COMMENTARY

A new tool to dissect the function of p70 S6 kinase

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Developing small-molecule inhibitors that are highly selective for specific protein kinases has been and remains a serious challenge. This especially applies to members of families of related kinases with overlapping substrate specificities, such as the serine/threonine kinases of the AGC family. In this issue of the Biochemical Journal, Dario Alessi’s group, in a collaboration with Pfizer, report on PF-4708671, a potent and highly selective inhibitor of S6K1 (p70 S6 kinase 1) in vitro and in cells. S6K1 is an AGC family member and a crucial effector of the mTORC1 (mammalian target of rapamycin complex 1) kinase. This is the first reported inhibitor that is highly selective for S6K1. This compound will help to understand the signalling and physiological roles of S6K1, and to dissect signalling downstream of mTORC1. S6K1 inhibitors may ultimately be useful in the treatment of diseases such as cancer where S6K1 is overexpressed, but most importantly in metabolic disease such as insulin resistance and obesity.

Key words: cancer, insulin resistance, mammalian target of rapamycin (mTOR), obesity, p70 S6 kinase 1 (S6K1), PF-4708671.

The AGC family of serine/threonine protein kinases comprises more than 50 different members, and includes isoforms of PKC (protein kinase C), PKB (protein kinase B, also known as Akt), SGK (serum- and glucocorticoid-induced protein kinase), p90Rsk (p90 ribosomal S6 kinase) and S6K (p70 S6 kinase) which are involved in diverse and important cellular functions, and which are regulated by various signalling pathways [1]. The development of selective inhibitors of AGC kinase family members has been challenging, and it is only recently that some inhibitors with the desired level of selectivity have been reported, such as the p90Rsk inhibitor BI-D1870 [2] and the PKB inhibitors MK-2206 and Akti-1/2 [3].

Mammals have two isoforms of S6K, called S6K1 and S6K2, which are encoded by separate genes giving rise (by alternative splicing) to cytoplasmic and nuclear variants of both S6K1 and S6K2 [1]. These S6Ks, which are closely related to RSks, are key downstream effectors of the PI3K (phosphoinositide 3-kinase)/PKB/mTORC1 (mammalian target of rapamycin complex 1) signalling pathway (Figure 1a). The mTORC1/S6K pathway forms a critical nodal point in a signalling network that regulates protein synthesis, cell growth, metabolism and aging. This pathway has therefore received huge attention, both in fundamental research and in drug development. It has been challenging, however, to narrow down which mTOR (mammalian target of rapamycin) S6K effectors play a role in each of these processes.

In response to various extracellular stimuli, including nutrients, energy stress and growth factors [insulin and IGF1 (insulin-like growth factor 1)], S6K is activated by co-ordinated phosphorylation (Figure 1b). These occur on Thr389 in the conserved hydrophobic motif in the non-catalytic region of S6K1 (by the mTORC1 serine/threonine kinase) and on Thr229 in the activation loop of the kinase domain [by the PDK1 (3-phosphoinositide-dependent kinase 1) serine/threonine kinase]. Once activated, S6K can phosphorylate numerous substrates (>10) in cells, many of which are components of the translational machinery, including rpS6 (40S ribosomal protein S6) (Figure 1a). However, it is not clear which of these proteins are genuine in vivo substrates of S6K1 or S6K2. Indeed, several AGC kinases can phosphorylate the same substrates as S6K, including rpS6, on the same sites. This also complicates the interpretation of S6K RNAi (RNA interference) or gene-KO (knockout) studies which allow compensatory signalling by other AGC kinases.

S6K1−/− mice are significantly smaller at birth due to a reduction in the size of all organs and also exhibit a selective decrease in pancreatic β-cell and myoblast size (reviewed in [4]). These studies identified S6K1 as a key player in the regulation of cell growth, in line with previous studies in Drosophila (which only has one isoform of S6K). S6K1 is also critical for glucose homeostasis, as S6K1−/− mice are glucose-intolerant, hypoinsulinaemic and are protected from diet-induced insulin resistance and obesity [5]. This suggests that S6K1 could be a drug target in metabolic disorders, such as Type 2 diabetes which is characterized by insulin resistance, and in obesity.

The underlying mechanism of the S6K1-null phenotype is in part explained by a negative-feedback loop whereby S6K1 turns off upstream signalling by nutrient overload. Indeed, under nutritional conditions, such as in the presence of excess amino acids or fat, S6K1 constitutively phosphorylates IRS-1 (insulin receptor substrate-1) at multiple sites (Figure 1b). This promotes the degradation of IRS-1, thereby shutting down the responsiveness of certain cells to insulin, ultimately dampening PI3K/PKB activation. Another contributor to the S6K1-null phenotype could be the reduced phosphorylation of rpS6. This is supported further by the observation that the metabolic effect of the S6K1-deficient mouse is phenocopied in a mutant mouse line in which endogenous rpS6 is converted into a mutant (called rpS6pS/pS−/−) that can no longer become phosphorylated, as a consequence of the introduction of a germine knockin mutation that converts all five phosphorylatable serine residues into alanine [6]. However, in these rpS6pS/pS−/− mice, the S6K1 negative-feedback loop is expected to occur given that S6K1 activity remains intact.

