90 ribosomal S6 kinase; TPA, tetradecanoylphorbol acetate.

Abstract. Hormones and growth factors induce the activation of a number of protein kinases that belong to the AGC subfamily, including isoforms of protein kinase A (PKA), protein kinase B (PKB also known as Akt), protein kinase C (PKC), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK) and the mitogen and stress activated protein kinase (MSK), which then mediate many of the physiological processes that are regulated by these extracellular agonists. It can be difficult to assess the individual functions of each AGC kinase because their substrate specificities are similar. Here we describe the small molecule BI-D1870, which inhibits RSK1, RSK2, RSK3 and RSK4 in vitro with an IC_{50} of 10-30 nM, but does not significantly inhibit 9 other AGC kinase members and over 40 other protein kinases tested at 100-fold higher concentrations. BI-D1870 is cell permeable and prevents the phorbol ester and EGF-induced phosphorylation of GSK3β and LKB1 in 293 and Rat-2 cells, which are mediated by RSK. In contrast, BI-D1870 does not affect the agonist-triggered phosphorylation of substrates for six other AGC kinases. Moreover, BI-D1870 does not suppress the phorbol ester or EGF-induced phosphorylation of CREB, consistent with the genetic evidence indicating that MSK, and not RSK, isoforms mediate the mitogen-induced phosphorylation of this transcription factor.

Introduction

Members of the AGC subfamily of protein kinases, including isoforms of cyclic AMP dependent protein kinase (PKA), protein kinase B (PKB), p70 ribosomal S6 kinase (S6K), protein kinase C (PKC), p90 ribosomal S6 kinase (RSK) and mitogen and stress activated protein kinase (MSK), mediate many of the cellular effects of extracellular agonists by phosphorylating key regulatory proteins. These protein kinases possess catalytic domains that are ~40 % identical to each other, but contain distinct non-catalytic domains, and participate in diverse signalling pathways. AGC kinases are also activated by different mechanisms. For example, agonists that stimulate adenylate cyclase induce the activation of PKA [1], while those that stimulate PI 3-kinase induce the activation of PKB, S6K and certain isoforms of PKC [2-4]. RSK isoforms are activated by the MAP kinase family members ERK1 and ERK2 in response to growth factors, phorbol esters and other agonists [5, 6], whereas MSK isoforms are activated in vivo by two different MAP kinase family members, namely ERK1/ERK2 and the stress and cytokine-activated p38 MAP kinase [7]. RSK and MSK isoforms are unusual in that they possess two catalytic domains in a single polypeptide. The N-terminal kinase domain is an AGC family member and catalyses the phosphorylation of all known substrates of these enzymes. The C-terminal kinase domain, which does not belong to the AGC family is required for the activation of the N-terminal kinase domain (reviewed in [8]).

In order to study the physiological roles of AGC kinases, a commonly used approach has been to overexpress the active forms in cells. However, due to the overlapping substrate specificities of many AGC kinases, it is likely that the overexpression of one member of this

kinase subfamily will result in the phosphorylation of substrates that are normally phosphorylated by another AGC kinase. Another strategy has been to overexpress catalytically inactive "dominant negative" mutants of AGC kinases in cells. However, such mutants are likely to interact with and inhibit the upstream protein kinase(s) that activates it, and thus prevent the "upstream" kinase(s) from phosphorylating other cellular substrates. For example, a dominant negative RSK may interact with ERK1/ERK2 preventing the activation of MSK isoforms and hence the phosphorylation of CREB [9]. Furthermore, overexpression in *S. cerevisiae* of catalytically inactive Rck2p, a kinase that binds to and is activated by the Hog1P MAPK, sequestered the substrate docking site of the Hog1P kinase, thereby preventing Hog1P from interacting with other substrates [10]. Thus catalytically inactive Rck2P is acting as a dominant negative mutant of Hog1P.

A few small cell-permeable molecules have been developed that inhibit the activation of AGC kinase members. For example, several compounds that prevent the activation of MAP Kinase Kinase-1 (MKK-1) have been described that potently inhibit the activation of ERK1/ERK2 and hence the activation of RSK and MSK isoforms [11, 12]. Inhibitors of PI 3-kinase have been employed to inhibit the activation of PKB and S6K [13], while rapamycin a drug that inhibits the protein kinase mTOR, prevents the activation of S6K [14]. Although these compounds have been valuable in providing evidence for or against a role of some AGC kinases in the regulation of particular cellular processes, the development of compounds that inhibit individual AGC kinase members specifically would be a major advance. However, thus far mainly rather non-specific AGC kinase inhibitors have been deployed for this purpose. For example, the use of Ro318220 has been described in over 500 publications to inhibit conventional PKC isoforms, but it also inhibits RSK and S6K with similar potency [15], as well as other kinases that are not AGC-family members [16]. H89, a compound originally identified as a PKA inhibitor, also inhibits MSK1, S6K and Rho-dependent protein kinase (ROCK) with a similar IC_{50} value to PKA [16, 17]. Here, we demonstrate that BI-D1870 is a remarkably specific inhibitor of RSK isoforms, with a >500-fold greater selectivity over 9 other AGC kinases tested. We also demonstrate that BI-D1870 inhibits RSK activity relatively specifically in cells.

Materials and Methods

Materials: BI-D1870 was synthesized as a racemate at Boehringer Ingelheim Pharma GmbH & Co. KG, PD 184352 was obtained by custom synthesis, LY 294002 was from Calbiochem, EGF and microcystin-LR were from Life Technologies Inc, foetal bovine serum (FBS) and other tissue culture reagents were from BioWhittaker, protease-inhibitor cocktail tablets were from Roche. Forskolin, tetradecanoylphorbol acetate (TPA), DMSO, antimycotic-antibiotic solution and dimethyl pimelimidate were from Sigma, while the precast 4-12% Bis-Tris gradient SDS-polyacrylamide gels were from Invitrogen. GST-ERK2 was expressed in *E.coli*

and activated with MKK1 [18] and His-PDK1 was expressed in Sf21 cells [19]. Apart from RSK3 (#14-462) and RSK4 (#14-702) that were purchased from Upstate, the protein kinases employed in Table 1 were generated in the Division of Signal Transduction Therapy Division University of Dundee. The protocols utilised to assay the 54 protein kinases listed in Table 1 are described in the Supplementary material section.

Buffers. Lysis Buffer: 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (by mass) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (by vol) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet per 25 ml). Buffer A: 50 mM Tris/HCl pH 7.5 and 0.1 mM EGTA, and 0.1% (by vol) 2-mercaptoethanol. Buffer B: 50 mM Tris/HCl pH 7.5 and 0.1 mM EGTA. SDS-Sample Buffer: 50 mM Tris/HCl pH 6.8, 2% (by mass) SDS, 10% (by vol) glycerol and 1% (by vol) 2-mercaptoethanol.

Expression, purification and activation of RSK1 and RSK2. cDNA clones encoding rat RSK1 were kindly provided by J. Avruch (Dept of Molecular Biology, Massachusetts General Hospital) and the human RSK2 cDNA was described previously [20]. The rat RSK1 sequence contained a HA-tag N-terminal to the initiating methionine and differed from the Genbank entry M99169, at three residues E551G, S637N, and G697A. The human RSK2 sequence differed from Genbank entry NM_004586 at one residue, V45G. In order to generate transfer vectors for baculovirus production, the RSK1 cDNA was cloned into the *Eco*RI site of pFastBAC-Hta vector (Life Technologies, Paisley, Scotland, UK), the resulting construct encodes RSK1 with an N-terminal hexahistidine tag. The RSK2 cDNA was cloned into the *Bam*HI site of a modified pFastBAC1 vector resulting in the residues MAHHHHHHGS being added N-terminal to Pro2 of the native sequence. The resulting constructs were then used to generate recombinant baculovirus using the Bac-to-Bac system (Life Technologies) following the manufacturer's protocol. The resulting baculovirus, were used to infect Sf21 cells (1.5 x 10^6/ml) at a multiplicity of infection of 5. The infected cells were harvested 72 hours post infection and His-tagged protein purified as previously described for His-PDK1 [19]. RSK1 and RSK2 were purified with yields of ~10 mg/L of infected cells and were greater than 85% homogeneous as judged by densitometric scanning of a Coomassie Blue-stained SDS polyacrylamide gels. The purified RSK1 and RSK2 isolated in this manner possessed a low basal activity of ~5 U/mg when assayed using Crosstide as a substrate [7], but could be activated by ERK2 to a specific activity of >300 U/mg [21]. In order to activate the purified RSK1 and RSK2, a reaction was set up (2.0 ml total volume) in Buffer A containing RSK1 or RSK2 (0.2 mg/ml), active 5U/ml GST-ERK2 and 3 μg/ml His-PDK1, 10 mM magnesium acetate, and 0.1 mM ATP. Following incubation for 1 h at 30 ºC, the specific activity of the RSK1 and RSK2 enzymes had increased to 150 and 370 Units/mg for RSK1 and RSK2, respectively. The reaction was terminated by adding EDTA to a concentration of 20 mM, and passed through a 0.5 ml column of glutathione-agarose equilibrated in Buffer A to remove

GST-ERK2. The eluate from this column, containing RSK1 and RSK2, was made 0.4 M NaCl and passed through a 0.5 ml Heparin-Sepharose column equilibrated in Buffer A containing 0.4 M NaCl to remove PDK1 which interacts with heparin-Sepharose under these conditions [22]. The eluate containing activated RSK1 and RSK2 was dialysed into 50 mM Tris-HCl pH 7.5, 270 mM sucrose, 150 mM NaCl, 0.1mM EGTA, 0.1 % 2-mercaptoethanol, 0.03 % Brij-35, 1 mM benzamidine and 0.2 mM PMSF, and aliquots snap frozen in liquid nitrogen and stored at – 80 ºC prior to use.

