Review

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Future Medicinal Chemistry

Pseudokinases: update on their functions and evaluation as new drug targets

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The pseudokinase complement of the human kinase superfamily consists of approximately 60 signaling proteins, which lacks one or more of the amino acids typically required to correctly align ATP and metal ions, and phosphorylate protein substrates. Recent studies in the pseudokinase field have begun to expose the biological relevance of pseudokinases, which are now thought to perform a diverse range of physiological roles and are connected to a multitude of human diseases, including cancer. In this review, we discuss how and why members of the 'pseudokinome' represent important new targets for drug discovery, and describe how knowledge of protein structure and function provides informative clues to help guide the rational chemical design or repurposing of inhibitors to target pseudokinases.

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Background

Protein pseudokinases are ubiquitous among the kingdoms of life, accounting for approximately 10% of a typical vertebrate kinome [1–4], increasing to >50% of the kinome in the unicellular protist *Giardia* [5]. Their catalytically active protein kinase counterparts are well-known regulators of many eukaryotic cellular processes, which tightly control intracellular signaling network by facilitating the catalytic transfer of phosphate from ATP to substrate proteins. With approximately 50% of all proteins thought to be phosphorylated in cells [6], it is not surprising that dysregulation of protein kinases is frequently a driving factor (or consequence) in a variety of human medical disorders including cancers [7,8], Type 2 diabetes [9], cardiovascular [10], neurodegenerative [11] and developmental diseases [12]. This has led to a considerable effort to develop small-molecule therapeutic agents to control aberrant protein phosphorylation, with notable early successes including Imatinib to inhibit BCR–ABL1 chronic myelogenous leukemia [13], vemurafanib to target V600E *BRAF*-mutant melanoma [14] and Lapatinib to treat *ERBB2/HER2*-amplified tumors [15]. Chemical structures of some of the kinase inhibitors discussed in this review are depicted in Figure 1.

In stark contrast, pseudokinases are much less-well characterized. They are traditionally defined as pseudoenzymes [16] lacking one or more of the canonical amino acids or motifs that are typically required to efficiently coordinate ATP and transfer phosphate in catalytically active kinase counterparts [3,17,18]. Although a loss of catalytic activity appears to be a feature of many pseudokinases, the ability to bind to and/or hydrolyze ATP (sometimes very weakly) can be detected in a number of cases [19], including CASK [20,21], TRIB2 [22], JAK2 [23], HER3 [24,25], with-no-lysine kinase (WNK) [26], STRADα [27], MLKL [28] and kinase suppressor of ras 1 and 2 (KSR1/2) [29]. A retained affinity for ATP

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indicates that nucleotide-dependent switching mechanisms may have been preserved in the fold in order to regulate a proportion of pseudokinase-dependent signaling, either driven through catalytic activity or by ATP-driven conformational transitions. Together, these requirements for signaling highlight pseudokinases as an important emerging class of drug targets [30]. Indeed, ATP binding is now understood to be essential for the biological activities of noncatalytic pseudokinases such as STRADα [27] and HER3 [31]. In contrast, several pseudokinases that possess a highly degraded ATP-binding site (such as vaccinia-related kinase 3 [VRK3]) appear to have lost the ability to bind ATP completely [4,19]. It is therefore now becoming clear that the rate-limiting cellular outputs of protein kinases, and in particular pseudokinases, are not restricted to intrinsic catalytic turnover [32]. This suggests that the development of ATP competitive, covalent or allosteric small molecules to modulate phosphorylation-independent cellular signaling by kinases and pseudokinases represents a new pharmacological challenge.

Disease-associated pseudokinases

Approximately two-thirds of pseudokinases have been implicated in a very diverse range of human diseases [30]. In the following section, we briefly discuss important disease-associated pseudokinases, whose biological activities might be susceptible to therapeutic intervention with small-molecule ligands.

Janus kinase-, hybrid kinase- and pseudokinase-signaling polypeptides

The JAK family of nonreceptor tyrosine kinases consist of JAK1, JAK2, JAK3 and TYK2 [33], which share seven regions of sequence termed Janus homology (JH) domains. JH1 consists of a conventional tyrosine kinase domain that becomes activated upon stimulation of type I/II cytokine receptors [33], and is involved in a variety of biological processes including hematopoiesis and the regulation of the immune system [34]. The JH1 domain is usually negatively regulated by JH2, a pseudokinase-containing domain that lacks the catalytic Asp residue in the HRD motif, which is typically required for catalytic transfer of the γ-phosphoryl group to the alcoholic substrate, but nonetheless still appears to regulate JAK2 signaling through ATP binding and/or weak catalytic activity [35]. In fact, JAK2 amino acid residues Ser523 and Tyr570 are potential substrate sites for the low activity JH2 pseudokinase domain, although targeted inactivation of the JH1 domain completely abolishes Tyr570 phosphorylation *in vitro*, suggesting a rate-limiting requirement for JH1 in this process [35–37]. Despite binding to ATP, the JH2 pseudokinase domains of JAK1 and TYK2 appear to be catalytically inactive, and neither carry conserved JH1 Ser523 and Tyr570 residues, potentially suggestive of a lack of phosphorylation-driven autoregulation [38,39]. However, ATP binding (which stabilizes the JH2 domain of TYK2 without significant structural reorganization) is still thought to serve a critical allosteric function in maintaining the tyrosine kinase domain in an auto-inhibited state [38]. The critical importance of JH2-dependent modulation of JAK kinase function is further emphasized by the marked enhancement in kinase activity upon deletion of the pseudokinase domains of JAK2 and 3 [36,40].

