

Ro 31-6045, the inactive analogue of the protein kinase C inhibitor Ro 31-8220, blocks in vivo activation of p70^{s6k}/p85^{s6k}: implications for the analysis of S6K signalling

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Abstract The mitogen-stimulated protein kinase p70^{s6k}/p85^{s6k} (S6K) plays an essential role in cell proliferation and growth, with inhibitors of the S6K signalling pathway showing promise as anti-tumour therapeutics. Here, we report that the bisindolylmaleimide derivative Ro 31-6045, previously reported to be inactive as a kinase inhibitor, inhibited S6K activity in vivo with an IC₅₀ = 8 μM. Structure/function analysis using mutant forms of S6K indicates that Ro 31-6045 inhibition is independent of the upstream activator mTOR. Ro 31-6045 will prove useful in elucidating the complex activation mechanism of S6K and its independence from mTOR will allow confirmation of functional data obtained using the mTOR inhibitor rapamycin. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protein kinase inhibitor; p70^{s6k}/p85^{s6k}; Bisindolylmaleimide; Ro 31-6045; mTOR

1. Introduction

The mitogen-stimulated protein kinase p70^{s6k}/p85^{s6k} (S6K) is a key player in the regulation of cell growth and proliferation. There are two isoforms of S6K, p70 and p85, which are generated from alternative translation start sites in the same transcript. The p85^{s6k} isoform is identical to p70^{s6k}, except for a 23 amino acid extension at the amino-terminus that specifically targets it to the nucleus [1]. Both isoforms play essential roles in cell growth [2,3] through the regulation of protein synthesis. Multiple phosphorylation of the 40S ribosomal protein S6 by S6K results in a specific increase of translation efficiency for the family of essential mRNAs containing an oligopyrimidine tract at their transcriptional start site (5'TOP mRNAs). This class of mRNAs encode many components of the protein synthetic machinery, i.e. ribosomal proteins, translation initiation and elongation factors associated with regulating general protein synthesis rates, and represent up to 20% of the total mRNA in the cell [4,5].

S6K is regulated by a complex mechanism involving multiple phosphorylation reactions. It is hypothesised that an initial yet-to-be-defined conformational change is required to break an inhibitory interaction between the C- and N-termini and thus allow phosphorylation at four sites within the C-ter-

минаl autoinhibitory domain (S411, S418, S424 and T421). This is followed by the sequential phosphorylation of T389 in a hydrophobic motif conserved in other AGC kinases and finally the activating phosphorylation at T229 by the phosphoinositide-dependent kinase 1 (PDK1) [6,7]. Despite intensive effort, the signalling mechanisms responsible for the initial priming event and subsequent autoinhibitory domain phosphorylation remain to be defined. Recently the NimA-related kinase, NEK6/7, has been implicated in the phosphorylation of T389 but a physiological role in the activation of S6K is yet to be proven [8].

Much of our understanding of this activation process stems from the use of inhibitors of S6K signalling. Rapamycin and its analogues inhibit the translational regulator mTOR that acts as a bifurcation point to two pathways. mTOR is required for activation of S6K and also phosphorylates eIF-4E-binding protein (4EBP-1), releasing its inhibitory interaction with eIF-4E to increase global translation rates [9]. The mechanism by which mTOR regulates S6K remains to be elucidated but its inhibition by rapamycin abolishes phosphorylation of the critical residues T389 and T229 [10]. Activation of S6K is also phosphatidylinositol 3-kinase (PI3K)-dependent with the PI3K inhibitor wortmannin potently inhibiting S6K [11] and this dependence is apparently mediated via PDK1 phosphorylating T229 [12]. Mutation of T389 to a glutamic acid residue renders the kinase rapamycin and wortmannin resistant [10,13], indicating that the two pathways converge to regulate T389 phosphorylation. Importantly however, deletion analysis of the N- and C-termini resulted in a mutant kinase that remains dependent on PI3K activity (wortmannin-sensitive) but is independent of mTOR (rapamycin resistant) [13]. This observation supports the existence of an additional input necessary for activation of the kinase.

