Pseudokinase drug intervention: a potentially poisoned chalice

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Abstract

Pseudokinases, the catalytically impaired component of the kinome, have recently been found to share more properties with active kinases than previously thought. In many pseudokinases, ATP binding and even some activity is preserved, highlighting these proteins as potential drug targets. In both active kinases and pseudokinases, binding of ATP or drugs in the nucleotide-binding pocket can stabilize specific conformations required for activity and protein–protein interactions. We discuss the implications of locking particular conformations in a selection of (pseudokinases and the dual potential impact on the druggability of these proteins.

Introduction

The eukaryotic cell is a staggeringly complex environment, with intricate regulatory mechanisms underpinning every aspect of its behaviour. At the heart of many of these regulatory mechanisms is the requirement for signals to be generated, amplified and relayed. One way in which cells achieve a regulated intracellular communication network is through protein phosphorylation. Responsible for this phosphotransfer is a group of 518 structurally related catalytic proteins called kinases [1]. Considering the importance of protein kinases in widespread cellular processes such as proliferation, migration, metabolism and cell death, it is of no surprise that breakthrough or hyperactivation of kinase signalling pathways can lead to diseases such as cancer [2].

The collection of all kinases in a cell, the kinome, is in flux through short-term mutational and long-term evolutionary processes. Mutagenic factors provide a daily strain on our genomes to maintain their integrity and to not let oncogenic mutations of proteins such as kinases, which can drive cancer formation, take hold. These mutations can take many forms. Gene amplification can increase the production of a particular protein as observed for HER2 (human epidermal growth factor receptor 2) in some forms of breast cancer [3,4]. Translocation can fuse two genes to create an entirely alien gain-of-function allele [4]. An example is the Philadelphia chromosome, which fuses the BCR (breakpoint cluster region) protein with the kinase Abl and presents a driving force in the development of CML (chronic myelogenous leukaemia) [5,6]. Additionally, single amino acid substitutions in particular kinases can cause hyperactivation [4,7]. An example of this is the activating V600E mutation in B-Raf, a common driving force in the formation of melanoma [8].

Oncogenic kinases present significant drug targets for directed chemotherapy [7]. Small-molecule inhibitors that prevent oncogenic kinases from binding ATP (and subsequently performing phosphotransfer) are in use in the clinic to good effect, combining potency with much higher selectivity than achieved with cytotoxic chemotherapy and therefore a lower side effect burden on the patient [7]. The evolutionary diversity of the kinome shows that, in the very long term, duplications and mutations that confer a functional advantage can cause divergence from a protokine into a family of similar, but functionally distinct, protein kinases [1]. An intriguing evolutionary path that close to 10% of the human kinome has followed is the loss of catalytic activity. These proteins, collectively referred to as pseudokinases, have lost one or several of the important motifs required for performing efficient phosphotransfer [1]. Pseudokinases are spread throughout the various evolutionary branches of the kinome [9]. Understudied for a long time, they have regained attention following the observation that many pseudokinases still retain ATP binding and, in some cases, even residual kinase activity [10–17]. The present review discusses the differences between pseudokinases and kinases on the basis of structural and biochemical evidence. Considering the efficacy of ATP-competitive kinase inhibitors and the ability of several pseudokinases to bind ATP, it might appear to be a sound strategy to develop small-molecule inhibitors for pseudokinases. We argue that there is certainly potential for pseudokinase interference with small-molecule inhibitors. However, we highlight a particular structural risk involved with this approach that needs to be considered carefully, namely through the counterintuitive activating effects that drug binding might have on pseudokinase function.

Key words: cancer, epidermal growth factor receptor (EGFR), kinase, kinase inhibitor, protein kinase A (PKA), pseudokinase.

Abbreviations used: EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; HER, human epidermal growth factor receptor; JAK, Janus kinase; JH, JAK homology; KSR1, kinase suppressor of Ras 1; PKA, protein kinase A; SIK1, STE20-related adaptor protein 1; VRK3, vaccinia-related kinase 3.