To date, JAKs have perhaps been the most intensively studied of the pseudokinases and this can be directly attributed to the occurrence of multiple disease-driving somatic mutations in JH2 domains [34]. Of particular note is a prominent gain of function oncogenic variant of JAK2 (V617F) [41–44] that has been strongly implicated in multiple myeloproliferative neoplasms [39] and is used diagnostically to guide therapeutic intervention strategies owing to its occurrence in approximately 95% of polycythemia vera and approximately 60% of essential thrombocythemia and primary myelofibrosis cases [43,45]. The consequence of this amino acid substitution is hyperactivation of JAK2 and constitutive downstream signaling due to a presumed destabilization of the auto-inhibitory JH1–JH2 interaction [46,47]. Interestingly, ATP binding to the pseudokinase domain of JAK2 was recently shown to be essential for the hyperactivation phenotype of pathogenic JH2 mutants such as V617F [48]. This clear association with human malignancies, combined with the documented ATP-mediated regulation of the pseudokinase domain, suggests that JH2 domains might be suitable small-molecule drug targets for the treatment of cancer and auto-immune diseases. In this regard, ruxolitinib, an orally available tyrosine kinase inhibitor that targets the JAK1 and JAK2 JH1 canonical kinase domains, was the first US FDA approved inhibitor for the treatment of myelofibrosis [49], and has also been approved for patients with polycythemia vera to control thrombotic events that fail to respond to hydroxurea [50]. Suppression of JAK signaling could also be achieved indirectly with small molecules that promote JH2 auto-inhibition of JH1 kinase output, which has already been suggested mechanistically for TYK2.

HER3/ErbB3 pseudokinases

The clinically significant pseudokinase HER3 belongs to the epidermal EGFR family of receptor tyrosine kinases. HER3 is a pseudokinase due to a lack of the canonical catalytic Asp residue [17]. In spite of this, HER3 is able to bind tightly to ATP (Kd ~1 μM),

and although it also exhibits very low tyrosine kinase activity [24], it appears unlikely that this vestigial phosphotransferase capacity is required for HER3 function in cells where this has been tested [25,51]. Instead, ligand-induced heterodimerization of HER3 with EGFR (HER1) or HER2 stimulates allosteric transactivation of these kinases and modulates a myriad of cellular responses, including downstream activation of the PI3K/AKT signaling pathway [52,53]. HER3 is also required for malignant progression in HER2-amplified breast cancers [54], which originally led to the idea that it might be targeted by conventional kinase inhibitors. Indeed, HER3 overexpression and mutation is associated with a multitude of human cancers [55,56] and it also represents a potential diagnostic marker in breast cancer, where it is amplified in around 60% of cases [57]. One of the major consequences of aberrant HER3 activation is the hyperactivation of PI3K/AKT signaling, which classically drives prosurvival pathways, and has been linked to the development of multidrug resistance in some cancers [58]. This is supported by the finding that antibody-mediated dual targeting of HER3 and EGFR might be a useful method to overcome acquired resistance elicited towards EGFR-targeted therapeutics [59]. Interestingly, several cancer-associated somatic mutations that confer a gain-of-function phenotype for HER3 have also been reported [55,60] and structure-based studies indicate that enhancement of HER3 allosteric activation function may underscore the molecular mechanisms of these 'activating' mutations [61]. In this regard, inhibitor compounds that specifically target the active signaling state of HER2 in the HER2–HER3 oncogenic complex [51] might have therapeutic potential.

STRADα

The catalytically inactive pseudokinase STRADα (and the closely-related, but enigmatic, pseudokinase STRADβ) contains a severely degraded kinase domain, which is devoid of canonical β3 Lys, DFG and HRD motif residues. STRADα functions as an allosteric regulator of the tumor-suppressor kinase LKB1, and its biological function is dependent on the adoption of a closed 'active' conformation, which is generated upon cooperative binding to ATP and the auxillary scaffolding protein MO25α [27,62]. Formation of the LKB1–STRADα–MO25α heterotrimeric complex results in LKB1-dependent phosphorylation of AMPK and the modulation of many cellular proliferation signaling pathways [27,63]. Several human cancers, and the rare inherited disease Peurtz-Jeghers syndrome, are the consequence of loss of function mutations in LKB1 that perturb binding and activation by STRADα and MO25α [62,64]. Furthermore, the effectiveness of the broad antitumor drug metformin appears to be contingent on an ability to stimulate LKB1-dependent activation of AMPK [65], which positions STRADα, as an important potential therapeutic target for the manipulation of downstream effectors of LKB1.

Kinase suppressor of Ras 1 & 2

KSR1 and 2 are important scaffolding pseudokinase proteins that coordinate the formation of the (oncogenic) Raf–MEK–ERK signaling complex [66,67] and are also important regulators of immune function and metabolism [68]. KSR 1 and 2 lack the canonical β3 Lys but are proposed to have dual scaffolding and catalytic roles, with established phosphorylated protein substrates including MEK1 [29,69] and C-RAF-1 [70,71] within this complex. However, the weak kinase activity of mammalian KSRs is likely dispensable for MEK phosphorylation and MAPK signaling in kinase-impaired mutants [72], which challenges the physiological importance of KSR catalytic activity. KSR1 is a prospective therapeutic target of Ras-driven tumors due to its oncogenic potential in human cells [67,73,74]. Disease associated mutations in KSR2 that disrupt KSR2 signaling through the Raf–MEK–ERK pathway have also been linked to obesity, insulin resistance and impaired cellular fuel oxidation [75].

MLKL

Despite lacking a Glycine-rich loop, and possessing noncanonical HGK and GFE residues in place of the typical DFG and HRD motifs, mixed lineage kinase domain like (MLKL) binds robustly to ATP in an obligate cation-independent manner. However, it appears unable to catalyze ATP hydrolysis [28,76], instead driving necroptosis (a form of regulated cell death) in a catalytically independent manner. MLKL function is indispensable for TNF-α-induced necroptosis, which proceeds via the obligate phosphorylation of two activation loop residues (Thr357 and Ser358) in MLKL by the canonical upstream kinase RIP3 [76,77]. Phosphorylation at these residues stabilizes the active conformation of MLKL, which facilitates release of the N-terminal domain four-helix bundle that drives MLKL oligomerization, membrane translocation and subsequent activation of necroptosis [78]. Under normal physiological conditions, necroptosis is triggered in response to physiological or pathophysiological stimuli as part of an innate defense against pathogens that suppress apoptosis [79]. However, deregulation of necroptosis is a risk factor in a multitude of autoimmune and inflammatory diseases [80], highlighting MLKL as a potential novel drug target. So far, only the biological

functions of MLKL in relation to necroptosis have been dissected, but a mutation (E351K) in the non-conventional GFE motif (which has evolved in place of the DFG motif) has also been described in human cancers, and could possibly be linked to an altered affinity or usage for nucleotides [28,81].