Selective inhibitors of protein kinases have been used to provide insight into both the mode of regulation and physiological roles of the targeted enzymes. Extensive screening of these compounds has revealed most have overlapping specificities, complicating interpretation of their effects on cell systems [14]. Rapamycin is one of the most selective but has still been implicated in the regulation of multiple pathways for example by altering the levels of cyclin D and the cdk inhibitor protein p27^{Kip1} in addition to its effects on mTOR, S6K and 4EBP-1 [15]. In this study searching for alternative inhibitors of S6K we found that the bisindolylmaleimide derivative Ro 31-6045, previously reported to be inactive as a kinase inhibitor [16], inhibited endogenous S6K activity in stimulated fibroblasts. Structure/function analysis using S6K mutants re-

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vealed Ro 31-6045 blocked activation of the kinase by inhibiting phosphorylation of T389 independently of mTOR and led us to propose that the inhibitor may target a novel activation pathway. Ro 31-6045 will prove useful in elucidating the complex activation mechanism of S6K and its independence from mTOR will allow confirmation of functional data obtained using the mTOR inhibitor rapamycin. Furthermore, Ro 31-6045 may provide the basis for the development of specific inhibitors of S6K of potential therapeutic importance.

2. Materials and methods

2.1. Materials

Ro 31-8220 and Ro 31-6045 were purchased from Alexis Corporation, UK. H89 and H85 were purchased from Seikagaku, USA. Extracellular growth factor (EGF) was purchased from Auspep, Australia, serum and tissue culture media from Gibco BRL, and general chemicals from Sigma.

2.2. Cell culture and preparation of cell extracts

HEK293 and Balb/c 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum at 37°C in a 5% CO₂ atmosphere. Balb/c 3T3 cells were plated at 2×10^6 per 10 cm plate for 24 h, then made quiescent by culturing for 24 h, in DMEM containing no serum. Serum-starved cells were rinsed twice with HEPES buffer solution containing 120 mM NaCl and 20 mM HEPES, pH 7.4. Cells were then treated at 37°C with 5 nM EGF for 5 min. Alternatively, the cells were pretreated for 20 min with varying concentrations of Ro 31-8220, H89, Ro 31-6045 or H85 as indicated. Following stimulation, the cells were rinsed twice with ice cold phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 1% Nonidet-P40, 1 mM EDTA, 50 mM NaF, 40 mM β -glycerophosphate, 0.1 mM sodium vanadate, 1 mM benzamide, 0.5 mM phenylmethylsulfonyl fluoride. Cell extracts were collected with a plastic scraper, and cleared by centrifugation at 4°C for 15 min at $12000 \times g$. Protein concentration was measured by the method of Bradford (Bio-Rad), with bovine serum albumin as standard. Aliquots of the supernatant were frozen in liquid nitrogen and stored at -70°C .

2.3. Transient transfections

HEK293 cells were plated at 1×10^6 per 10 cm plate and 24 h after plating were transfected with p70^{S6K} expression constructs tagged with the myc epitope using the calcium phosphate method as described previously [17] or with myc epitope-tagged PDK1 [18]. The constructs used were wild-type (2B4); constitutively active, rapamycin and wortmannin resistant (dED3E) with T389 and T421 mutated to glutamic acid and S411, S418, and S424 mutated to aspartic acid residues; the rapamycin resistant, wortmannin-sensitive double truncation mutant ($\Delta\text{N}\Delta\text{C}$) missing residues 1–54 and 389–502 and $\Delta\text{N}\Delta\text{C}$ with T389 mutated to glutamic acid ($\Delta\text{N}\Delta\text{C}389\text{E}$). Twenty-four hours after transfection, the cells were serum-starved for 24 h. Following serum-starvation the cells were rinsed with HEPES buffer and stimulated with 20% serum for 20 min. Alternatively the cells were pretreated with varying concentrations of Ro 31-8220, Ro 31-6045, H89 or H85 as indicated. Following stimulation the cells were washed and harvested as above.

2.4. S6K, Akt and PDK1 activity assays

Endogenous S6K activity was assayed as described previously [19] using 40S ribosomes as substrate. Myc-tagged p70^{S6K} was immunoprecipitated and assayed as described previously [10]. Akt activity was assayed as described previously using RPRAATF peptide as substrate [20]. The results are expressed in units of activity per mg protein lysate or as % of control values. One unit of activity results in the transfer of 1 pmol of $^{32}\text{P}_i$ to the respective substrate per minute. Autophosphorylation activity of myc-tagged PDK1 was determined essentially as reported previously [21]. Briefly, myc-PDK1 was immunoprecipitated from 250 μg of extracts from transiently transfected HEK293 cells and the washed immunocomplexes incubated for 20 min at 30°C in a total volume of 20 μl of 25 mM Tris-HCl pH 7.5, 0.5 mM dithiothreitol, 0.5 mM benzamide, 50 μM [γ - ^{32}P]ATP (5000 cpm/pmol) and 10 mM MgCl₂. The reaction was stopped by boiling in SDS-polyacrylamide

gel electrophoresis (SDS-PAGE) sample buffer and proteins resolved by SDS-PAGE. Phosphorylation of PDK1 was visualised by autoradiography of the dried gel and quantitated by liquid scintillation counting of excised bands.

