COMMENTARY

A sharper instrument for dissecting signalling events: a specific AGC kinase inhibitor

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Small-molecule inhibitors are now widely used to try to dissect regulatory signalling events. Many of these interfere with the function of protein kinases, often as part of signalling cascades. In addition to their utility as tools for the researcher, the long-term aspiration is that certain of these compounds may be useful as therapeutic agents for the treatment of conditions that arise from the dysregulation of specific signalling pathways. In this issue of the *Biochemical Journal*, Sapkota and colleagues report the

identification and initial validation of a compound that inhibits the RSK (p90 ribosomal S6 kinase) group of protein kinases, which are members of an important family of kinases (the ‘AGC kinases’) that have overlapping specificities.

Key words: AGC kinase, mitogen-activated protein kinase (MAPK), p90 ribosomal S6 kinase (RSK), protein kinase inhibitor, small-molecule inhibitor.

There are four closely related RSK (p90 ribosomal S6 kinase) genes in the human genome (RSK1–4), which belong to the AGC family of protein kinases. RSKs phosphorylate a number of protein substrates *in vitro*: as with many phosphorylation events, the challenges are to confirm which kinase mediates them *in vivo* and to establish their relevance for the regulation of the substrate in a cellular context. This is especially important for enzymes such as the RSKs, for at least two major reasons. First, the substrate specificities of the RSKs resemble those of certain other AGC family kinases, such as isoforms of PKB (protein kinase B; also termed Akt) and of SGK (serum- and glucocorticoid-induced kinase) [1]. All phosphorylate serine or threonine residues in motifs of the general format RXXR(S/T), at least *in vitro*, and this potentially overlapping specificity makes it important to be able to distinguish which kinases act on which substrates *in vivo*. Secondly, the RSKs are activated by the ‘classical’ MAPKs (mitogen-activated protein kinases), ERK1/2 (extracellular-signal-regulated kinases 1 and 2). Several other kinases are also turned on by ERK signalling [e.g. the MSKs (mitogen- and stress-activated kinases) and MNKs (MAPK-integrating kinases)], again making it important to have specific tools to allow one to distinguish which ‘downstream’ kinases mediate which effects of the ERKs. Distinguishing functions of MSKs from those of RSKs is especially challenging, as they are closely related members of the AGC family of kinases.

In fact, the RSKs have the unusual feature of possessing two functional catalytic domains. ERK1/2 actually phosphorylate the C-terminal (non-AGC family) domain. Once activated, this domain is thought to phosphorylate a serine residue in the region linking the two catalytic domains, allowing a kinase called PDK1 (phosphoinositide-dependent kinase 1) to dock and phosphorylate a serine residue in the N-terminal domain. This step turns on the activity of RSKs towards other substrates [2]. Indeed, numerous potential RSK substrates have been described. Which are really RSK substrates *in vivo* and, given the multiplicity of signalling pathways, under what conditions?

Investigators often use ‘dominant-negative’ mutants of kinases to investigate their roles in cellular regulation. For enzymes in signalling cascades such as the ERK pathway, this approach is of limited value: downstream targets of ERKs, for example, bind the upstream ERK proteins. Thus overexpressing a ‘dominant-negative’ (e.g. kinase-dead) variant will probably bind much of the ERK in the cell, thus preventing activation of other ERK targets. For example, overexpression of one RSK isoform may well block activation of MSKs and MNKs, as well as other RSK isoforms. A much better approach would be to use small-molecule inhibitors of defined and narrow specificity.

A major challenge is to design inhibitors that are truly specific for individual protein kinases (or kinase subfamilies). This is especially tricky, as most protein kinase inhibitors act in a competitive manner with respect to the substrate ATP; thus one must exploit potential small differences in the ATP-binding pocket to create inhibitors that are selective, and hopefully specific, for the kinase under study. In the past, this has proven difficult.