Tribbles pseudokinases

Three human Tribbles (TRIB) homologs (TRIB1, TRIB2 and TRIB3) are characterized by an N-terminal PEST region, a pseudokinase domain containing an atypical DFG metal-binding motif (E[S/N]LED) and a C-terminal COP1-binding region [22,82]. TRIB proteins are important regulators of many divergent cellular processes including lipoprotein metabolism, immune function and cellular differentiation and proliferation [83]. This is achieved via two major modes of action; the first involving the E3-ligase-dependent ubiquitination of their protein substrates, and the second by modulation of MAPK and AKT signaling modules [82,84–87]. The recently solved crystal structure of TRIB1 reveals how the SLE motif adopts a unique 'inactive'-like conformation that obstructs nucleotide binding in the TRIB1 ATP-binding site and potentially also represents a physical barrier to ATP-competitive inhibitors [88]. This adaption presumably helps TRIB1 to function as an efficient regulator of ubiquitin-mediated substrate degradation and limits or rules out ATP binding and hydrolysis. In contrast, TRIB2 and 3 demonstrate very weak ATP affinity and ATP hydrolysis *in vitro* [22]. Dysregulation of TRIB proteins has been implicated in a variety of human cancers, including acute myeloid leukemia (AML) [89,90], lung [91], hepatic cancers [92] and melanoma [93]. In the case of TRIB2, these malignancies are primarily thought to proceed via TRIB-mediated degradation of the C/EBPα transcription factor. Interestingly, TRIB2 requires an intact nucleotide-binding site in order to drive AML in mice [89], which presents a potential opportunity for pharmacological intervention with compounds that target the TRIB2 ATP-binding site. In this regard, it is noteworthy that our lab has recently identified small-molecule ligands from a library of kinase inhibitors that interact with TRIB2, although the precise binding modes have yet to be characterized [82].

As well as being linked to AML, TRIB1 has been identified in Down's syndrome-related human acute megakaryocytic leukemia [94]. In this case, the cancer-associated TRIB1 mutant, R107L, enhances the degradation of C/EBPα through an unknown mechanism. Although TRIB3 does not modulate the stability of C/EBPα it has been implicated in the degradation of acetyl-CoA carboxylase [95]. In addition TRIB3 has a role in glucose-induced insulin resistance in diabetic rats [96].

WNK

The WNK family of Ser/Thr pseudokinases (comprising WNK1–4) [97] are defined because they lack the conserved β3 lysine, which was long assumed to be indispensable for nucleotide binding and stabilization of the active kinase conformation [26]. Despite this apparent handicap, WNK family kinases readily demonstrate phosphorylation-dependent regulation of a host of intracellular substrates. This innovation is made possible by the evolution a novel mechanism of catalysis; the terminal residue in the glycine-rich loop (often a Gly in kinases) is conserved as a Lys residue in WNKs and this residue provides the compensatory charge to support the binding of ATP [26]. It has been proposed that the site normally occupied by the β3 lysine functions as a chloride sensor by binding to halide ions and inhibiting WNK activation by autophosphorylation, thus facilitating a feedback mechanism for the regulation of Cl^- levels [98]. Consistently, in its active state, WNKs phosphorylate and activate SPAK and the oxidative stress response kinase (OSR1), which in turn modulate the downstream activities of Na+-driven, Cl^--importing NKCCs (Na+/K+/Cl^- cotransporters) in order to modulate blood pressure and ion homeostasis [97].

WNK family members are differentially expressed in tissues and the deregulated function of neuronal WNK isoforms has been implicated in various diseases, including hereditary neuropathy and glioma [97]. In addition, mutations in WNK1 and WNK4 have been linked to hereditary hypertension [99]. WNK1 and WNK3 also stimulate proliferative and invasive activity in glioma cells [100–102]. Conversely, WNK2 predominantly plays a role as a tumor suppressor and downregulates cellular proliferation by increasing and decreasing activity of Rac1 and RhoA respectively [103].

Protein kinase & pseudokinase small-molecule inhibitors

The majority of protein kinase inhibitors are reversible ATP-competitive molecules that can broadly be classified into two major groups: type 1 inhibitors, which bind to the ATP-binding sites of targets assuming a closed 'active' conformation (where the αC helix and DFG motif are in an inward orientation) and type II inhibitors that bind to- and lock kinases in a more open 'inactive' state by occupying a hydrophobic groove adjacent to the ATP-binding site that only becomes accessible in the 'DFG-out' conformation [104]. Achieving selectivity among the large kinase superfamily, which all share a high degree of

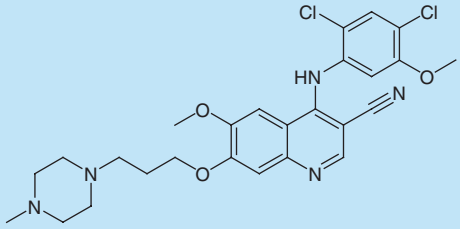
sequence conservation in the active site, has proven extremely challenging, and is only truly possible for ATP-competitive inhibitors through the exploitation of unique features found in regions surrounding the ATP-binding site. For example, the pyridinyl-imdazole SB 203580 was an early example of a partially selective type I inhibitor that targets p38 MAPK α and β isoforms, but not the very closely related γ or δ isoforms, and this specificity among MAP kinase isoforms was deduced to be absolutely dependent on the presence of a bulky 'gatekeeper' Met in the latter, which imparts steric hindrance to inhibitor binding [105,106]. In actual fact, the use of promiscuous inhibitors that simultaneously target multiple dysregulated signaling pathways might actually be advantageous from an oncological perspective [107], but clearly potentially increases the risk of adverse side-effects. Such compounds are also much less practical for the treatment of (noncancer) diseases, where logical mechanistic approaches have historically been preferable, reflecting the likelihood for long-term therapeutic maintenance and a high therapeutic index. In this regard, specific targeting of pseudokinases may indeed be useful, in large part because these proteins as a class characteristically possess unusual amino acid compositions and structural architecture in the ATP-binding site, be it functional or not [18,30]. Exploiting these evolutionary differences might facilitate the generation of highly specific ligands that are directed against pseudokinases, especially those whose regulatory functions are linked to adaptions around the nucleotide-binding site. Alternatively, chemical genetic 'bump-and-hole' approaches, such as those pioneered by Shokat and colleagues [108] might be useful approaches for swiftly evaluating pseudokinase signaling with modified kinase inhibitors after mutagenesis of the pseudokinase gatekeeper [22,109].