The GST-RSK2[1-389, S381E] was expressed in *E.coli* and purified on glutathione-Sepharose as described previously [6]. In order to activate this fragment of RSK2, a reaction was set up (1.0 ml total volume) in Buffer A containing GST-RSK2[1-389,S381E] (0.4 mg/ml) and 50 μg/ml His-PDK1, 10 mM magnesium acetate, and 0.1 mM ATP. Following incubation for 1 h at 30 ºC the specific activity of the GST-RSK2[1-389,S381E] was 54 U/mg. The reaction was terminated by addition of EDTA to 20 mM, sucrose added to 0.27 M and aliquots of the activated GST-RSK2[1-389,S381E] snap frozen in liquid nitrogen and stored at – 80 ºC prior to use.

Antibodies: Antibodies recognising LKB1 phosphorylated at Ser431 were raised in sheep [23]. The antibodies that we raised previously against mouse LKB1 protein [23], did not immunoprecipitate human LKB1 and we therefore raised a further antibody against human LKB1 expressed in *E.coli* with an N-terminal glutathione S-transferase (GST) tag, using a cDNA construct generously provided to us by Dr Nicoletta Resta (University of Bari, Italy). The antibodies were affinity purified on CH-Sepharose coupled covalently to human GST-LKB1 and passed through a column of CH-Sepharose coupled to GST to remove anti-GST antibodies. The antibody that did not bind, was selected. This antibody immunoprecipitates human LKB1 efficiently and was used to immunoprecipitate LKB1 from human 293 cells.

Antisera recognising the phosphorylated forms of ribosomal S6 protein (Ser235; available from Upstate) were raised in sheep using the peptide AKRRRLSpSLRASTS, where Sp indicates phosphoserine. The antibody was affinity purified on CH-Sepharose that had been coupled covalently to the phosphorylated peptide, then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptide. The flow-through fractions were collected.

The antibody used to immunoprecipitate and immunoblot RSK isoforms, was raised against the peptide RNQSPVLEPVGRSTLAQRRGIKK (corresponding to residues 712 to 734 of human p90RSK2), whilst the antibody used to immunoprecipitate MSK1 was raised against the peptide FKRNAAVIDPLQFHMGVER (corresponding to residues 384 to 402 of MSK1). The following antibodies were from Cell Signalling Technology with the catalogue numbers indicated in parenthesis: antibodies for immunoblotting ERK1/ERK2 (#9102), ribosomal S6 protein (#2212s) and CREB (#9192), as well as the phospho-specific antibodies recognising the phosphorylated forms of ERK1/ERK2 (#9106S) and GSK3α/GSK3 (#9331S). The monoclonal antibody recognising the PH domain of PKB (#05-591), as well as the phospho-

specific antibody recognising CREB phosphorylated at Ser133 (#06-519) and the phospho-specific antibody recognising the forkhead in rhabdosarcoma transcription factor (FKHR) phosphorylated at Thr24 (#07-162) were from Upstate Inc. In order to immunoblot GSK3α and GSK3 , we combined antibodies raised against the C-terminal residues of rat GSK3α (residues 471-483, QAPDATPTLTNSS) and an antibody raised against the GSK3 protein. The antibody used to immunoprecipitate FKHR has been described previously [24]. Secondary antibodies coupled to horseradish peroxidase for use in immunoblotting were from Pierce.

Protein kinase assays: Purified His-RSK1, His-RSK2 or GST-RSK2$_{1-389}$ (1-2 U/ml) were assayed for 10 min at 30ºC in a 50 μl assay mixture in Buffer A containing 30 μM substrate peptide (KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK), 10 mM magnesium acetate, 100μM [-^{32}P]ATP (200 cpm/pmole). Reactions were terminated and analysed as described previously [25]. One Unit of activity (U) was that amount of enzyme, which catalysed the phosphorylation of 1 nmole of substrate peptide in 1 min.

In order to assay RSK and MSK1 in human 293 or Rat-2 cell lysates, these kinases were immunoprecipitated from the cell lysates (0.1 mg lysate protein for RSK and 0.3 mg for MSK1) and assayed as described previously, except that for RSK assays the immunoprecipitates were washed twice with Buffer A containing 1 mM ATP and twice with Buffer A prior to the assay, as a precaution to ensure dissociation of BI-D1870 from the RSK isoforms.

Cell Culture, stimulation and cell lysis. The rat embryo fibroblast cell line termed Rat-2 cells was obtained from the European Collection of Cell Cultures. Rat-2 cells were cultured on 10 cm-diameter dishes in Dulbecco's Modified Eagle's medium supplemented with 10% FBS. HEK 293 cells were cultured on 10 cm-diameter dishes in Dulbecco's Modified Eagle's medium supplemented with 10% FBS and 1X antimycotic-antibiotic solution. Prior to stimulation, cells were cultured in the absence of serum for 16 h. Inhibitors were dissolved in DMSO at a 1000-fold higher concentration than that at which they were used. These inhibitors, or the equivalent volume of DMSO as a control, were added to the tissue culture medium 30 min prior to stimulation unless indicated otherwise. The final concentration of DMSO in the culture medium (0.1% by vol) had no effect on agonist-induced activation or phosphorylation of any substrate examined. The cells were stimulated with the indicated agonists and lysed in 1 ml of ice cold Lysis Buffer and centrifuged at 4ºC for 5 min at 16,000 x g. The supernatants were frozen in liquid nitrogen and stored at -80ºC until use. Protein concentrations were determined using the Bradford method and bovine serum albumin was employed as the standard.

Immunoprecipitation of LKB1 and FKHR. 1 mg of the polyclonal antibodies raised against human LKB1 or mouse LKB1 or FKHR were coupled covalently to protein G-Sepharose (1 ml) using dimethyl pimelimidate [26]. Rat-2 (0.5 mg) or 293 (1 mg) cell lysate protein was incubated for 60 min at 4 ^{0}C with the anti LKB1 or anti-FKHR Protein G-Sepharose conjugates

(5 μl). The immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and washed twice with Buffer B. For immunoblot analysis, the beads were resuspended in SDS Sample Buffer that did not contain 2-mercaptoethanol.

Immunoblotting. For blots of total cell lysates, 20 μg of lysate protein was used. All samples were subjected to SDS/polyacrylamide gel electrophoresis, and transferred to nitrocellulose. For experiments in which LKB1, RSK2, FKHR, GSK3α/GSK3 isoforms and the phospho-ribosomal protein S6 and phospho-LKB1 were immunoblotted, the membranes were incubated in 50 mM Tris/HCl pH 7.5, 0.15 M NaCl, 0.5 % (by vol) Tween (TBS-Tween) and 10% (by mass) skimmed milk for 7 h at 4 ºC in the presence of 1 μg/ml antibody. Prior to immunoblotting with the S431-P antibody or the phospho-ribosomal protein S6 antibody, the antibodies were incubated with the non-phosphorylated forms of the peptide (10 μg/ml) antigen used to raise the antibodies. All the other antibodies obtained commercially were used a 1000-fold dilution of the stock and 5% (by mass) bovine serum albumin was used in place of skimmed milk as the blocking agent. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Results

BI-D1870 is a potent inhibitor of RSK isoforms. BI-D1870, derived from a novel series of dihydropteridinones (Figure 1A) was identified in a kinase selectivity screen performed in the Division of Signal Transduction Therapy in Dundee, as a potent inhibitor of RSK1. We found that BI-D1870 inhibited RSK1 and RSK2 with IC_{50} values of 10 nM (Figure 1B) and 20 nM (Figure 1C), respectively, when the kinase assays were performed with 100 μM ATP. When the assays were performed at a 10-fold lower ATP concentration, the IC_{50} of BI-D1870 was reduced to 5 nM for RSK1 and 10 nM for RSK2. These observations indicate that BI-D1870 is a potent ATP-competitive inhibitor of RSK isoforms. An active mutant of RSK2 lacking the C-terminal kinase catalytic domain (RSK2[1-389,S381E] [20]), was inhibited by BI-D1870, with an IC_{50} of ~30 nM (Figure 1D), indicating that BI-D1870 inhibits the N-terminal AGC-kinase domain. BI-D1870 also inhibited RSK3 and RSK4 with similar potency to RSK1 and RSK2 (Table 1 and 2).