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Architecture of (pseudo)kinases

Kinases have a highly conserved structure with a few key motifs required for binding ATP, transferring ATP $\gamma$-phosphate to substrate hydroxy groups and general structural stability [18–20]. The kinase domain consists of two lobes, with a nucleotide-binding pocket in between. The various highly conserved residues are highlighted on the canonical structure of PKA (protein kinase A) in Figure 1 [20]. This PKA structure and the numbering for the key conserved residues is used throughout the present review as a point of reference. The ATP-binding pocket is bordered at the bottom by a collection of $\alpha$-helices in the C-terminal lobe. To the top there is a $\beta$-sheet containing a glycine-rich loop (following the GXGXXG consensus) and a highly conserved lysine residue (Lys$^{72}$ in PKA numbering), which co-ordinate ATP binding. To the side of the nucleotide-binding pocket is the $\alpha$-C helix, which is typically involved in a conformational change when a kinase takes on an active state. For an inactive kinase conformation, the $\alpha$-C helix is tilted out of the pocket, whereas it is tucked in when a kinase adopts an active conformation. The $\alpha$-C helix contains a conserved glutamate residue (Glu$^{91}$ in PKA) that, with the $\alpha$-C helix in the active conformation, is able to form a salt bridge with Lys$^{72}$, adding to the structural stability of the pocket. In an inactive conformation, the position of Glu$^{91}$ may be too far from Lys$^{72}$ to support this conserved salt bridge. In addition, there are two aspartate-containing regions of high importance for activity, the HRD (His-Arg-Asp) and DFG (Asp-Phe-Gly) motifs. The HRD [YRD (Tyr-Arg-Asp) in PKA] motif contains an aspartate residue (Asp$^{166}$) that acts as the catalytic base for the transfer of an ATP $\gamma$-phosphate to a substrate. The aspartate residue (Asp$^{184}$) in the DFG motif is responsible for pocket co-ordination of the divalent metal ions (e.g., $\text{Mg}^{2+}$ or $\text{Mn}^{2+}$) associated with $\text{Me}^{2+}$-ATP binding and is required for catalysis to occur.

The pseudokinase subset of the kinome was originally identified on the basis of the loss of the equivalent of the Lys$^{72}$, Asp$^{166}$ or Asp$^{184}$ residues [1,9]. These sequence analyses did not include the glycine-rich loop or Glu$^{91}$ equivalent. In the present review, we provide a categorization of the 48 members of the pseudokinome, incorporating all five conserved motifs in a classification based on which residues are retained (Figure 2). With 31 out of 48 pseudokinases still retaining two

Figure 1 | The five canonical kinase motifs highlighted on the structure of PKA (PDB code 1ATP) [20]
(a) Binding of ATP in the kinase domain pocket is co-ordinated by the G-loop, Lys$^{72}$ and the HRD and DFG motifs. Additional stability is provided by a salt bridge between Lys$^{72}$ and Glu$^{91}$ in the $\alpha$-C helix. (b) Schematic representation of (a).
Figure 2 | Classification of the 48 members of the pseudokinome according to their retention of canonical kinase motifs

Colour coding corresponds to structural features in Figure 1. The G-loop was scored as positive when all three glycine residues in the GXGXXG motif were conserved. An arginine substitution of the Lys\textsuperscript{72} equivalent residue was not considered to be a positive score.

| ANPa | KSR2 | ANPB | cask | HER3 | ILK | KSR1 | PSKH2 | Sgk495 | VACAMKL | CCK4 | EphA10 | GCN2 | HSER | IRAK2 | JAK1 | JAK2 | JAK3 | SCYL2 | Sgk071 | Sgk196 | Slob | Trb1 | Trb2 |
|------|------|------|------|------|-----|------|-------|--------|---------|------|--------|------|------|-------|------|------|------|-------|-------|--------|------|------|------|------|
| Trb3 | VRK3 | ULK4 | CYGD | CYGF | EphB6 | MLKL | RSKL1 | RSKL2 | SgK223 | SgK269 | SgK307 | SgK396 | STRAD\textalpha | STRAD\textbeta | NRPB1 | NRPB2 | SCYL1 | SCYL3 | SgK424 | TBCK | TRRAP |

or more of the essential motifs in various combinations, it is not unlikely that there could be a number of pseudokinases still able to bind nucleotides and even perform limited catalysis, blurring the line between pseudokinase and active kinase.

Kinases domains in pseudokinases

Despite the impaired catalytic potential of pseudokinases compared with the majority of the active kinome, pseudokinases still play important roles in the cell. Particularly from crystallographic evidence, a pattern of behaviour has emerged where the structural conformation of a pseudokinase may be integral to its function.

In the case of the pseudokinase STRAD\textalpha (STE20-related adaptor protein \textalpha), ATP binding has been shown to stabilize a particular conformation that allows it to sustain a complex with LKB1 and MO25 [17,21]. STRAD\textalpha has retained the position 91 equivalent and has an arginine residue instead of Lys\textsuperscript{72}, which are the only two conserved residues of the five kinase motifs (Figure 3a). It does still bind ATP, which locks it into a conformation similar to that of an active regular kinase, with a tucked in \(\alpha\)-C helix [17,21]. This stabilizes an interaction surface, allowing STRAD\textalpha to maintain the complex with LKB1 and MO25.