Type I & II ATP-competitive inhibitors: insights from kinase structures

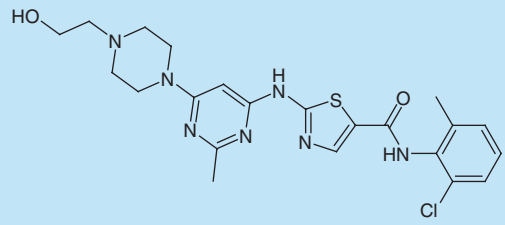
Currently, it is estimated that approximately 40% of the pseudokinome retains the ability to interact with adenine-based nucleotides, and most, if not all, of these proteins might theoretically be modulated by ATP-competitive inhibitors [19,110]. To test this assertion, two highly generic kinase inhibitors, DAP and/or VI16832, were shown to bind to nearly a third of a sample set of human pseudokinases [19], building upon the very limited small-molecule interactions described for pseudokinases, which with the exception of ErbB3/HER3 have often been omitted from biochemical or cellular screening approaches. Indeed, the pan-tyrosine kinase inhibitor Bosutinib (SKI-606) was one of the first examples of a clinically approved multikinase ATP-mimetic compound capable of modulating the function of the HER3, which it binds to with subnanomolar affinity [111,112]. A low nanomolar HER3 affinity for other kinase inhibitors, including familiar clinical compounds such as Dasatinib, and the covalent compound Neratinib, have also been described in the literature [112]. In addition, HER3 kinase activity has been successfully targeted with compounds such as Compound 2 (Figure 1) [51], or shown to bind to ATP-competitive compounds such as KIN001–051 [25]. Paradoxically, binding of bosutinib to the ATP-binding pocket of HER3 actually increases the capacity of the pseudokinase to operate as an allosteric activator of the 'receiver' kinase EGFR in a heterodimer, most likely by stabilizing the 'active' HER3 conformation [111]. Although in this specific example bosutinib fails to elicit a phenotypic 'inhibitory response', the data clearly support the hypothesis that pseudokinases can be targeted (and perhaps regulated) by ATP-competitive ligands. This finding also highlights the potential risk (or advantage, depending upon one's point of view) of small molecules behaving as allosteric activators depending on the binding mode and induced effects to the kinase fold [113]. This phenomenon has previously been observed for catalytically active kinases such as B-RAF, for which binding of certain inhibitors stabilizes its active state and promotes homo- and hetero-dimerization (with C-RAF), stimulating MAPK kinase signaling in the process [114,115]. In contrast, inhibitors such as Vemurafenib that stabilize an inactive conformation do not promote B-RAF/C-RAF heterodimerization [114]. As discussed previously, the adoption of a ligand-induced 'active' conformation in STRADα is a critical step in the allosteric activation of LKB1 tumor suppressor function [62]. Development of compounds that lock STRADα in an appropriate conformation might therefore be an effective therapeutic strategy to inhibit downstream proliferative pathways in specific tumors.

The TYK2 pseudokinase domain is also likely to be amenable to similar targeting by ATP-competitive therapeutics, as the TYK2 JH2 domain has recently been cocrystalized in complex with small-molecule inhibitors that stabilize the intradomain auto-inhibitory interaction with the tyrosine kinase domain, and block downstream signaling cascades [38,116]. Although currently scarce, pseudokinases whose biological functions are linked to ATP hydrolysis and direct substrate phosphorylation, such as WNK, are also obvious targets for traditional ATP-competitive inhibitors. As discussed previously, KSR2 exhibits dual scaffold and catalytic activity, and this latter functionality can be modulated by pan kinase inhibitors such as ASC24, which greatly reduces KSR2-dependent phosphorylation of MEK1 at non-BRAF phosphorylated Ser resi-

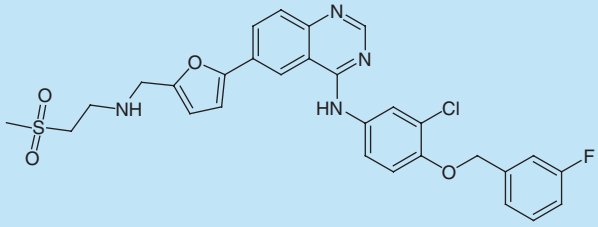
Bosutinib (HER3)



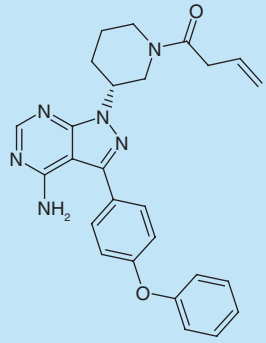
Dasatinib (HER3)



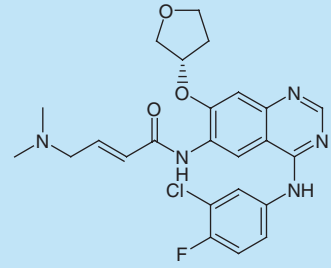
Lapatinib (HER2)



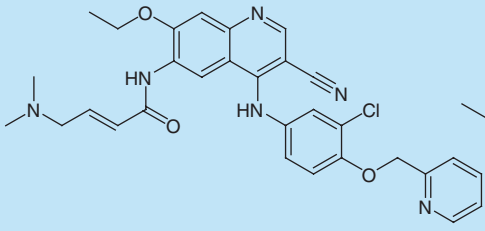
Ibrutinib (BTK covalent)



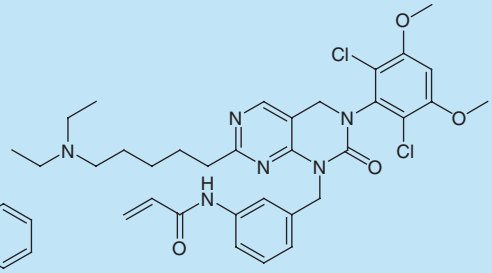
Afatinib (HER2 covalent)