BI-D1870 is a specific inhibitor of RSK isoforms. In order to investigate the specificity of BI-D1870, we studied the effect of 10 μM, 0.1 μM and 0.01 μM BI-D1870 at ATP concentrations which approximate the Km constant for ATP, towards a panel of 54 protein kinases which included 13 AGC kinase members (RSK1, RSK2, RSK3, RSK4, MSK1, PKBα, PKB , S6K1, SGK1, PKCα, PDK1 and ROCK-II), as well as three tyrosine kinases (Table 1). At 0.1μM BI-D1870, a concentration that inhibited RSK1 and RSK2 by over 98%, most enzymes on the panel were not affected significantly apart from polo-like kinase whose activity was reduced 83% and Aurora B, DYRK1a, CDK2/cyclin A, Lck, casein kinase-1, and GSK3 , whose

activities were reduced by 40-70% (Table 1). PLK1 was inhibited by BI-D1870 with an IC50 of 100 nM, whilst the IC50 values for Aurora B, DYRK1a, CDK2/cyclin A, Lck, casein kinase-1, and GSK3 were 10 to 100-fold higher than that of RSK isoforms (Table 2). Importantly, the activity of MSK1, the kinase most closely related to RSK, was not inhibited significantly by 0.1μM BI-D1870.

Evidence that BI-D1870 inhibits RSK activity specifically in 293 cells. To examine whether BI-D1870 could inhibit RSK activity in cells, we investigated the effect of BI-D1870 on the RSK-catalysed phosphorylation of its known substrates in 293 cells, using TPA as an agonist to activate ERK1/ERK2 and RSK isoforms. We first incubated the cells with 10 μM BI-D1870, for periods of 15 min to 4 h prior to stimulation with TPA, and investigated the phosphorylation of GSK3α at Ser21 and GSK3 at Ser9, which is mediated by RSK under these conditions [27]. These experiments demonstrated that incubation of cells with BI-D1870 greatly inhibited the TPA-induced phosphorylation of GSK3α and GSK3 (Figure 2A). In contrast, BI-D1870 had little effect at any time point on the TPA-induced activation of ERK1/ERK2, which is catalysed by Raf and MKK1, or the phosphorylation of CREB at Ser133 which is largely catalysed by MSK1 and MSK2 isoforms in fibroblasts [9]. We next studied the effect of varying the concentration of BI-D1870 on the TPA-induced phosphorylation of GSK3 isoforms and another RSK substrate, the protein kinase LKB1 (which is phosphorylated by RSK at Ser431 [23]) in 293 cells. These experiments showed that the phosphorylation of both proteins was inhibited in 293 cells with an IC_{50} of about 1 μM (Figure 2B).

Stimulation of 293 cells with TPA also activates S6K whose principal cellular substrate is thought to be the ribosomal protein S6 [2]. Incubation of cells for up to 4h with 10 μM BI-D1870 did not affect the TPA-induced phosphorylation of the ribosomal S6 protein (Figure 2), indicating that BI-D1870 does not inhibit the activation or activity of S6K in cells. In 293 cells, TPA also induces a weak activation of p38 MAP kinase which was also unaffected by BI-D1870 (Figure 2B).

In order to test whether BI-D1870 affected the activation of RSK isoforms, 293 cells were stimulated with TPA in the presence or absence of BI-D1870. RSK1 and RSK2 isoforms were immunoprecipitated with an antibody that recognises both forms of RSK, the immunoprecipitates washed and assayed for activity in the absence of BI-D1870. Treatment of the cells with BI-D1870 did not markedly affect the large TPA-induced activation of ERK1/ERK2, RSK isoforms or the phosphorylation of RSK at Ser381 and Thr 574, two phosphorylation sites required for the activation of RSK [5] (Figure 3A and 3B). BI-D1870 also did not affect the TPA-induced activation of MSK1 in 293 cells (Figure 3C).

We also compared the effectiveness of BI-D1870 to inhibit the phosphorylation of GSK3 isoforms with that of PD 184352, a potent inhibitor of the activation of MKK1 [12] (Figure 3A and 3B). BI-D1870 (10 μM) inhibited the TPA-induced phosphorylation of GSK3 isoforms to the same extent as PD 184352 (2 μM). As expected, and in contrast to BI-D1870, PD 184352

also prevented the phosphorylation of ERK1/ERK2 and the phosphorylation and activation of RSK and MSK isoforms as well as the phosphorylation of CREB.

Further evidence that BI-D1870 is a specific RSK inhibitor. We next assessed the effectiveness of BI-D1870 as an inhibitor of RSK activity in EGF stimulated Rat-2 cells. As observed in 293 cells, BI-D1870 inhibited the EGF-induced phosphorylation of LKB1 at Ser431 with an IC_{50} of ~ 1 μM. Furthermore, BI-D1870 did not affect the activation of ERK1/ERK2 and MSK1, nor did it inhibit the phosphorylation of CREB (Figure 4).

In contrast to TPA, EGF not only activates the isoforms of RSK, MSK, S6K [23], but also triggers the activation of PKB as judged by using phospho-specific antibodies that recognise PKB phosphorylated at Thr308 and Ser473 (Figure 4). Since PKB phosphorylates GSK3α and GSK3 at the same residues as RSK in vivo (reviewed in [27]), it would be expected that, as shown previously in Swiss 3T3 cells [28], blockade of PKB activation as well as blockade of RSK activity would be needed to prevent the EGF-induced phosphorylation of GSK3 isoforms. Consistent with this notion, treatment of Rat-2 cells with BI-D1870 alone or the PI 3-kinase inhibitor LY294002 alone did not affect the EGF-induced phosphorylation of GSK3 isoforms, but when added to Rat-2 cells together, the EGF-induced phosphorylation of GSK3α or GSK3 was blocked completely (Figure 4 & 5). Similarly, addition of both PD 184352 and LY 294002 to inhibit the activation of RSK and PKB, respectively, but not either inhibitor alone or a combination of PD184352 and BI-D1870, prevented EGF-induced phosphorylation of GSK3 (Figure 5). As expected, LY 294002, inhibited the activation of PKB and the phosphorylation of two of its substrates, the forkhead transcription factors (FKHR and FKHRL1), which are not phosphorylated by RSK [29]. BI-D1870, did not affect the activation of PKB or the phosphorylation of the FKHR isoforms in EGF-stimulated cells (Fig 5), indicating that this compound does not affect the PKB pathway.

BI-D1870 does not inhibit PKA activity in vivo. PKA also phosphorylates GSK3α and GSK3 at Ser21 and Ser9 [30], and LKB1 at Ser431 [23, 31], in the presence of agonists that elevate cAMP. In order to verify that BI-D1870 does not inhibit the activity of PKA in vivo, we stimulated 293 and Rat-2 cells with forskolin, an activator of adenylate cyclase which elevates the level of cyclic AMP and hence the activity of PKA in the presence or absence of 10 μM BI-D1870. We then measured the phosphorylation of GSK3α, GSK3 and LKB1 at the sites that are phosphorylated by PKA. BI-D1870 (10 μM) did not inhibit the forskolin-induced phosphorylation of GSK3 isoforms or LKB1 significantly (Figure 6).

BI-D1870 induces the activation of ERK1/ERK2 in Rat-2 cells, but not in 293 cells. Interestingly, the incubation of Rat-2 cells with BI-D1870 in the absence of EGF induced a time-dependent (Figure 7A) and dose-dependent (Figure 7B) phosphorylation of ERK1/ERK2. Treatment of cells with 10 μM BI-D1870, began to induce ERK1/ERK2 phosphorylation after 5 min, with maximal activation being attained between 20 and 80 min and a slow decline thereafter (Figure 7A). Incubation of Rat-2 cells with 10 μM BI-D1870 for 30 min, resulted in

the phosphorylation of ERK1 and ERK2 to ~50% of the level that is observed following stimulation of cells with EGF (Figure 7B). Consistent with a significant activation of ERK and hence MSK1, BI-D1870 induced the phosphorylation of CREB at Ser133. In contrast, 10 μM BI-D1870 did not stimulate a detectable increase in ERK phosphorylation in 293 cells (Figure 2B). These results are considered further in the discussion.