Nucleotide binding seems to have a similar role in the pseudokinase ILK (integrin-linked kinase), which binds ATP [14]. ILK lacks the glycine-rich loop and the aspartate residues in the DFG and HRD motifs, but retains Lys\textsuperscript{72} [14]. A number of non-canonical contacts from other residues (Asn\textsuperscript{200}, Asn\textsuperscript{202}, Ser\textsuperscript{204}, Asp\textsuperscript{339} and Lys\textsuperscript{341}) have taken over the responsibility of ATP co-ordination in the absence of the other classical motifs [14] (Figure 3b). ILK forms protein–protein interactions with \(\alpha\)-parvin and plays a part in focal adhesion assembly [14,22]. Mutation of the Lys\textsuperscript{72} equivalent residue in ILK inhibits its association with \(\alpha\)-parvin and gives a severe kidney agenesis phenotype in mice [22]. Evidently the conformation of this pseudokinase is critical to its function.

The family of JAKs (Janus kinases) have a particularly intriguing architecture with two kinase domains, the active JH (JAK homology) 1 and the pseudokinase JH2 domain. Recent work has shown that the JAK2 JH2 domain has interesting structural features [16]. The ‘apo’ form of the JAK2 JH2 domain (where the nucleotide pocket is unoccupied) has a very similar conformation to that of the ATP-bound structure, because additional interactions between the C- and N-lobes of the pseudokinase domain (Arg\textsuperscript{715}–Thr\textsuperscript{555} and between the Lys\textsuperscript{72} and Asp\textsuperscript{184} (DPG (Asp-Pro-Gly) motif in JAK2 JH2) equivalent residues stabilize the protein in the presence or absence of ATP [16] (Figure 3c).

A non-canonical interaction between the Lys\textsuperscript{72} and Asp\textsuperscript{184} equivalent residues can also be found in HER3, a pseudokinase in the EGFR (epidermal growth factor receptor) family of receptor tyrosine kinases [10,11] (Figure 3d). HER3 plays an important role in oncogenic signalling through heterodimerization with HER2 [23]. Activation in the EGFR family occurs through an asymmetric dimer in which the first partner (‘activator’) allows the second partner (‘receiver’) to become active [24]. In this heterodimer, the ‘receiver’ kinase is the only one that needs catalytic activity [24]. HER3 is able to fulfil the ‘activator’ role, sustaining the activity of its heterodimer partner [10]. It is not currently known whether HER3 ATP binding contributes to sustaining this heterodimer.

Several pseudokinases that have lost important motifs compensate using non-canonical contacts, perhaps none more so than VRK3 (vaccinia-related kinase 3), which does not bind ATP and lacks the glycine-rich loop and both aspartate residues [15]. Instead of binding ATP, the VRK3 nucleotide-binding pocket has multiple hydrophobic residues in the area where the ATP adenosine rings would normally sit [15] (Figure 3e). This hydrophobic spine contributes to increased protein rigidity to such an extent that VRK3 is more thermostable in the absence of ATP than VRK1 and VRK2 are in the presence of it [15].

Although alignments of primary sequences are a good initial tool to classify the various levels of conservation of the five canonical kinase motifs, many pseudokinases have non-canonical substitutions that (partly) take over the role
Figure 3 | Structural features of particular interest in selected pseudokinases

(a) STRADα shows nucleotide binding in a similar conformation to that of an active kinase (PDB code 2WTK [17]). (b) In ILK, several non-canonical motifs take over the co-ordination of ATP in the nucleotide-binding pocket (PDB code 3KMW [14]). (c) The Jak2 JH2 pseudokinase domain in its apo form (without nucleotide bound) shows structural stability in the absence of nucleotide through two additional salt bridges (PDB code 4FVP [16]). (d) The pseudokinase HER3 has an additional polar contact between the DFG motif and Lys72 equivalent (PDB code 3KEX [10]). (e) VRK3 has lost the ability to bind nucleotide, but non-canonical hydrophobic residues fill the space the adenosine rings of ATP would have otherwise occupied. This hydrophobic spine gives VRK3 high stability in the absence of nucleotide binding (PDB code 2JII [15]). (f) Structural overlay of EGFR with type I inhibitor erlotinib (red) and type II inhibitor lapatinib (green) bound. The G-rich loop, DFG motif and \( \alpha \)-C helix are highlighted. Type II inhibitors such as lapatinib occupy an additional pocket created when the \( \alpha \)-C helix is in the ‘out’ position (PDB codes 1M17 [25] and 1XKK [34]). Nucleotide is coloured ochre in relevant panels.

of the canonical residues. These substitutions would be difficult to identify \textit{a priori} on the basis of primary sequence considerations. The above examples highlight the importance of structural information in evaluating the involvement of non-canonical motifs.