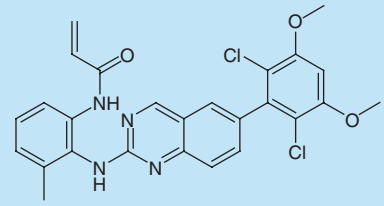
Neratinib (HER2 covalent)



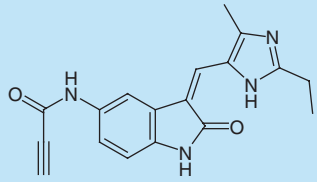
FIIN-1 (FGF1-3 covalent)



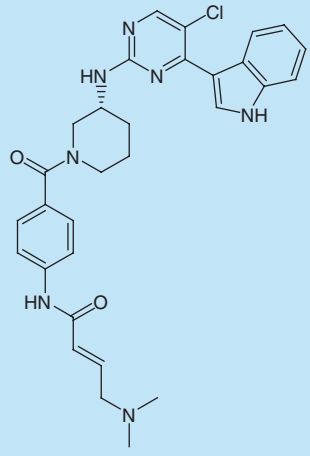
BLU9331 (FGFR4 covalent)



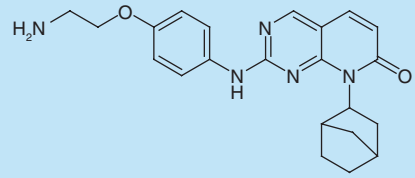
JH295 (NEK2 covalent)



THZ531 (CDK12/13 covalent)



VI6832 (broad pseudokinase)



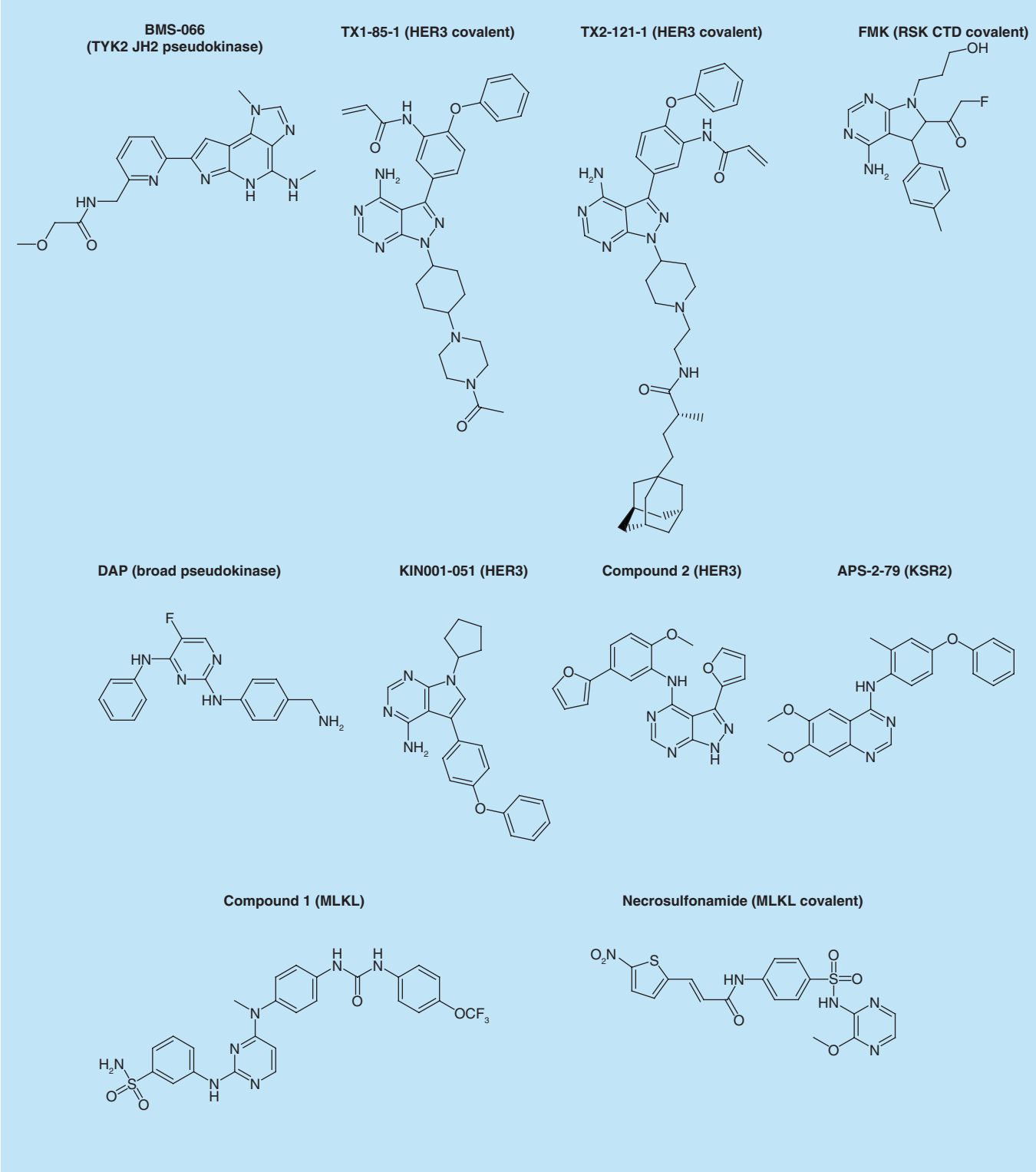


Figure 1. Chemical structure of selected ATP-competitive and covalent kinase and pseudokinase small-molecule inhibitors (cont. from facing page). Major kinase and pseudokinase targets are shown, where these have been reported in the literature. Several covalent compounds that target Cys residues in kinases or pseudokinase are noted.

dues [29]. However, considering the scant evidence for physiological catalytic outputs from pseudokinases such as KSR and JAK JH2 domains, the effectiveness of this approach may be limited.