Discussion

The data presented in this study indicate that BI-D1870 is a highly specific cell-permeable inhibitor of RSK that is an ATP competitive inhibitor of the N-terminal AGC kinase domain of RSK (Figure 1). BI-D1870 is cell permeable because at 10 μM it inhibits the phosphorylation of three known RSK substrates (GSK3α, GSK3 and LKB1) in response to agonists that induce the activation of RSK. BI-D1870 is stable in tissue culture medium as it inhibited the phosphorylation of RSK substrates efficiently even after 4 h, without affecting the TPA-induced phosphorylation of ERK1/ERK2 (Figure 2A). BI-D1870 inhibited all RSK isoforms, with IC_{50} values of 10-40 nM at 20-100 μM ATP (Figure 1 and Tables 1 & 2). However, our in vivo data indicate that to completely inhibit the phosphorylation of RSK substrates in cells a concentration of 10 μM BI-D1870 is required (Figure 2B and 4A). The requirement for higher compound concentration to suppress the activity of a protein kinase in vivo compared to those required in vitro is frequently observed with many ATP-competitive inhibitors of protein kinases. One reason is the far higher mM intracellular concentration of ATP in cells compared to the in vitro assay, while a second is that there may be some barrier to the penetration of cells by BI-D1870. A third possibility is that RSK is a relatively abundant and active protein kinase, and inhibiting its activity partially, may be insufficient to prevent the phosphorylation of substrates. A fourth possibility is that the concentrations of TPA and EGF employed in this study induced maximal activation of ERK1/ERK2 and RSK isoforms; lower levels of BI-D1870 may inhibit RSK more effectively after stimulation with lower agonist concentrations. A remarkable feature of BI-D1870 is its very high specificity for RSK isoforms. BI-D1870 inhibited RSK isoforms >500-fold more potently than the 9 other related AGC kinases that were tested in vitro. We have also verified that BI-D1870 is a highly specific RSK inhibitor in vivo, because treatment of cells with 10 μM BI-D1870 did not significantly inhibit the phosphorylation of the ribosomal S6 protein by S6K, the phosphorylation of GSK3 isoforms or FKHR1/FKHRL1 by PKB, the phosphorylation of CREB by MSK1, the phosphorylation of GSK3 isoforms or LKB1 by PKA, the PDK1 mediated activation of S6K1 and PKBα, or the PKC-mediated TPA-induced activation of ERK1/ERK2. It will be important in future work to perform a detailed structural analysis of BI-D1870 bound to RSK to understand the basis for the high specificity of BI-D1870. Understanding how BI-D1870 inhibits RSK activity at the structural level may provide important insights into how specific inhibitors of other AGC kinases could be developed.

Recently it has been reported that an acetylated flavonol glycoside isolated from the tropical plant *Forsteronia refracta* termed SL0101, inhibited RSK2 with an IC_{50} of 90 nM [32]. The selectivity of this compound is uncertain, as it has not yet been tested against a large panel of other kinases, but was reported not to significantly inhibit MSK1, S6K1, PKA and the FAK kinase. Employing an elegant structure-based design strategy, Taunton and colleagues have synthesised an inhibitor termed fmk, that interacts covalently with and modifies covalently the C-terminal kinase domain of RSK isoforms [33]. In cell extracts fmk, formed a specific covalent interaction with RSK isoforms, and also inhibited the phosphorylation of histone H3 at Ser10, in cells that overexpress RSK2. However, the specificity of fmk towards a panel of protein kinases was not assessed [33], and whether it is really a specific inhibitor of RSK has yet to be rigorously evaluated.

As discussed in the Introduction, it was originally shown that a dominant negative mutant of RSK inhibited the growth factor-induced phosphorylation of the transcription factor CREB at Ser133 and that the overexpression of RSK in cells induced the phosphorylation of CREB at this residue [34]. This was originally interpreted to indicate that RSK was catalysing the phosphorylation and hence the activation of CREB. However, the finding that CREB was a vastly more efficient substrate for MSK1 than RSK in vitro [7] and that the compound H89, at concentrations which inhibits MSK isoforms but not RSK, prevents growth factor-induced CREB phosphorylation [23, 35], indicated that MSK1, rather than RSK, may mediate the phosphorylation of CREB in cells. The finding that the growth factor and phorbol ester induced phosphorylation of CREB is greatly reduced, whilst phosphorylation of GSK3 or LKB1 [23] mediated by RSK is unaffected, in mouse embryonic stem cells lacking MSK1 [36] and mouse embryonic fibroblast cells lacking both MSK1 and MSK2 [9] strongly support the conclusion that MSK1/MSK2 mediate the phosphorylation of CREB. Interestingly, trace residual growth factor-induced phosphorylation of CREB that occurs in the fibroblast cell lines lacking MSK1 and MSK2, is not prevented by 10 μM BI-D1870, indicating that this is not catalysed by RSK isoforms [9]. The finding in this study that the RSK inhibitor BI-D1870 does not inhibit the phosphorylation of CREB detectably in cells, but completely inhibits the phosphorylation of GSK3 isoforms and LKB1 (Figures 2 & 4), provides further evidence that RSK is not rate limiting for the phosphorylation of CREB in the cells, under the conditions that we have examined. Moreover, the observation that addition of BI-D1870 to Rat-2 cells (in the absence of any other agonist), stimulates ERK1/ERK2 phosphorylation, resulting in the activation of MSK1 and CREB phosphorylation, but not the phosphorylation of the GSK3 isoforms (Figure 7), also strongly supports the conclusion that MSK1/MSK2 (rather than RSK isoforms) mediates the phosphorylation of CREB.

The observation that BI-D1870 alone, significantly activated the ERK1/ERK2 pathway in Rat-2 cells (Figure 7), indicates that RSK activity controls a negative feedback loop that regulates ERK1/ERK2 activation in certain cell types. This conclusion is also suggested by the finding

that, in mouse embryonic stem cells lacking PDK1 and in which RSK is therefore inactive, ERK1/ERK2 phosphorylation in unstimulated cells, is elevated significantly [37]. It is therefore possible, that in unstimulated Rat-2 cells, basal levels of RSK activity suppress the activity of a protein that stimulates the ERK1/ERK2 pathway. The inhibition of RSK that occurs when cells are incubated with BI-D1870 may switch off this negative feedback loop. Douville and Downward [38] reported that in PC12 cells, RSK phosphorylates and inactivates mSOS, the guanine nucleotide exchange factor for Ras. More recently RSK has been shown to inhibit ERK-MAP kinase signalling during *Drosophila* development although the precise mechanism by which RSK inhibited ERK in this system was not defined [39]. The role of RSK in regulating the activity of ERK1/ERK2 is likely to be cell specific, as the addition of 10 μM BI-D1870 to unstimulated 293 cells does not induce detectable activation of ERK1/ERK2, p38 (Figure 2B) or JNK (GPS, data not shown). RSK is thought to play an important role in stimulating cell growth and promoting cell survival [8, 40], and is therefore a potential target for an anti-cancer drug. However, the observation that inhibition of RSK in some cells promotes the activation of ERK1/ERK2, which might therefore stimulate rather than inhibit cell growth, is potentially an adverse side effect. Furthermore, the discovery that mutation of the RSK2 gene in humans results in Coffin-Lowry syndrome, characterised by mental retardation, facial dysmorphisms and skeletal deformations [41], also indicates that drugs that inhibit RSK isoforms could have undesired effects.

RSK has been suggested to phosphorylate a large number of cellular substrates and to play an important role in promoting cell growth and survival (reviewed in [8, 40]). However, the evidence for an involvement of RSK in regulating these processes in many cases is based largely on the overexpression of constitutively active and dominant negative RSK mutants in cells, which, as discussed earlier, can lead to erroneous conclusions. The activation of RSK also requires its phosphorylation at one site by PDK1. Thus a dominant negative mutant of RSK could also interact with PDK1, preventing PDK1 from activating a number of other AGC kinase members. Small cell permeable inhibitors of RSK, such as BI-D1870, should not suffer from these problems and have the potential to facilitate the investigation of the cellular roles of RSK in the same way that inhibitors of MKK-1 activation (PD 98059, U0126 and PD 184352) and the p38 MAP kinase inhibitor (SB 203580) have enabled many roles of particular MAP kinase pathways to be defined. In order to determine whether a substrate is phosphorylated by RSK in cells, it will be necessary in the future to demonstrate that it is inhibited by BI-D1870, as well as by inhibitors of the activation of MKK-1. Furthermore, the phosphorylation of the substrate should not occur in cells that lack PDK1, which also lack RSK activity [37]. Thus far the only RSK substrates that have met all of these criteria are GSK3α, GSK3 and LKB1. It will be important to deploy BI-D1870 to re-evaluate the role that RSK plays in mediating phosphorylation of the myriad of other substrates [8, 40] that have been suggested for this protein kinase.