Many widely used protein kinase inhibitors have been shown to lack specificity when tested against panels of members of the main protein kinase superfamily (e.g. see [3,4]). A good (or perhaps bad!) example of this, as Sapkota and colleagues point out, is Ro31-8220, which has often been used by investigators (including the present authors; [5]) as a specific inhibitor of certain protein kinase isoforms. However, Ro31-8220 actually interferes with the activity of many members of the AGC family of kinases, often at similar potency to its effects on other AGC kinases, and indeed also inhibits more distantly related protein kinases [4]. It is clearly not the signaller’s equivalent of a sharp scalpel!

The AGC family of protein kinases contains more than 50 different members, and includes enzymes involved in very diverse aspects of cellular regulation, and which are regulated by diverse signalling pathways (see www.cellsignal.com/reference/kinase/kinome.html). In addition to the N-terminal kinase domain of the RSKs, the group includes the cyclic-nucleotide-activated kinases,

Abbreviations used: ERK, extracellular-signal-regulated kinase; GPCR, G-protein-coupled receptor; GSK3, glycogen synthase kinase 3; MEK, MAPK/ERK kinase; MNK, MAPK-integrating kinase; MSK, mitogen- and stress-activated kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; RSK, p90 ribosomal S6 kinase; SGK, serum- and glucocorticoid-induced kinase.

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protein kinase A and protein kinase G; the PKB α - γ (or Akt1–3) group, which are regulated via PI3K (phosphoinositide 3-kinase); the related group of SGKs (SGK1–3); the seven-member GPCR (G-protein-coupled-receptor) kinase subfamily, which modulate GPCRs; and nine isoforms of protein kinase C, some of which are controlled by Ca²⁺ ions, as well as by products of phospholipase action. Several other AGC kinases play other key roles in cell regulation, e.g. Rho-kinase and myotonin-related Cdc42-binding kinase, which regulate contractility, and the related myotonic dystrophy kinases. The MSKs, as mentioned, are ERK-activated members of this family, and are closely related to the RSKs. PDK1, another AGC kinase, is a 'master regulator' of multiple kinases. Thus if an AGC kinase inhibitor also interferes with PDK1 function, this would have extensive effects on signalling pathways!

It would clearly be very valuable to have truly specific inhibitors of these enzymes, even if simply as research tools. Given the clear links between, e.g. dysregulation of PI3K signalling or GPCR signalling and certain diseases, appropriate compounds could be effective therapeutic agents.

The AGC family also includes the ribosomal protein S6 kinases, which lie downstream of the mammalian target of rapamycin. Rapamycin is a highly specific inhibitor of mTOR (mammalian target of rapamycin), a protein kinase, but actually does not block the kinase active site, but rather binds (together with an immunophilin, FKBP12) to a region adjacent to the active site. One could argue that, rather than exemplifying the ease of creating kinase inhibitors, the mode of action of rapamycin actually illustrates the challenge – inhibiting mTOR specifically does not involve a compound that hits the active site. Indeed, it is now clear that certain functions of mTOR are immune to inhibition by rapamycin [6]. Indeed, targeting features other than binding of ATP can work to inhibit kinase function, as indicated by compounds such as PD098059, which inhibits the ERK pathway at the level of MEK (MAPK/ERK kinase), but appears to act by preventing activation of MEK rather than by blocking its catalytic function [7]. This may help explain its good specificity, although even these compounds are not specific to one pathway, as they also interfere with activation of MEK5 in the ERK5 pathway [8]. Thus the paper by Sapkota et al. [9] potentially represents a key step forward in two major respects.