Although mitigation of aberrant pseudokinase signaling through type-1 inhibition is a potentially attractive prospect, realizing target selectivity will nearly always be a barrier for effective cellular analysis using chemical biology approaches [109], and certainly more so than for many clinical evaluations, where efficacy and safety can often trump the need for detailed mechanistic understanding. The pursuit of type II kinase inhibitors might therefore be an alternative approach to achieve greater target specificity, especially considering the reduced amino acid conservation in the areas immediately surrounding the ATP pocket (which only becomes exposed in the DFG-out state) and also the greater conformational variability that is observed between kinases in their inactive states [117]. The development of compounds that trap or block these different induced conformations is a strategy that was exploited to a great effect in the development of imatinib, which stabilizes ABL in the DFG-out conformation [118,119] and is approved for the treatment of patients with BCR–ABL positive chronic myeloid leukemia [120,121]. Importantly, the extent to which pesudokinase activity is regulated by dynamic conformational transitions is not yet fully resolved. This notwithstanding, many of the hydrophobic amino acid residues that control the conformations of canonical kinases are also conserved in numerous pseudokinases, which predicts that an ability to adopt some form of regulatory active and/or inactive state upon nucleotide or ligand binding has at least been partially retained [122]. For example, crystal structures of RNase L, which contains a catalytically inactive pseudokinase domain [123–125], reveal a rigid closed structure that nonetheless bears similarity to the canonical kinase 'DFG-in' fold. Of potential regulatory relevance, this architecture is stabilized when bound to the nonhydrolyzable ATP analog AMP–PNP, but becomes severely disrupted in the absence of nucleotides, reverting to an inactive conformation. Back-to-back homodimerization and consequently activation of the RNase domain was shown to be entirely dependent on nucleotide binding and the adoption of the closed conformation. It is also predicted that non-enzymatic activity of MLKL is dependent on a conformational change to an active form brought about by RIPK3-mediated phosphorylation of the activation loop [76]. Catalytic-independent STRADα signaling also requires a conformational transition to an active 'closed' state that is induced by ATP binding and through complex formation with the MO25 scaffold protein [27]. Based on these and other examples, it is apparent that the noncatalytic activities of many pseudokinases may be closely linked to an active 'DFG-in' conformation, and inhibitors that select for or stabilize inactive conformations could potentiate phosphorylation independent signaling networks. As discussed previously, Bosutinib behaves as an allosteric activator of HER3, which would seem to indicate that HER3 signaling is at least partially dependent on conformational transition states [111]. It seems reasonable to hypothesize that compounds that sequester HER3 as an inactive monomer could begin to display the qualities of *bona fide* inhibitors. In support of this, antibodies that trap HER3 in an inactive conformation or directly inhibit its association with active kinase family members neutralize several facets of HER3 dependent signaling [126,127]. Recently, an ATP-competitive inhibitor (APS-2-79) of KSR2 was shown to stabilize the pseudokinase in an inactive state and modulate KSR2-dependent MAPK signaling by antagonizing RAF heterodimerization in a manner independent of KSR2 catalytic activity [128]. Interestingly, MEK phosphorylation by KSR2 is also stimulated by an RAF-mediated allosteric transition of KSR2 suggesting that both the catalytic and scaffold output of the protein could be pharmacologically targeted [29]. Molecular switching of MLKL to a pseudoactive state is a determining factor for its oligomerization and thus membrane translocation, which drives necroptosis. Necroptosis was inhibited by a small ATP-competitive molecule, termed compound 1, although it is not clear if this occurs via the stabilization of an inactive or active state [129], and the target specificity and mechanism of action have recently been re-evaluated [130]. To our knowledge, the only example of a molecule driving type II inhibition with a pseudokinase is that described for KSR2. However, this important study serves to expose the potential vulnerability of the pseudokinome in general to small-molecule intervention. It is also worth considering that stabilization of a 'native' DFG-out state is not necessarily the only route via which inhibition can be achieved, as exemplified by various classes of ATP-competitive inhibitors that induce an unusual inactive conformation in Aurora A that is not associated with 'normal' catalytic function [131,132].

Type III 'allosteric' inhibitors

Two additional modes of kinase inhibition have also been described; type III inhibitors that bind to sites outside of the ATP-binding pocket to drive conformational changes, and type IV (covalent) cysteine-driven interactions. These compounds can offer a distinct efficacious and selective advantage over noncovalent type I and II inhibitors, which target highly conserved regions of the

ATP-binding site, and under physiological conditions have limited potency in the presence of high intracellular concentrations of ATP [133]. In contrast, type III kinase inhibitors exert their (allosteric) effects either by indirectly targeting catalytic activity or by modulating noncatalytic function, and generally achieve excellent target precision through binding to less conserved, non-ATP-binding sites [134]. For example, GNF-5 binds to the myristate pocket of ABL and inhibits the kinase by allosterically inducing a restructuring of the ATP-binding site [135]. In contrast, AKT can be locked in an inactive conformation by compounds which bind at the interface between the kinase domain and the pleckstrin homology domain [136,137], and PDK1 can be targeted by allosteric ligands binding in the PIF-binding pocket in the kinase N-lobe that communicates with the ATP-binding site when occupied [138]. Unfortunately, most allosteric inhibitors appear to have been discovered serendipitously, and the rational design of new target-specific compounds requires a detailed structural and biomolecular understanding of the protein and its regulatory mechanisms. Indeed, only approximately 25% of mammalian pseudokinase domains have been structurally characterized (Table 1). Although no proof of concept for type III inhibition currently exists in the public domain for pseudokinases, the growing number of pseudokinases observed to undergo 'kinase-like' conformational changes in response to appropriate cellular cues is a promising indication of the potential of this area for biomedical research. Indeed, the interesting effects of ATP-competitive allosteric regulators of KSR2 on distinct inhibitors of MEK signaling in RAS-mutant cancer cells lends further credence to this theory [128].