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Table 1. **Effect of BI-D1870 on 54 protein kinases**. Results are presented as percentage of kinase activity compared to that in control incubations in which BI-D1870 was omitted. Protein kinases were assayed as described in the supplementary section, in the absence or presence of the indicated amount of BI-D1870. As ATP concentrations can affect the apparent IC50 value of ATP-competitive inhibitors, assays were undertaken at approximately the Km concentration of ATP, namely 5 μM (p38δ, PKBα, GSK3 , p38γMAPK, CK2, ERK8, MKK1, PIM2, IKK , PRK2, MARK3, EF2K and PLK1), 20 μM (JNK1, PRAK, ROCK-II, P38 2, CDK2, CHK1, CHK2, MSK1, CSK, S6K1, PKA, CK1, MAPKAP-K2, SGK1, PKCα, PDK1, NEK7, MAPKAP-K3, Aurora B and MST2) and 50 μM (p38α, Lck, AMPK, ERK2, DYRK1a, PHK, RSK1, RSK2, NEK6, NEK2a, CAMK-1, PKD1, SRPK-1, JNK3, MNK1, MNK2, Src, PKB and smMLCK). The results are an average of a triplicate determination ± standard deviation. Abbreviations not defined in main text: PKBα-ΔPH, PKBα lacking the pleckstrin homology domain; SGK1, serum and glucocorticoid-induced kinase-1; ROCKII, Rho-dependent protein kinase-II; MKK1, MAP-kinase kinase-1; M-K2, MAPK-activated protein kinase-2 (MAPKAP-K2); M-K3, MAPK-activated protein kinase-3 (MAPKAP-K3); MNK, MAP kinase interacting Kinase; PRAK, p38-regulated/activated protein kinase; CAMK-1, Calmodulin dependent kinase 1; smMLCK, smooth muscle light chain kinase; EF2K, Elongation factor kinase; PRK2, PKC-related kinase-2; AMPK, AMP-activated protein kinase; CHK1, cell cycle checkpoint kinase-1; CK, Casein kinase; PHK, phosphorylase kinase; Lck, lymphocyte kinase; CSK, C-terminal Src kinase; CDK2-A, cyclin dependent kinase-2 complexed with cyclinA; DYRK, dual specificity tyrosine phosphorylated and regulated kinase; NEK, NIMA related Protein Kinase; SRPK1, Serine Arginine Protein Kinase; MARK3, Microtubule affinity regulating kinase; MST2, Mammalian sterile 20-like 2; PKD1, Protein kinase D 1; PLK1, Polo like kinase 1. Kinase families are defined as in [42].

		% Remaining Kinase Activity		
Protein kinase	**Kinase family**	D1870 10μM	D1870 0.1μM	D1870 10 nM
RSK1	AGC	3±0	1±1	15±3
RSK2	AGC	2±1	2±0	32±3
RSK3	AGC	1±1	13±2	65±6
RSK4	AGC	1±1	12±3	55±5
MSK1	AGC	71±7	87±2	95±5
PKBα	AGC	95±8	116±12	110±5
PKB	AGC	95±7	97±1	100±4
S6K1	AGC	54±2	68±1	83±4
SGK1	AGC	67±11	83±4	99±3
PKCα	AGC	37±6	42±2	103±5
PKA	AGC	66±6	94±2	91±4
PDK1	AGC	79±7	86±1	99±4
ROCKII	AGC	70±4	80±0	104±2
PRK2	AGC	68±6	72±1	80±6
MKK1	STE	49±1	76±2	103±4
MST2	STE	13±1	49±4	65±8
JNK1	CMGC	83±9	87±6	98±5
JNK3	CMGC	72±1	76±0	95±5
p38α	CMGC	77±11	86±6	89±3
p38ß2	CMGC	91±9	86±2	90±0
p38γ	CMGC	89±4	93±5	99±9
p38δ	CMGC	89±0	86±6	89±1
ERK2	CMGC	74±0	86±1	96±2
ERK8	CMGC	54±8	110±4	94±1
GSK3	CMGC	21±3	29±3	84±0
CDK2-A	CMGC	30±7	38±10	89±5
DYRK1a	CMGC	46±6	57±5	91±3

		% Remaining Kinase Activity		
Protein kinase	**Kinase family**	D1870 10μM	D1870 0.1μM	D1870 10 nM
SRPK1	CMGC	80±10	87±3	98±2
M-K2	CAMK	90±8	98±10	93±6
M-K3	CAMK	92±4	99±1	58±5
CAMK-1	CAMK	60±9	67±4	94±5
smMLCK	CAMK	46±6	67±1	106±4
MARK3	CAMK	37±2	39±4	101±1
PKD1	CAMK	53±5	49±3	90±6
AMPK	CAMK	68±2	70±2	101±3
CHK1	CAMK	74±7	88±9	99±8
CHK2	CAMK	65±2	79±2	97±9
PHK	CAMK	100±5	104±2	77±2
MNK1	CAMK	76±0	80±0	98±6
MNK2	CAMK	86±4	92±1	122±4
PRAK	CAMK	74±5	74±5	85±8
PIM2	CAMK	61±4	71±0	99±5
CK1	CK1	32±0	51±3	94±0
CK2	CK1	100±3	95±0	101±3
EF2K	atypical	96±1	101±0	108±3
NEK2a	NEK	81±0	87±0	94±5
NEK6	NEK	92±1	100±1	89±4
NEK7	NEK	76±4	83±12	107±9
IKK	IKK	75±4	83±6	95±5
Aurora B	Aurora	5±0	42±4	54±6
PLK1	PLK	15±2	17±1	33±1
Lck	TK	35±2	45±1	90±3
CSK	TK	81±12	92±7	91±2
Src	TK	60±4	69±0	98±2

Table 2. IC_{50} values of D1870 for kinases inhibited by BI-D1870. The indicated kinases were assayed in the presence of concentrations of BI-D1870 ranging from 1 nM to 100 μM. IC_{50} values in which 50% of the kinase was inhibited compared to that in control incubation in which BI-D1870 was omitted, were extrapolated from this data. RSK isoforms were all assayed at 100 μM ATP, as the Km for ATP for RSK3 and RSK4 have not been determined. Other kinases were assayed at approximately the Km concentration of ATP. Protein kinases were assayed as described in the supplementary section and abbreviations are defined in the legend to Table 1. The results are the mean of two separate experiments with each assay condition was performed in triplicate.

Protein kinase	Kinase family	ATP Concentration in assay (μM)	IC_{50} (μM)
RSK1	AGC	100	0.031±0.005
RSK2	AGC	100	0.024±0.005
RSK3	AGC	100	0.018±0.003
RSK4	AGC	100	0.015±0.007
MST2	STE	20	0.86±0.028
GSK3β	CMGC	5	1.59±0.021
MARK3	CAMK	5	2.20±0.247
CK1	CK1	20	0.45±0.042
Aurora B		20	0.34±0.141
PLK1		50	0.10±0.029

Figure Legends.

Figure 1 Effect of BI-D1870 on RSK activity in vitro. The ATP-competitive kinase inhibitor, BI-D1870 (A), derived from a novel chemical series (WO 03/020722), selectively and potently inhibits RSK1/2. Activated His-RSK1 (B) or His-RSK2 (C) were assayed in the absence or presence of the indicated concentration of BI-D1870 at a concentration of 100 μM ATP (closed symbols) or 10 μM ATP (open symbols). The results are presented as percentage kinase activity relative to the control measured in the absence of BI-D1870. (D) As in B and C except that an active fragment of RSK comprising the isolated N-terminal catalytic domain (RSK[1-389,S381E] activated by phosphorylation with PDK1 as described under Materials and Methods, was assayed in the presence or absence of BI-D1870 at 100 μM ATP. The results presented are the average of 2 experiments with each determination carried out in triplicate. The error for each data point is less than 5%.

Figure 2. BI-D1870 inhibits RSK activity in vivo. (A) 293 cells that had been deprived of serum for 16 h, were incubated in the absence or presence of 10 μM BI-D1870 for the indicated times, then stimulated with TPA (400 ng/ml) for 20 min. The cells were lysed and the lysates were immunoblotted with the indicated antibodies as described in the Materials and Methods. (B) As in A except that cells were incubated for 30 min with the indicated concentrations of BI-D1870 prior to stimulation with TPA. Similar results were obtained in 2 separate experiments. Phosphorylation of GSK3 isoforms was quantitated by densitometric analysis of autorads.