2.5. Western blotting

Whole protein extracts (50 μg) were subjected to SDS-PAGE on 15% gel slabs with 5% stacking gels at 200 V for 1 h. Proteins were transferred onto Immobilon-P PVDF membrane (Millipore) using the Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 12 V (~ 100 mA) for 30 min. Following transfer, the membrane was blocked for 30 min in PBS containing 5% (w/v) skim milk powder and 0.5% (v/v) Tween-20 (PBSM). The membrane was incubated at room temperature for 1 h with either the phospho-4EBP-1 (Cell Signalling #9451), actin (ICN #691001), phospho-T389 (Cell Signalling #9205F) or 9E10 anti-myc tag antibodies diluted 1:1000 in PBSM. After washing the blot was incubated with horseradish peroxidase anti-rabbit or anti-mouse secondary antibodies diluted 1:2000 at room temperature for 1 h, the immunoblot complex visualised by the enhanced chemiluminescence method (NEN Life Science) and detected by exposure to X-ray film (Kodak).

3. Results

3.1. Effects of Ro 31-8220, H89, Ro 31-6045 and H85 on in vivo activation of S6K

In order to search for selective inhibitors of S6K signalling we have investigated the in vivo potency of two commonly used inhibitors of AGC family kinases, the protein kinase C (PKC) inhibitor Ro 31-8220 and the protein kinase A (PKA) inhibitor H89 that have been shown to inhibit S6K in vitro [14,22]. Their inactive analogues Ro 31-6045 [16] and H85 were incorporated as specificity controls (Fig. 1). Ro 31-8220 and H89 potently inhibited EGF-induced activation of S6K in fibroblasts with IC₅₀ values of 600 nM and 3 μM respectively. Surprisingly, Ro 31-6045 also inhibited S6K with IC₅₀ = 8 μM and H85 with IC₅₀ = 25 μM . None of these

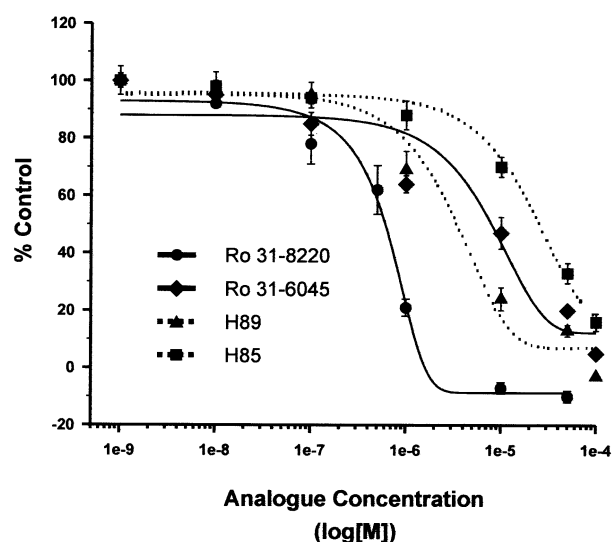


Fig. 1. Effects of Ro 31-8220, H89, Ro 31-6045 and H85 on in vivo activation of S6K. Balb/c 3T3 cells were plated, serum-starved for 24 h, pretreated with varying concentrations of Ro 31-8220, H89, Ro 31-6045 and H85 as indicated for 20 min, then stimulated with EGF (5 nM) for 5 min and harvested. S6 kinase assay was performed as described under Section 2, normalised to control (EGF-stimulated) and graphed (mean \pm S.E.M.) with increasing concentrations of drug. The IC₅₀ values are: Ro 31-8220 – 6×10^{-7} M, H89 – 3×10^{-6} M, Ro 31-6045 – 8×10^{-6} M and H85 – 2.5×10^{-5} M.