Drug binding in kinases

Oncogenic kinases have become significant targets for therapeutic interventions with small-molecule inhibitors [7]. The inhibitors that compete with ATP binding to the kinase can be roughly classified into two types. Type I inhibitors bind the active kinase conformation, with the \( \alpha \)-C helix in an inward position [7]. An example is erlotinib, which binds EGFR in the active conformation [25]. There are also inhibitors that bind kinases in the inactive conformation where the \( \alpha \)-C helix is pushed outwards, opening up an additional hydrophobic pocket termed the allosteric site [7]. Because these inhibitors, termed type II, inhabit an additional pocket combined with the ATP-binding site, they generally show higher specificity [26]. Indeed, lapatinib, a type II inhibitor of EGFR, shows low non-specific binding across the kinome [26]. Comparing the crystal structures of EGFR with erlotinib and lapatinib shows the different conformations of EGFR that these drugs bind (Figure 3f). It was reported for EGFR, for which a large selection of specific inhibitors exist, that type I EGFR inhibitors erlotinib and gefitinib, but not the type II inhibitor lapatinib, are able to induce homodimerization of EGFR in the absence of ligand [27]. This drug-induced dimerization depended on EGFR being locked in an active conformation by type I inhibitors, as the dimerization was abolished by mutating the ‘activator’ and ‘receiver’ interfaces of an active EGFR homodimer [27].

As noted above for EGFR, stabilizing a particular protein conformation can have repercussions on the effect a drug has on a protein, separate from its ability to block ATP binding and phosphotransfer. Our previous work on PKCε (protein kinase Cε) has shown that occupation of the nucleotide-binding pocket offers the structural rigidity required for stable priming phosphorylation [28]. This priming occurred regardless of whether the nucleotide-binding pocket was occupied by ATP or inhibitor, separating out a structural role for pocket occupation separate from the catalytic role that ATP binding also plays [28].

Analogously, it was found that inhibitors directed at the oncogenic V600E mutation of B-Raf are dually able to inhibit the mutant, but activate the wild-type kinase [29,30]. Inhibitors are able to activate the downstream pathway by stabilizing B-Raf’s active conformation, allowing it to structurally sustain a heterodimer with c-Raf causing...
transactivation and consequent MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/ERK activation [29,30]. This inhibitor-induced activation of the pathway can be attenuated by inhibitor-driven heterodimerization of B-Raf and the pseudokinase KSR1 (kinase suppressor of Ras 1), which competes with c-Raf [31].

Perspective

Recently, several pseudokinases, including HER3, KSR1, KSR2, JAK2 JH2 and CASK (Ca\(^{2+}\)/calmodulin-dependent serine protein kinase) have been shown to retain a measure of phosphotransferase activity under various conditions [11–13,32,33]. This flurry of activity has raised the question of whether these pseudokinases represent viable drug targets. We do not discount the potential importance of residual activity for information relay, but urge caution; the function of many pseudokinases appears to be very much dependent on conformation, often promoting specific protein–protein interactions. Evidence from small-molecule inhibitors directed at various active kinases indicates that these inhibitors are able to lock kinases in particular conformations, with sometimes unexpected consequences for their interactions with other proteins.

When it comes to designing small-molecule inhibitors for pseudokinases, these conformational considerations become highly relevant. In pseudokinases acting as scaffolds to facilitate protein–protein interactions, we predict that kinase inhibitors might disrupt or stabilize these functions with due consequences. For example, there exists a dangerous potential of designing inhibitors that show excellent in vitro binding to HER3, but in fact stabilize, rather than inhibit, signalling heterodimer formation with HER2. Conversely, we expect that pseudokinases might present viable drug targets for inhibitors that lock them in a conformation where they are not able to exert their (oncogenic) protein–protein interactions. This should be carefully considered on a case-by-case basis, where the read-out of assays testing potential pseudokinase inhibitors is based not only on binding to the pseudokinase, but also on how inhibitor binding affects functional protein–protein interactions associated with the pseudokinase being targeted.

Funding

We acknowledge funding from Cancer Research UK.

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The Authors Journal compilation © 2013 Biocological Society

Received 3 May 2013
doi:10.1042/BST20130078