First, they have identified a compound that inhibits the activity of a discrete subset of AGC family kinases, which will be invaluable in dissecting their roles in cellular regulation. BI-D1870 inhibits RSK activity more than 500 times more potently (in terms of the IC₅₀ value) than any of the other AGC family kinases tested. It is especially important that BI-D1870 has essentially no effect on other kinases that are activated by the ERKs, such as MSK1 (another AGC family kinase, and one that is very closely related to the RSKs) and MNK1/2. It also fails significantly to inhibit kinases downstream of other MAPKs, such as MK2 and MK3 (also known as MAPKAPK2 and 3, or MAPK-activated protein kinases 2 and 3), which are activated by p38 MAPKs α/β , and PRAK (p38-regulated/activated protein kinase; also known as MAPKAPK5), which is activated by ERK5.

The results described above were derived from *in vitro* assays. It is clearly crucial to validate inhibitor action in living cells, because: (i) cells contain many more than the 54 kinases tested here (which amount to approx. 10% of the known protein kinases); (ii) interactions between signalling pathways often occur, and may complicate the effects of inhibitors; and (iii) since inhibitors of this type are competitive with respect to ATP, higher concentrations must be employed in cells than those used *in vitro* (since cellular ATP concentrations may be up to 50 times higher than the standard concentration of 100 μ M used *in vitro*). There are also, of course,

issues concerning the ability of inhibitors to enter cells, and their stability/metabolism.

The compound described by Sapkota et al. [9] passes these tests with flying colours. Importantly, BI-D1870 blocks the ERK-dependent phosphorylation of known or probable RSK targets, such as GSK3 (glycogen synthase kinase 3) and LKB1. The compound is thus cell-permeant and, as inhibition persisted for at least 4 h, BI-D1870 seems to be reasonably stable. For example, BI-D1870 does not appear to affect the activation of the upstream kinases, i.e. ERKs, in HEK-293 cells, indicating that it does not interfere with upstream ERK signalling, a key point for any inhibitor. The fact that BI-D1870 did cause increased ERK activity in another cell line (Rat-2 cells) may indicate some kind of feedback inhibition from RSKs to upstream components of ERK signalling that operates only in some cell types.

The availability of a specific RSK inhibitor clearly has immediate practical utility. Indeed, Sapkota et al. [9] exploit this compound to test which kinases mediate the phosphorylation of GSK3 at the regulatory N-terminal sites. In HEK-293 cells, BI-D1870 inhibited the PMA-induced phosphorylation of GSK3, which was indeed thought to be mediated by RSKs, although other AGC kinases (e.g. PKB and S6 kinases) can also phosphorylate these proteins at the same site. On the other hand, BI-D1870 does not block the phosphorylation of CREB (cAMP-response-element-binding protein) at a site (Ser¹³³) that can be phosphorylated *in vitro* by both MSK1/2 and RSKs. These data are consistent with earlier findings, and define BI-D1870 as a very useful tool for discriminating between events due to different AGC kinases in living cells.

Secondly, this report represents an important conceptual advance, as it shows that it is possible to generate compounds which exhibit good selectivity for different members of the same family of protein kinases. This should, and no doubt will, encourage the search for specific inhibitors of other AGC family kinases, and indeed for agents suitable for distinguishing the functions of members of other protein kinase subfamilies.

Cohen et al. [10] previously reported a selective RSK inhibitor ('fmk'), but this targets the C-terminal domain, which is not in the AGC family. Its specificity and efficacy have not been fully documented. BI-D1870 offers the advantage that it inhibits the N-terminal catalytic domain of RSKs, which catalyses the phosphorylation of other substrates, rather than the C-terminal domain.

Interestingly, BI-D1870 shows differential potency even within the RSK group, having noticeably greater activity against RSK1 and 2 as compared with RSK3 and 4. Thus it may even prove possible to generate compounds that distinguish between closely related AGC subfamily members. A co-crystal structure of BI-D1870 with an RSK protein (especially RSK1 or RSK2) would probably be very informative in terms of understanding its specificity and in helping to design potential inhibitors of other AGC family kinases. Such inhibitors will be very valuable in the research laboratory, and perhaps, ultimately, in the clinic too.

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