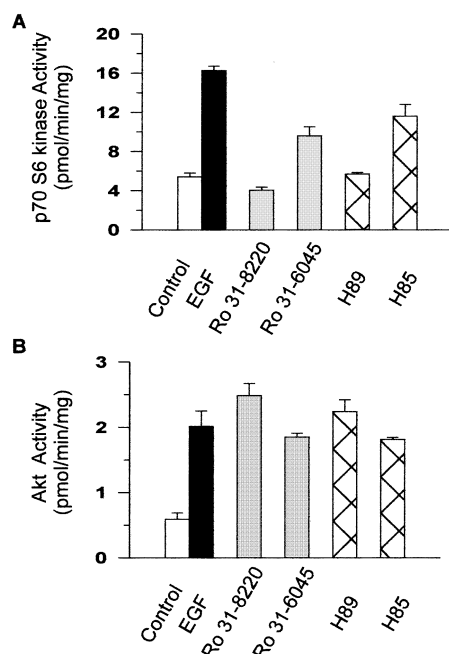


Fig. 2. Effects of Ro 31-8820, H89, Ro 31-6045 and H85 on in vivo activation of S6K and Akt activity. Balb/c 3T3 cells were plated, serum-starved for 24 h, pretreated with 5×10^{-6} M Ro 31-8820, H89, Ro 31-6045 or H85 for 20 min, then stimulated with EGF for 5 min and harvested. S6K and Akt assays were performed as described under Section 2. The data shown are the mean \pm S.E.M. of the Akt and S6K activity in pmol P_i /mg protein/min.

compounds inhibited the related kinase Akt at 5 μ M, a concentration that significantly inhibited S6K activation (Fig. 2A,B). Interestingly, both Ro 31-8820 and H89 reproducibly enhanced Akt activity.

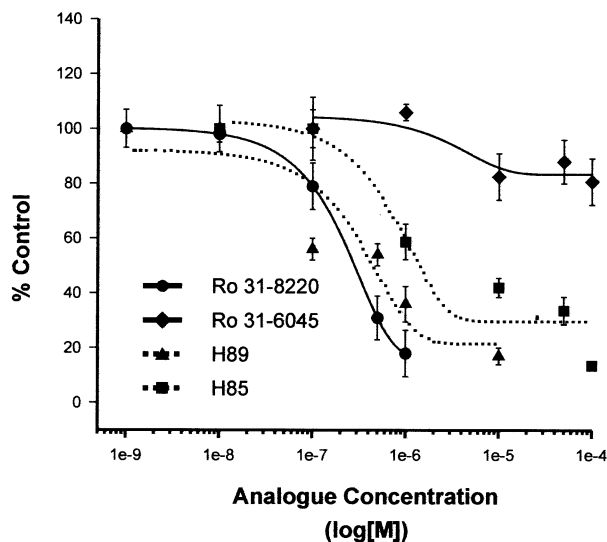


Fig. 3. Effects of Ro 31-8820, H89, Ro 31-6045 and H85 on in vitro activation of S6K. HEK293 cells were plated, transfected with wild-type myc-tagged S6K for 24 h before being serum-starved for 24 h, then stimulated with serum for 20 min and harvested. Myc-tagged S6K was immunoprecipitated and the precipitated kinase incubated with increasing doses of each inhibitor as indicated for 20 min then assayed as described under Section 2. The results were normalised to control (serum-starved) and graphed (mean \pm S.E.M.) with increasing concentrations of drug. The IC_{50} values are: Ro 31-8820 – 3×10^{-7} M, H89 – 5×10^{-7} M and H85 – 1.5×10^{-6} M.

3.2. Effects of Ro 31-8820, H89, Ro 31-6045 and H85 on in vitro activity of S6K

Both the bisindolylmaleimide (Ro 31-8820 and Ro 31-6045) and isoquinolinesulfonamide analogues (H89 and H85) were designed to compete with ATP for binding to the active site of kinases. In order to determine whether all the inhibitors directly affected S6K activity, epitope-tagged wild-type S6K was expressed in serum-stimulated HEK293 cells, immuno-purified and assayed for activity at varying doses of each inhibitor (Fig. 3). While Ro 31-8820, H89 and H85 all inhibited S6K in vitro, Ro 31-6045 had no effect up to 100 μ M, indicating this compound may be acting via inhibition of an upstream signalling pathway.

3.3. Effects of Ro 31-6045 on PDK1 signalling

PDK1-catalysed phosphorylation of T229 in the activation loop of S6K is the best characterised activating mechanism for the kinase. PDK1 also activates other AGC kinase family members including Akt, PKA and PKC via phosphorylation of homologous residues [23]. While PKA and PKC have been