Figure 3 Effect of BI-D1870 on activation of RSK and MSK1. (A) 293 cells that had been deprived of serum for 16 h, were incubated in the absence or presence of 10 μM BI-D1870 or 2 μM PD 184352 as indicated. After 30 min the cells were stimulated with TPA for 20 min, the cells lysed and RSK isoforms immunoprecipitated with an antibody that recognises RSK1 and RSK2, washed to remove BI-D1870 (see Methods) and assayed in the absence of BI-D1870. (B) As in A except that the cell lysates were immunoblotted with the indicated antibodies, as described in Materials and Methods. (C) As in (A), except that MSK1 was immunoprecipitated from the lysates and assayed. In A & C the results are presented as the average ± SEM of 2 separate experiments with each determination carried out in triplicate.

Figure 4 Effect of BI-D1870 on EGF-stimulated Rat-2 cells. Serum-deprived Rat-2 cells were incubated for 30 min in the presence of the2 indicated concentrations of BI-D1870. The cells were then stimulated with EGF (100 ng/ml) for 10 min, lysed and immunoblotted with the indicated antibodies.

Figure 5. Effect of BI-D1870, PD 184352 and LY294002 on signalling pathways in Rat-2 cells. Serum-deprived Rat-2 cells were incubated for 30 min in the absence or presence of 10

μM BI-D1870, 2 μM PD 184352 or 100 μM LY 294002 as indicated. The cells were then stimulated with EGF (100 ng/ml) for 10 min, lysed and the cell lysates were immunoblotted with indicated antibodies as described in the Materials and Methods. Similar results were obtained in 2 separate experiments.

Figure 6. BI-D1870 does not inhibit PKA in vivo. Serum-deprived Rat-2 cells or 293 cells were incubated for 30 min in the absence or presence of 10 μM BI-D1870. The cells were either left unstimulated or stimulated with 20 μM forskolin for 10 min. The cells were lysed and the lysates immunoblotted with the indicated antibodies. Similar results were obtained in 2 separate experiments.

Figure 7. BI-D1870 activates ERKs and p38 MAP kinase in Rat-2 cells. (A) Serum-deprived Rat-2 cells were incubated for the indicated times the presence of 10 μM BI-D1870 in the absence of EGF. The cells were lysed and the lysates immunoblotted with the indicated antibodies (B) As in A, except that cells were incubated with the indicated concentrations of BI-D1870 for 30 min and then either left unstimulated or stimulated with 100 ng/ml EGF for 10 min. For the immunoblotting studies similar results were obtained in 2 separate experiments.

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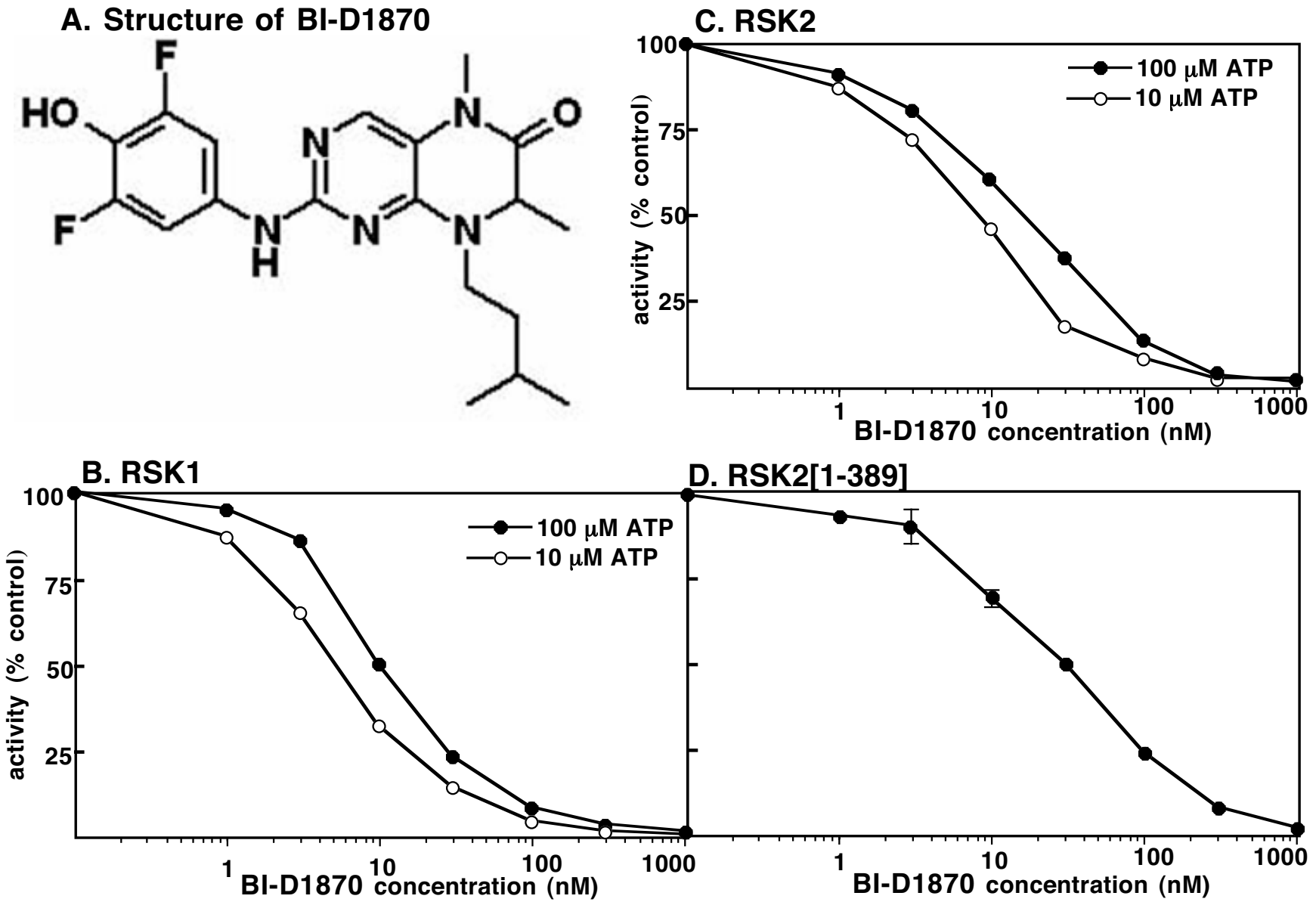

Figure 1

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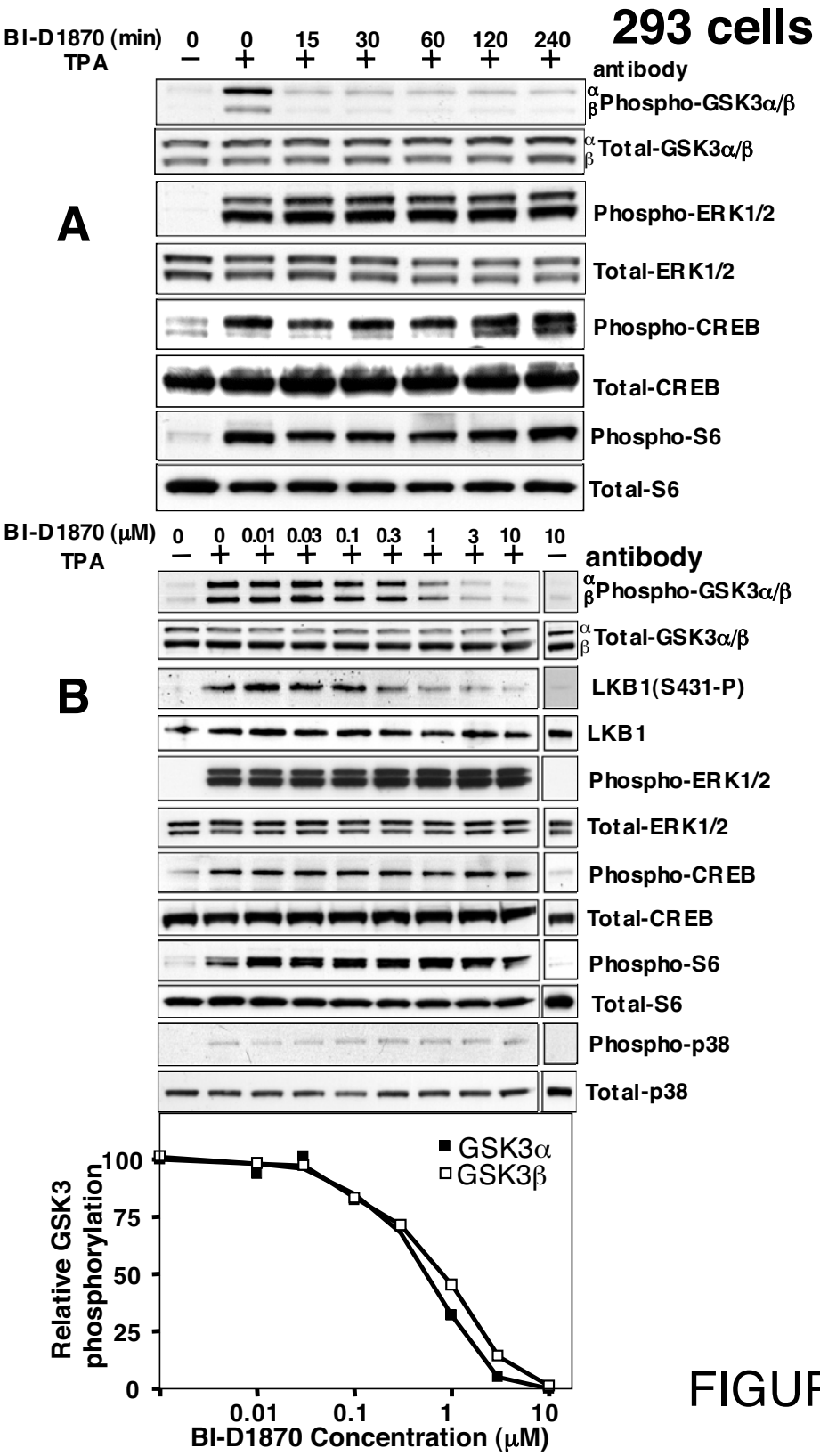