Abbreviations used: 4EBP1, eukaryotic initiation factor 4E-binding protein 1; HEK, human embryonic kidney; IGF1, insulin-like growth factor 1; IRS-1, insulin receptor substrate-1; KO, knockout; MSK1, mitogen- and stress-activated protein kinase 1; mTOR, mammalian target of rapamycin; mTORC, mTORC complex; p90Rsk, p90 ribosomal S6 kinase; PD3K, 3-phosphoinositide-dependent kinase 1; PKB, protein kinase B; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; rpS6, 40S ribosomal protein S6; S6K, p70 S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase.

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and it is not entirely clear how the lack of phosphorylatable serine residues in rpS6 is able to phenocopy the effect of S6K1 deficiency (discussed in [6]).

Little is known about S6K2. S6K2\(^{-/-}\) mice, unlike S6K1\(^{-/-}\) mice, do not have any apparent phenotypes. However, S6K1\(^{-/-}\)/S6K2\(^{-/-}\) double KO results in perinatal lethality, indicating that S6K2 may have a critical function in development which may be taken over by S6K1 in the absence of S6K2 [7]. In other words, the lack of a phenotype in S6K2-KO mice could be a limitation that is often seen in KO studies, whereby a compensatory mechanism by a related kinase can mask a phenotype. This may, in fact, also be the case in S6K1\(^{-/-}\)/S6K2\(^{-/-}\) mice as they have elevated S6K2 expression [8]. Genetic strategies whereby inactivation of the kinase activity (made by introduction of an inactivating mutation) while retaining its expression (so-called knockin mice), can circumvent this problem [9]. Clearly, this mode of kinase inactivation is also achieved by a small-molecule inhibitor which, unlike a KO allele, inactivates the kinase, but does not wipe out its expression (although a kinase inhibitor is more acute compared with the constitutive kinase-dead mice). S6K1 kinase-dead knockin mice have not been reported so far. The availability of a pharmacological tool with selectivity for S6K1, as reported in the work by Pearce et al. [10] in this issue of the Biochemical Journal, is clearly an advantage in this type of studies.

To study S6K signalling, researchers have often resorted to the use of rapamycin, an allosteric inhibitor of the S6K activator mTORC1 (Figure 1a), which is widely used as an immuno-suppressant to prevent graft rejection. However, this inhibitor also blocks mTORC1 effectors other than S6Ks such as 4EBP1 (eukaryotic initiation factor 4E-binding protein 1), a regulator of cap-dependent translation. Rapamycin also does not distinguish between inhibition of S6K1 and S6K2. Furthermore, prolonged treatment with rapamycin can also block mTORC2 activity in a subset of cancer cell lines [11] (Figure 1b).

Pearce et al. [10] have developed and characterized a cell-permeant S6K1-specific inhibitor, called PF-4708671. In vitro, PF-4708671 inhibits S6K1 at mid- to low-nanomolar concentrations (IC\(_{50}\) of 160 nM). In a panel of ~90 protein and lipid kinases, this compound was found to be highly selective for S6K1 over its most closely related AGC family members, including S6K2 on which this inhibitor is 400-fold less potent. Furthermore, PF-4708671 does not hit PDK1, which acts as a ‘master regulator’ of several AGC kinases, including S6K1, RSKs, PKB, SGK and PCKs (Figure 1b). PF-4708671 was found to inhibit S6K1 activity upon stimulation with IGF1 and PMA (two separate pathway activators) in HEK (human embryonic kidney)-293 cells. In these cells, protein kinase selectivity was confirmed by assessing phosphorylation of different AGC kinase substrates, with only the S6K substrate rpS6 being affected. This confirms earlier studies that S6K1 is the primary kinase phosphorylating rpS6. Among the AGC kinases, MSK1 (mitogen- and stress-activated protein kinase 1) is the next most sensitive to PF-4708671, with an in vitro IC\(_{50}\) of 950 nM. It is possible that inhibition of MSK1, an ERK (extracellular-signal-regulated kinase)-activated member of this family and closely related to the RSKs, may contribute to the cellular effects of PF-4708671 and this will need to be addressed in the future. However, PF-4708671 does not appear to inhibit MSK1 in cells at doses where it strongly inhibits S6K1 (e.g. 10 \(\mu\)M). To address this specificity issue of PF-4708671 in cells, it will be useful to treat S6K1-null cells with this compound to further assess potential off-target effects of this drug. Vice versa, as the authors point out, treating S6K2-null cells with this inhibitor could identify genuine S6K1 substrates [10].