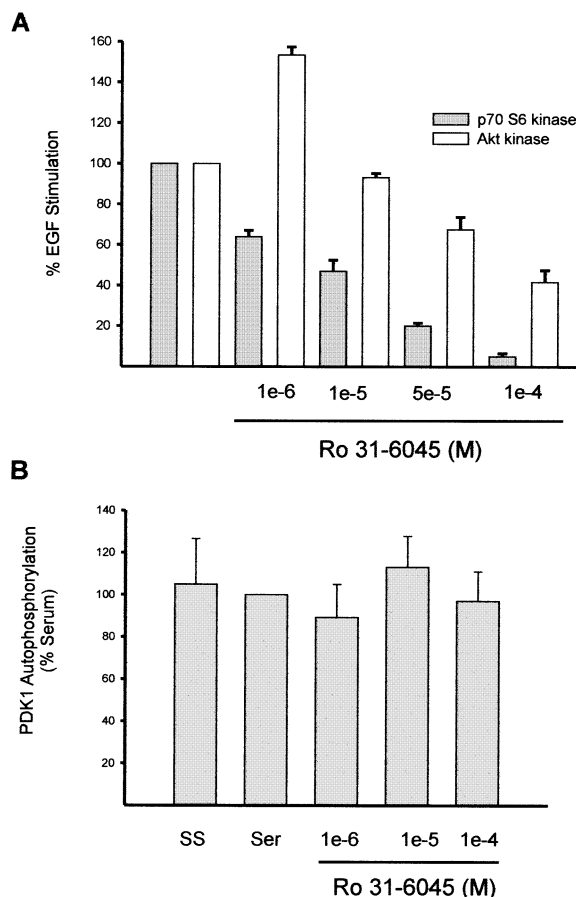


Fig. 4. Effects of Ro 31-6045 on PDK1 signalling. A: Balb/c 3T3 cells were plated, serum-starved for 24 h, pretreated with varying concentrations of Ro 31-6045 as indicated for 20 min, then stimulated with EGF (5 nM) for 5 min and harvested. S6 kinase and Akt assays were performed as described under Section 2, normalised to control (EGF-stimulated) and graphed (mean \pm S.E.M.) with increasing concentrations of drug. B: HEK293 cells were plated, transfected with myc-tagged PDK1 for 24 h and then serum-starved for 24 h, pretreated with varying concentrations of Ro 31-6045 as indicated for 20 min, then stimulated with serum for 20 min and harvested. Myc-tagged S6K was immunoprecipitated and assayed for autophosphorylation activity as described under Section 2.

shown to be resistant to Ro 31-6045 up to 100 μ M [16], it is theoretically possible that Ro 31-6045 inhibits PDK1 and the resistant kinases require lower levels of PDK1 activity. In order to test this possibility, a detailed dose–response curve of the effects of Ro 31-6045 on Akt, the kinase most closely related to S6K, was carried out (Fig. 4A). Akt was weakly inhibited with an $IC_{50} > 50 \mu$ M, 10-fold higher than for S6K. Furthermore, Ro 31-6045 had no effect on the activity of PDK1 exogenously expressed in HEK293 cells at concentrations up to 100 μ M (Fig. 4B), consistent with the resistance of PKA and PKC and indicating that Ro 31-6045 does not act via PDK1.

3.4. Effects of Ro 31-6045 on in vivo activation of S6K mutants

In order to further delineate the mechanism of action of Ro 31-6045 we determined the effect of this inhibitor on the ac-