Covalent kinase inhibitors & the human protein kinome cysteinome

The majority of covalent 'type IV' kinase inhibitors are versions of established kinase inhibitors that have undergone structure-guided modification, specifically with the addition of an electrophilic 'warhead' to target the nucleophilic thiol groups of cysteine residues found in their targets [150]. Originally thought to lack clinical utility, in part due to issues with promiscuity, compounds containing warheads that target Cys residues have seen a resurgence [151,152], driven by the approval of the nonspecific Bruton's tyrosine kinase inhibitor Ibrutinib [153] and the discovery of more potent and selective, compounds such as acalabrutinib (ACP-196) [154]. Although Cys residues encoded in the human kinome have not been reported to serve a direct catalytic role, in marked contrast to protein phosphatases, they are still found in and around the ATP pocket in relatively high abundance, providing opportunities for redox regulation of the catalytic output or protein interactomes of a large proportion of protein kinases [155,156]. Indeed, based on structural- and sequence-based analysis, it has previously been estimated that as many as 39% of protein kinases have accessible noncatalytic cysteines in their active sites [150,157] and there has been considerable effort dedicated to developing inhibitors that block access to the ATP pocket by covalently modifying such residues. Covalent inhibitors have several desirable characteristics compared with reversible inhibitors, especially in instances where target specificity can be increased, or perhaps even guaranteed, based on the distribution of Cys residues in the kinase domain. The reactions are energetically favorable, requiring lower inhibitor concentrations to achieve high efficacy, and once bound the covalent molecule provides terminal inhibition, meaning new protein synthesis must occur to restore kinase function. Importantly, because targeted Cys residues are not uniformly conserved among kinases and pseudokinases, they act as specificity filters to limit inhibitor promiscuity. Moreover, with deeper analysis of kinase conformational changes, thiol groups that are differentially exposed in active and inactive kinase states will likely produce a diverse and versatile reservoir of rather specific drug targets [150]. From a clinical perspective, one potential downside of using irreversible inhibitors is unanticipated toxicity from concentration dependent off-target adduct formation with hyper-reactive cysteines of unrelated proteins, an occurrence that will have to be evaluated on a case-by-case basis [133,158,159]. The following section describes how our understanding of kinase structures has guided the design of irreversible covalent inhibitors with useful target specificity, and how this might be applied as a framework for the design of similar compounds directed against pseudokinases.

The term 'cysteinome' describes a convenient methodology for the categorization of targetable cysteine loci in and around the ATP pocket (Figure 2) [157]. To date, most attention has been directed to the development of compounds that target Cys on the hinge region amino acids that connect the N- and C-terminal kinase lobes. For example, the noncatalytic hinge-region cysteine (Cys481) of Bruton's tyrosine kinase is the target of Ibrutinib [160], an irreversible inhibitor which is used for the treatment of B-cell malignancies chronic lymphocytic leukemia [161] and mantle cell lymphoma [162]. Sequence alignment reveals that all four other TEC family kinases (BMX, ITK, TEC and TXK), in addition to BLK, JAK3, MKK7 and ErbB family members (EGFR, HER2 and HER4), possess an equivalent cysteine at this position, and display cross-reactive liabilities toward certain compounds [157,159]. As discussed earlier, the JAKs are highly attractive therapeutic targets for the treatment of numerous human diseases, but development of compounds with strong selectivity within this family has been impeded by the extremely high sequence

Table 1. Publically available structural information obtained from crystallized human pseudokinase domains.

Name of pseudokinase	Crystal structure of pseudokinase domain, amino acid residues and PDB ID	Known function(s) in human model cell systems	Diseases associated with vertebrate deregulation	Ref.
ADCK3	Pseudokinase domain (256–647, PDB ID: 4PED)	UbiB family member with a putative protein kinase like fold that forms an electron-transferring membrane complex involved in isoprenoid lipid biosynthesis in the mitochondria	Cerebellar ataxia, estrogen receptor-positive breast tumors, glioblastoma multiforme and steroid-resistant nephrotic syndrome	[139]
CASK	CaM pseudokinase domain (1–337, PDB ID: 3MFT) CaM pseudokinase domain (1–337) bound to 3′-AMP (PDB ID: 3C0G) CaM pseudokinase domain (1–337) bound to ADP (PDB ID: 3C0H) CaM pseudokinase domain (1–337) bound to ANP (PDB ID: 3MFR)	Contributes to neural development and regulation of gene expression	Neurological diseases	[20,21]
HER3	Pseudokinase domain 698–1019 with Mg^{2+}-ANP (PDB ID: 3KEX), 684–1020 with Mg^{2+}-ANP (PDB ID: 3LMG) 698–1020 domain bound to bosutinib (PDB ID: 4OTW) EGFR/HER3 heterodimer with Mg^{2+}-ADP/ANP (682–1022, 698–1020, PDB ID: 4RIW)	Binds to neuregulin 1, forms heterodimers with other HER family members and activates PI3K/AKT signaling	Lung, melanoma, breast, pancreatic, prostate, ovarian, colon and gastric cancers	[24,31,61,111]
ILK	Pseudokinase domain/alpha-parvin core complex (183–452, PDB ID: 3KMU)	Regulates actin polymerization by linking integrins to the actin cytoskeleton	Breast, prostate, brain and colon cancers	[140]
JAK1	JH2 domain, V658F (561–860, PDB ID: 4L01) and nonmutated (561–860, PDB IDs: 4L00)	JH2 pseudokinase domain of JAK1 facilitates regulatory functions and is needed both for suppression and full activation of JH1 domain catalytic activity. V658F fund in T-cell leukemia	T-cell leukemia, gynecological tumors rheumatoid arthritis, Crohn's disease, multiple sclerosis; myelofibrosis and psoriasis	[141]
JAK2	JH2 domain (536–812, PDB ID: 4FVP) JH2 domain bound to Mg-ATP (536–812, PDB ID: 4FVQ) V617F mutant JH2 domain bound to Mg-ATP (536–812, PDB ID: 4FVR) E596A V617F mutant JH2 domain bound to Mg-ATP (535–812, PDB ID: 5I4N)	JH2 domain is catalytically active and phosphorylates two negative regulatory sites in JAK2 (Ser523 and Tyr570) to suppress JAK-STAT signaling. The pathogenic V617F mutation promotes a confirmation in the JH2 α-C helix that enables stimulatory interactions with the JH1 domain	Polycythemia vera, acute myeloid leukemia and somatic erythrocytosis	[142,143]

Pseudokinase boundaries used for protein expression, and details of ligands observed after x-ray crystallography are provided with relevant links to available PDB files. A brief description of the biological function and potential disease links is also provided, alongside the key primary references.
AML: Acute myeloid leukemia; NKCC: $Na+/K+/Cl^-$ cotransporter; T-ALL: T-cell acute lymphoblastic leukemia.