Surprisingly, the authors found that, in cells, PF-4708671 very rapidly (within 1 min) promoted phosphorylation of S6K1 on its activating sites Thr\(^{389}\) and Thr\(^{229}\) inside cells [10]. Upon immunoprecipitation of S6K1 from these cells, and extensive washing to remove the inhibitor, drug-treated S6K1 was indeed found

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**Figure 1** mTORC1/S6K signalling pathway

(a) The serine/threonine kinase mTOR exists in two structural and functional complexes, mTORC1 and mTORC2. Among other components, mTORC1 contains raptor (regulatory associated protein of mTOR), is sensitive to rapamycin and is regulated by growth factors and nutrients. mTORC1 has S6K1 and S6K2 and 4EBP1 as its main substrates. mTORC2 contains rictor (rapamycin-insensitive companion of mTOR) instead of raptor, and is rapamycin-resistant, although long-term treatment of some cells with this agent can inactivate mTORC2. mTORC2 activation seems to be mediated only by growth factors and phosphorylates PKB on Ser\(^{473}\) in is hydrophobic motif, and can also phosphorylated PKC\(_{\alpha}\) in the same motif. elf4E, eukaryotic initiation factor 4E; (b) mTORC1 phosphorylates S6K1 at Thr\(^{389}\) in the hydrophobic motif, enabling PDK1 to phosphorylate Thr\(^{229}\) in the activation loop of S6K1, leading to full activation of S6K1. Activated S6K1 triggers a feedback loop that negatively affects PI3K signalling through various mechanisms. The key pathway is via S6K1-mediated phosphorylation of IRS-1 at multiple sites, which leads to a significant attenuation of the insulin and IGF1 effect, thereby damping PDK1/PKB activation. This explains why inhibition of mTORC1, which stops the negative-feedback loop, can enhance PDK1/PKB signalling. AGC kinases are coloured pink. ERK, extracellular-signal-regulated kinase.
to be more active [10]. Similar effects have been described previously for PKB inhibitors which promote PKB hyperphosphorylation on its activating sites Ser473 and Thr308 [12]. At present, the mechanism of this drug-induced S6K1 phosphorylation is unclear. However, this finding raises some concern that these types of inhibitors have the potential to activate the kinase they are designed to inhibit. It remains to be seen whether non-ATP-competitive compounds that interact with regulatory pockets on AGC kinases would lead to similar drug-induced phosphorylation.

An important question is whether there would be a potential therapeutic benefit of inhibiting S6K1 using compounds such as PF-4708671, for example in cancer and metabolic diseases, on the basis of the role of S6K1 in cell growth and glucose homeostasis. Rapamycin and its analogues (known as Rapamune or sirolimus) are currently progressing through clinical trials in a number of cancer types, although the clinical outcome is unpredictable and, in most instances, not effective. Limitations of the clinical use of rapamycin include immune suppression and the abrogation of the negative-feedback loop, ultimately resulting in enhanced PKB signalling and therefore promoting survival rather than reduction of cell proliferation.

As far as we are aware, the immune phenotype in S6K1-KO cells has not been reported. It is therefore not clear whether S6K1 inhibitors will have an immunosuppressive activity and thus could be different from rapamycin in this respect. On the other hand, inhibiting S6K1 only is expected to have the same disadvantage as rapamycin because S6K1 is the main player involved in this feedback mechanism. Pearce et al. [10] did not investigate in detail whether PF-4708671 would shunt the negative-feedback loop, but mention that the compound does not stimulate PKB phosphorylation and activation in HEK-293 cells. It remains to be determined whether PF-4708671 enhances PKB signalling in other cancer cells. In summary, it is not yet clear whether inhibition of S6K1 alone pharmacologically will result in the abrogation of the feedback loop as seen with rapamycin or in S6K1 KO cells. In some cancer cells, S6K appears to be overexpressed [13], and inhibiting S6K activity selectively in this context might be beneficial.

On the other hand, interfering with the negative-feedback loop could be advantageous in the treatment of metabolic disorders such as insulin-resistance-induced obesity and diabetes, a context in which enhanced signalling could be beneficial. Whether or not S6K1 inhibition would have similar side effects as rapamycin, such as hyperlipidaemia and deleterious effects on pancreatic islets, remains to be determined [14,15].

In conclusion, the study by Pearce et al. [10] demonstrates that it is possible to target, with potency and high selectivity, S6K1, a kinase belonging to the family of closely related kinases, the AGC kinase family. The recently reported crystal structures of inactive and partially activated forms of S6K1 [16] may aid further S6K inhibitor design. It will now be important to test the impact of S6K1 inhibitors on biological outcomes such as cell size, proliferation or protein synthesis. We anticipate that this tool will allow many outstanding questions in the mTORC1 signalling field to be answered.

ACKNOWLEDGEMENTS
We thank Alex Sullivan and Neil Torbett for critically reading this paper.

FUNDING
Work in the laboratory of B. V. is supported by the Medical Research Council [grant number G0700755] and Cancer Research UK [grant number C23338/A10200].

REFERENCES


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Received 8 September 2010; accepted 10 September 2010
Published on the Internet 28 September 2010, doi:10.1042/BJ20101445