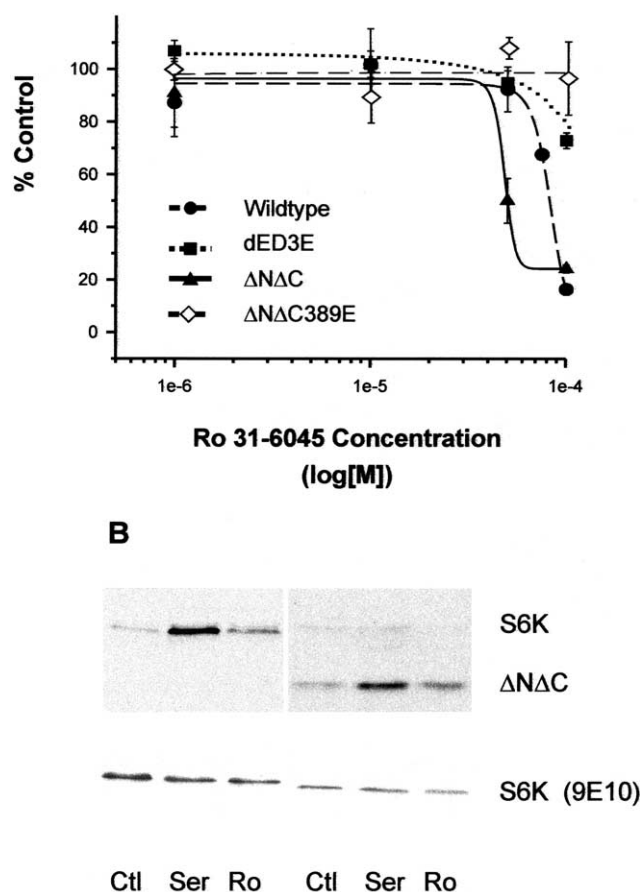


Fig. 5. Effects of Ro 31-6045 on in vivo activation of S6K mutants. HEK293 cells were plated, transfected with wild-type, dED3E, Δ N Δ C and Δ N Δ C389E myc-tagged S6K for 48 h before being serum-starved for 24 h. The cells were then pretreated with varying concentrations of Ro 31-6045 as indicated for 20 min then stimulated with serum for 20 min and harvested. A: Myc-tagged S6K was immunoprecipitated and assayed as described under Section 2. The results were normalised to control (serum-starved) and graphed (mean \pm S.E.M.) with increasing concentrations of drug. The IC_{50} for wild-type S6K was 8.5×10^{-5} M and 5×10^{-5} M for Δ N Δ C. B: Cells expressing wild-type S6K (lanes 1–3) or Δ N Δ C (lanes 4–6) were serum-starved (Ctl), serum-stimulated (Ser) or treated with 100 μ M Ro 31-6045 (Ro) as described above, extracted and subjected to Western blot analysis for S6K phosphorylated on T389, or for S6K expression using the 9E10 α -myc tag monoclonal antibody as described under Section 2.

tivity of some S6K structure/function mutants. S6K is activated by a complex mechanism involving wortmannin-sensitive activation of PI3K and signalling via the rapamycin-sensitive kinase mTOR. A mutant form of S6K with the hydrophobic motif phosphorylation site and three autoinhibitory sites substituted with acidic residues (dED3E) is resistant to inactivation by both wortmannin and rapamycin [10,13]. Deleting portions of the C- and N-termini of the kinase results in a mutant (Δ N Δ C) that remains resistant to rapamycin but sensitive to wortmannin [13], indicating a clear difference between the two processes. As both mutants are phosphorylated on T229, the target site of the PI3K-dependent activator PDK1, it is likely that this double deletion mutant reveals a novel signalling pathway [24]. Because these mutants apparently distinguish the two activating pathways, each was tested for its sensitivity to inhibition by Ro 31-6045 (Fig. 5A). Like wortmannin, Ro 31-6045 failed to significantly inhibit the activity of the constitutively active dED3E mutant at concentrations that reduced the activity of both the double truncation mutant and wild-type enzyme by 80%. Western blot analysis of these extracts using the phospho-T389 antibody revealed that the inhibition of wild-type and Δ N Δ C by Ro 31-6045 was accompanied by the reduction of Thr-389 phosphorylation to levels similar to baseline (Fig. 5B), consistent with the inhibitor targeting phosphorylation of this key regulatory site. Indeed, substitution of T389 with glutamic acid renders Δ N Δ C totally resistant to Ro 31-6045 (Fig. 5A), confirming T389 phosphorylation as the critical target for inhibition.

4. Discussion

In the present study we have shown that two commonly used inhibitors of AGC family kinases, the PKC inhibitor Ro 31-8220 and the PKA inhibitor H89, also inhibit S6K in vivo, consistent with previous reports that they inhibited S6K in vitro [14,22]. Surprisingly, their inactive analogues Ro 31-6045 and H85 also inhibited EGF-induced activation of S6K in fibroblasts. In fact, the 8 μ M IC_{50} determined for Ro 31-6045 is comparable to that of H89 (3 μ M) and consistent with the low micromolar IC_{50} values reported for other kinases in cell-based assays [14]. These observations reinforce the need for careful controls in interpreting the results of inhibitor studies in cell-based assays. Clearly, even the use of inactive analogues can be complicated by altered inhibitor specificity. The gold standard for these studies will be the ability of drug resistant forms of the kinase to reverse the effects of the inhibitor. It is perhaps not surprising that H85 shows some inhibitory activity as although it does not inhibit PKA, it does target other kinases including Ca^{2+} /CaM kinase II, MLCK and casein kinase 1 [25]. On the other hand Ro 31-6045 had no effect on PKC, PKA or Ca^{2+} /CaM kinase II even at 100 μ M [16].