FIGURE 2

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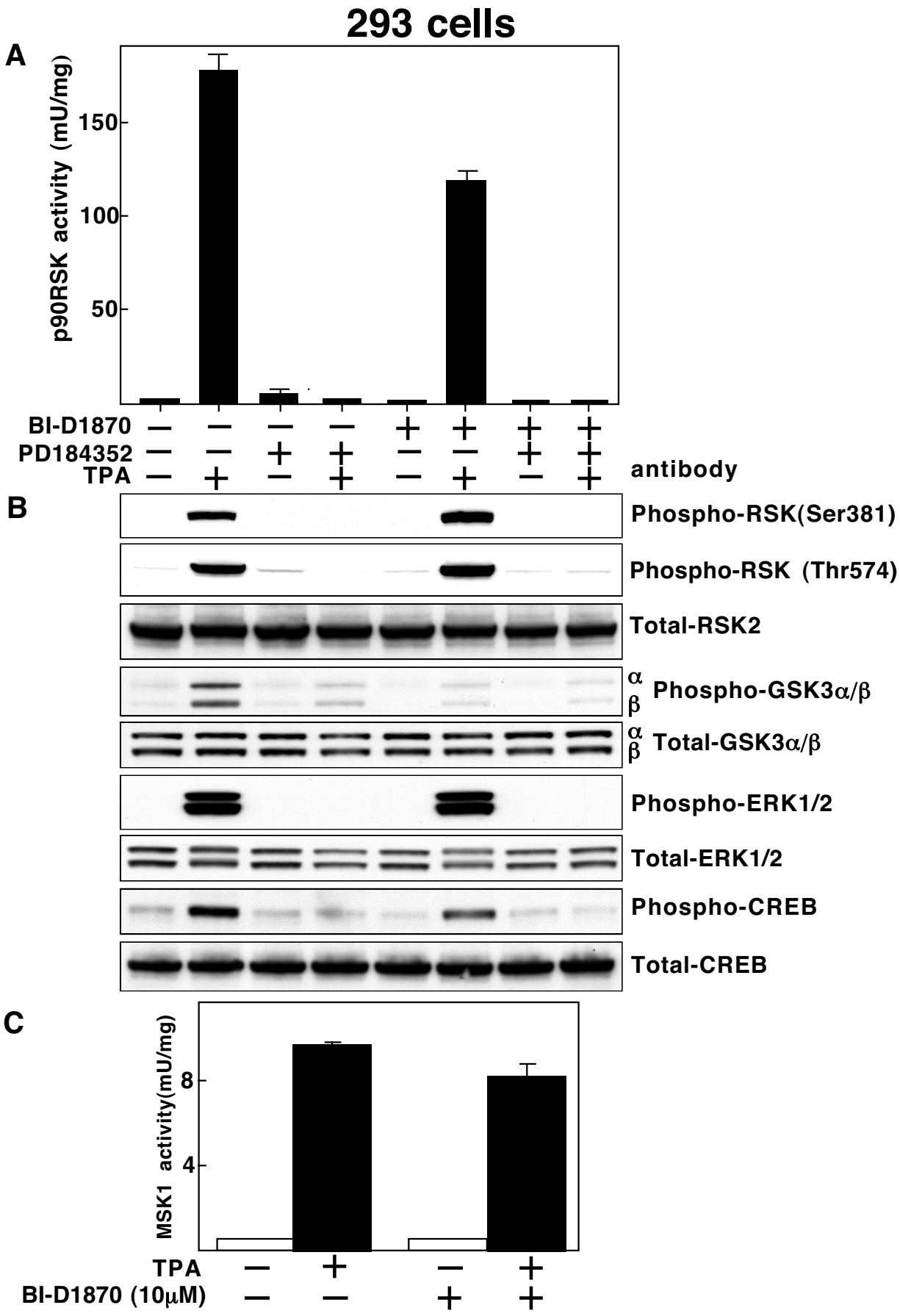

Figure 3

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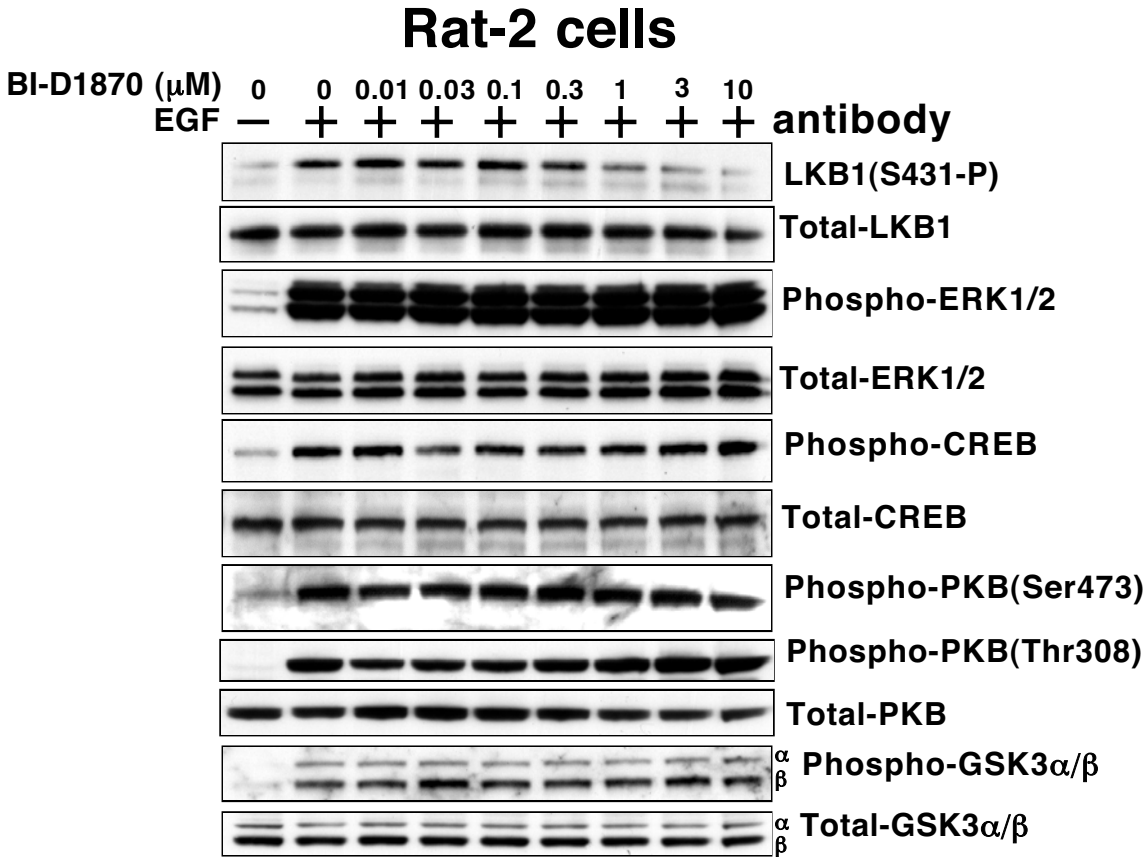