Unlike the other three compounds, Ro 31-6045 did not inhibit S6K in vitro, implying it targeted an upstream activation pathway. Analysis of its effects on the mutant forms of the kinase revealed the inhibition was associated with dephosphorylation of T389. Thus Ro 31-6045 apparently targets a T389 kinase but this is independent of mTOR signalling as the rapamycin resistant mutant Δ N Δ C is sensitive to Ro 31-6045. As the utility of this inhibitor to assess S6K signalling independent of rapamycin is a key finding of this study, we used a

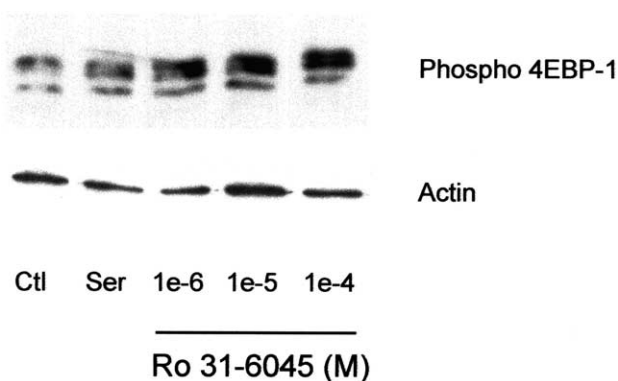


Fig. 6. Effects of Ro 31-6045 on phosphorylation of eIF-4EBP-1. Balb/c 3T3 cells were plated, serum-starved for 24 h, pretreated with varying concentrations of Ro 31-6045 as indicated for 20 min, then stimulated with EGF (5 nM) for 5 min. Extracts were prepared and subjected to Western blot analysis for phosphorylated eIF-4EBP-1, or actin as a loading control as described under Section 2.

phospho-specific antibody to the alternative mTOR substrate 4EBP-1 to confirm Ro 31-6045 did not affect mTOR-catalysed phosphorylation of 4EBP-1 up to 100 μ M (Fig. 6).

This inhibition is not mediated via PDK1 as Ro 31-6045 does not inhibit the kinase at concentrations up to 100 μ M, consistent with the resistance of PKA and PKC reported previously [16]. Furthermore, Ro 31-6045 does not inhibit dED3E, despite this mutant requiring T229 phosphorylation by PDK1 for activity. It is likely that the weak effect of the inhibitor on Akt activity is mediated via the regulatory mechanism targeting S473, the carboxy-terminal hydrophobic phosphorylation site highly homologous to T389 in S6K.

Ro 31-6045 thus provides a new tool for examining the mechanism of activation and signalling of S6K. In particular, it will be important to assess the effects of Ro 31-6045 on the newly discovered potential T389 kinase NEK6/7 and the inhibitor may provide a useful tool for probing the physiological role of this enzyme in activating S6K. Given Ro 31-6045 is a bisindolylmaleimide analogue of staurosporine it is likely to be acting as a competitive inhibitor of ATP-binding and thus may be a suitable compound for affinity purifying the putative mTOR-independent T389 kinase. The utility of this approach was initially shown for ATP itself coupled to an agarose support [26].

The observation that overexpression of wild-type S6K in HEK293 cells increases the IC_{50} 10-fold from that of endogenous S6K in fibroblasts raises the possibility that Ro 31-6045 also targets the inactive form of the kinase, as reported for the inhibition of MKK1 by U0126 and PD 184352 [14] and SAP-K2a by SB 203580 [27]. Alternatively, this difference may reflect inherent differences of drug sensitivity of each cell line, or perhaps is due to the variety of growth factors used. Nonetheless, Ro 31-6045 provides the facility for probing S6K function independent of rapamycin inhibition of mTOR in cell-based experiments. Although rapamycin is one of the most selective signalling inhibitors reported [14] it has still been implicated in the regulation of multiple pathways. The availability of an Ro 31-6045-independent mutant of S6K will allow convenient validation of the implied roles of the kinase in the control of a cellular process.

In addition to aiding in the analysis of S6K signalling, Ro 31-6045 may provide a lead compound for development of

S6K signalling-specific inhibitors of therapeutic importance. Maintenance of a sustained elevated rate of protein synthesis is a fundamental requirement for tumour formation. Over the last year analogues of the S6K signalling pathway antagonist, rapamycin have been tested in pre-clinical and phase I studies with preliminary evidence of anti-tumour effects being observed in renal cell carcinoma and non-small cell lung cancer [28]. In fact, a specific role for S6K in breast cancer has been inferred from the observation that the S6K gene is amplified and the protein overexpressed in a number of breast cancer cell lines and 8% (68/698) of primary tumours [29].

In summary, we have identified the bisindolylmaleimide derivative Ro 31-6045 as an *in vivo* inhibitor of S6K. Structure/function analysis using mutant forms of S6K indicates that Ro 31-6045 targets T389 phosphorylation independent of the upstream activator mTOR. This inhibitor will prove useful in elucidating the complex activation mechanism of S6K, and in combination with a drug resistant mutant of S6K (dED3E), this reagent will allow rapamycin-independent analysis of S6K signalling in cell-based systems. In addition, Ro 31-6045 may provide a lead in the development of S6K-specific inhibitors of tumour growth.

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References

- [1] Reinhard, C., Thomas, G. and Kozma, S.C. (1992) *Proc. Natl. Acad. Sci. USA* 89, 4052–4056.
- [2] Lane, H.A., Fernandez, A., Lamb, N.J. and Thomas, G. (1993) *Nature* 363, 170–172.
- [3] Reinhard, C., Fernandez, A., Lamb, N.J. and Thomas, G. (1994) *EMBO J.* 13, 1557–1565.
- [4] Jefferies, H.B., Fumagalli, S., Dennis, P.B., Reinhard, C., Pearson, R.B. and Thomas, G. (1997) *EMBO J.* 16, 3693–3704.
- [5] Jefferies, H.B., Reinhard, C., Kozma, S.C. and Thomas, G. (1994) *Proc. Natl. Acad. Sci. USA* 91, 4441–4445.
- [6] Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A. and Thomas, G. (1998) *Science* 279, 707–710.
- [7] Moser, B.A., Dennis, P.B., Pullen, N., Pearson, R.B., Williamson, N.A., Wettenhall, R.E., Kozma, S.C. and Thomas, G. (1997) *Mol. Cell Biol.* 17, 5648–5655.
- [8] Belham, C., Comb, M.J. and Avruch, J. (2001) *Curr. Biol.* 11, 1155–1167.
- [9] Gingras, A.C., Raught, B. and Sonenberg, N. (2001) *Genes Dev.* 15, 807–826.
- [10] Pearson, R.B., Dennis, P.B., Han, J.W., Williamson, N.A., Kozma, S.C., Wettenhall, R.E. and Thomas, G. (1995) *EMBO J.* 14, 5279–5287.
- [11] Han, J.W., Pearson, R.B., Dennis, P.B. and Thomas, G. (1995) *J. Biol. Chem.* 270, 21396–21403.
- [12] Alessi, D.R., Kozlowski, M.T., Weng, Q.P., Morrice, N. and Avruch, J. (1998) *Curr. Biol.* 8, 69–81.
- [13] Weng, Q.P., Andrabi, K., Kozlowski, M.T., Grove, J.R. and Avruch, J. (1995) *Mol. Cell Biol.* 15, 2333–2340.
- [14] Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000) *Biochem. J.* 351, 95–105.
- [15] Hashemolhosseini, S., Nagamine, Y., Morley, S.J., Desrivieres, S., Mercep, L. and Ferrari, S. (1998) *J. Biol. Chem.* 273, 14424–14429.
- [16] Twomey, B., Muid, R.E., Nixon, J.S., Sedgwick, A.D., Wilkinson, S.E. and Dale, M.M. (1990) *Biochem. Biophys. Res. Commun.* 171, 1087–1092.
- [17] Pearson, R.B. and Thomas, G. (1995) *Prog. Cell Cycle Res.* 1, 21–32.
- [18] Park, J., Hill, M.M., Hess, D., Brazil, D.P., Hofsteenge, J. and Hemmings, B.A. (2001) *J. Biol. Chem.* 276, 37459–37471.

- [19] Lane, H.A. and Thomas, G. (1991) *Methods Enzymol.* 200, 268–291.
- [20] Bozinovski, S., Cristiano, B.E., Marmy-Conus, N.J. and Pearson, R.B. (2002) *Anal. Biochem.*, in press.
- [21] Wick, M.J., Dong, L.Q., Riojas, R.A., Ramos, F.J. and Liu, F. (2000) *J. Biol. Chem.* 275, 40400–40406.
- [22] Alessi, D.R. (1997) *FEBS Lett.* 402, 121–123.
- [23] Toker, A. and Newton, A.C. (2000) *Cell* 103, 185–188.
- [24] Dennis, P.B., Pullen, N., Pearson, R.B., Kozma, S.C. and Thomas, G. (1998) *J. Biol. Chem.* 273, 14845–14852.
- [25] Chijiwa, T. et al. (1990) *J. Biol. Chem.* 265, 5267–5272.
- [26] Haystead, C.M., Gregory, P., Sturgill, T.W. and Haystead, T.A. (1993) *Eur. J. Biochem.* 214, 459–467.
- [27] Frantz, B. et al. (1998) *Biochemistry* 37, 13846–13853.
- [28] Dancey, J. (2001) *ASCO Meeting Highlights*, 17.
- [29] Barlund, M. et al. (2000) *J. Natl. Cancer Inst.* 92, 1252–1259.