Figure 4

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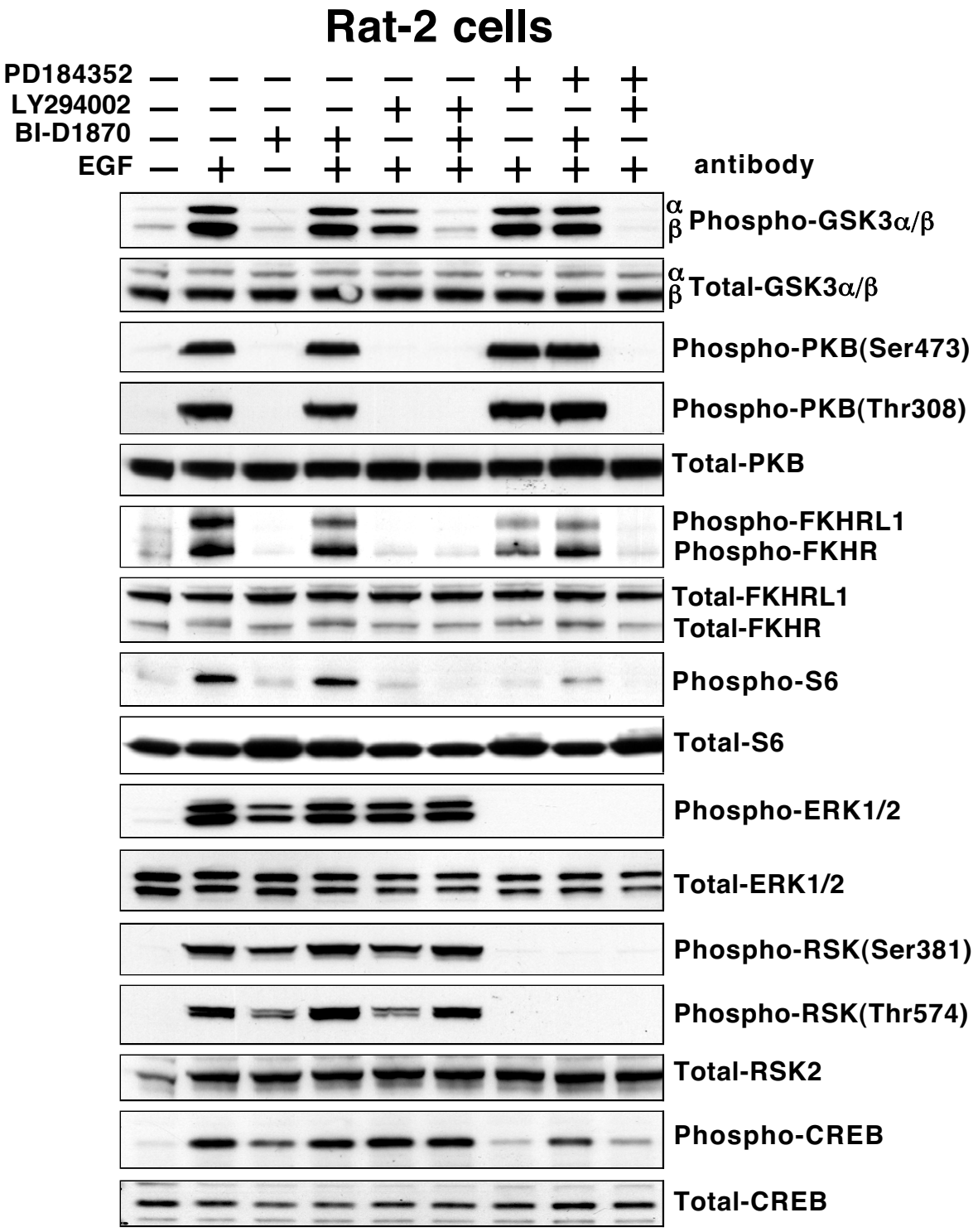

Figure 5

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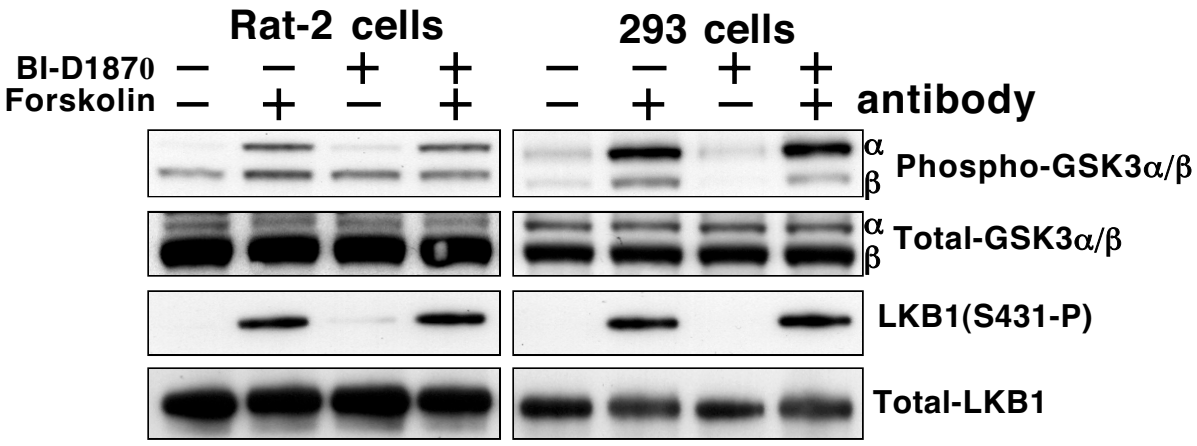

Figure 6

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A

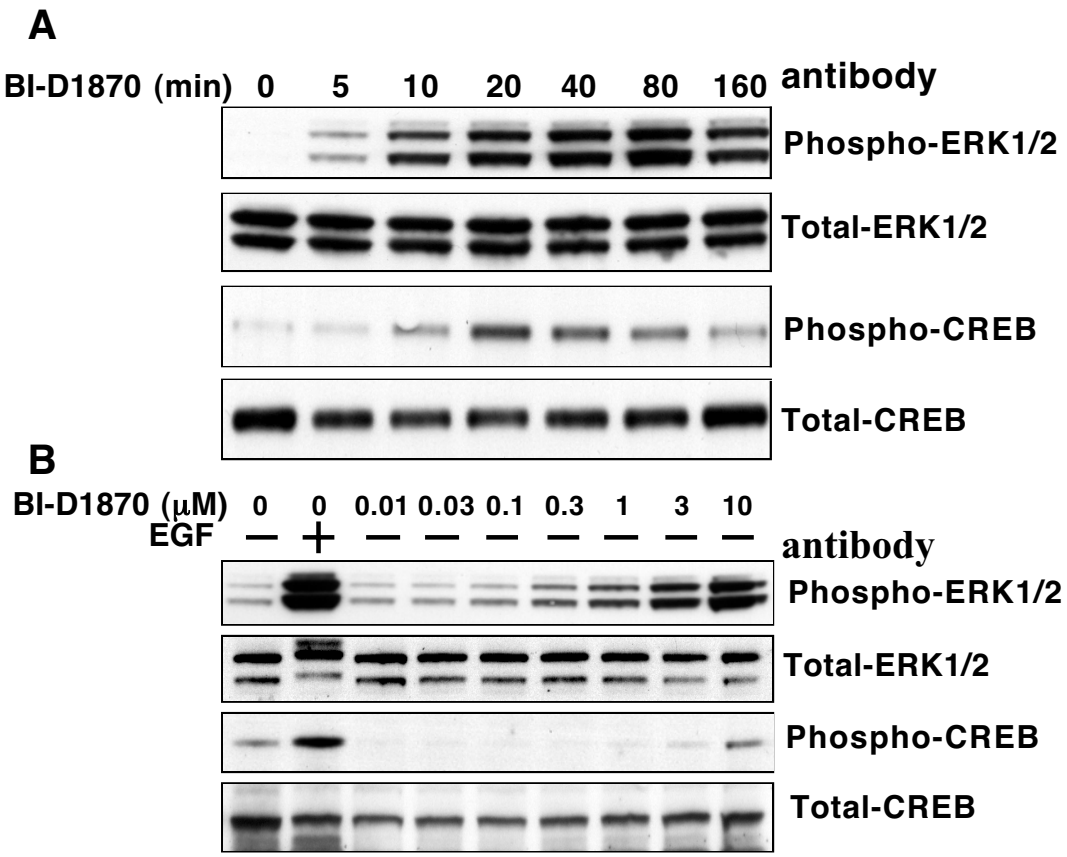

Figure 7