Table 1. Publically available structural information obtained from crystallized human pseudokinase domains (cont.).

Name of pseudokinase	Crystal structure of pseudokinase domain, amino acid residues and PDB ID	Known function(s) in human model cell systems	Diseases associated with vertebrate deregulation	Ref.
KSR2	Heterodimeric pseudokinase (634–950) in complex with MEK1 (1–393) and APS-2-79 ligand (PDB ID: 2Y4I)	Scaffolding pseudokinase that coordinates the formation of the Raf-MEK-ERK signaling complex. Phosphorylates MEK1 at non-canonical sites upon in a complex with BRAF	Obesity, Type 2 diabetes and tumor cell transformation	[29]
MLKL	Pseudokinase domain (183–471, PDB ID: 4MWI). Pseudokinase domain (179–471, PDB ID: 4M67). No ATP mimetic bound	Phosphorylated by RIP3 to mediate downstream necroptosis signaling. Pseudokinase domain controls 4 Helix bundle output domain	Autoimmune diseases, inflammatory bowel disease and neoplastic diseases	[28,144]
PAN3	Drosophila melanogaster Pseudokinase domain bound to AMP phosphoramidate (349–790, PDB ID: 4BWP)	Forms a poly(A)-specific RNase complex with the exonuclease PAN2, which functions in mRNA deadenylation	Unknown	[145]
RNase L	Mammalian pseudokinase domains (365–586 in humans) included as part of RNase L polypeptide (4OAU, 4OAV, 4O1P) with ANP, ACP and ADP	Inteferon regulated endoribonuclease that blocks viral infection in vertebrates through sensing of 2′,5′-oligoadenylate (2–5A) viral second messengers	Prostate cancer and chronic fatigue syndrome	[123–125]
ROR2	Pseudokinase domain (464–751, PDB ID: 3ZZW), 452–753 (PDB ID: 4GT4), no ATP mimetic	Modulates Wnt signaling upon receptor stimulation	Renal cell carcinoma, melanoma, osteosarcoma, colon cancer, head and neck squamous cell carcinoma and breast cancer	[146]
STRADα	STRADα C terminal pseudokinase domain (59–431) in complex with MO25α, LKB1 and ANP (PDB ID: 2WTK)	Binds to ATP and complexes with MO25α to allosterically stimulate LKB1 and other AMPKs	Peutz-Jeghers syndrome and sporadic cancers	[62]
TITIN	Pseudokinase domain 32,172–32,492 (PDB ID: 1TKI), 32,172–32,492 (PDB ID: 4JNW)	Regulator of muscle contraction	Human skeletal tibial muscular dystrophy, dilated and cardiomyopathy hypertrophic cardiomyopathy	[147–149]
TRIB1	Pseudokinase domain (83–343, PDB ID: 5CEK), Pseudokinase and COP1-binding C-terminal tail (83371, PDB ID: 5CEM), no ATP mimetic	Binds to substrates (e.g., C/EBPα) and E3 ubiquitin ligases (e.g., COP1). Interacts with MKK proteins to modulate MAPK signaling	AML, CML, T-ALL, megakaryocytic leukemia, fatty liver disease and coronary artery disease	[88]

Pseudokinase boundaries used for protein expression, and details of ligands observed after x-ray crystallography are provided with relevant links to available PDB files. A brief description of the biological function and potential disease links is also provided, alongside the key primary references.
AML: Acute myeloid leukemia; NKCC: Na+/K+/Cl^- cotransporter; T-ALL: T-cell acute lymphoblastic leukemia.

Table 1. Publically available structural information obtained from crystallized human pseudokinase domains (cont.).

Name of pseudokinase	Crystal structure of pseudokinase domain, amino acid residues and PDB ID	Known function(s) in human model cell systems	Diseases associated with vertebrate deregulation	Ref.
TYK2	JH2 domain bound to 5′ ATP (556–871, PDB ID: 5C03). Pseudokinase domain bound to BMS-066 (575–869, PDB ID: 4WOV)	JH2 domain binds to ATP but is catalytically inactive, which is functionally important in JH1 domain regulation	Autoimmune disorders including systemic lupus erythematosus	[38,116]
VRK3	Pseudokinase domain without ATP mimetic (146–474, PDB ID: 2JII)	VRK3 negatively regulates MAPK signaling by promoting vaccinia H1-related (VHR) dependent inactivation of ERK in the nucleus	Alzheimer's disease and Parkinson's disease	[4]
WNK1	Pseudokinase domain without ATP mimetic (210–482, PDB ID: 4PWN)	Stimulates activities of Na^+ driven, Cl^- importing NKCCs ($Na^+/K^+/Cl^-$ cotransporters	Hereditary neuropathy, migration in glioblastoma and hereditary hypertension	[98]

Pseudokinase boundaries used for protein expression, and details of ligands observed after x-ray crystallography are provided with relevant links to available PDB files. A brief description of the biological function and potential disease links is also provided, alongside the key primary references.
AML: Acute myeloid leukemia; NKCC: Na+/K+/Cl- cotransporter; T-ALL: T-cell acute lymphoblastic leukemia.

identity shared across their conserved tyrosine kinase active sites. The hinge region around Cys909 of the JAK3 JH1 (kinase) domain is unique among JAKs, and provided a potential selectivity handle to develop irreversible inhibitor compounds which specifically target JAK3 with little cross-reactivity with other JAK kinases or kinases carrying analogous Cys residues [163]. The equivalent cysteine (Cys797) of EGFR is also of clinical significance, and is covalently targeted by clinically approved covalent inhibitors such as Afatanib for the treatment of non-small-cell lung cancer [164]. Interestingly, a nonconserved and extremely rare hinge-region cysteine was exploited to design highly specific inhibitors against EphB3. Only two other human kinases, LKB1 and PINK1, share this residue and in these cases the Cys appears less accessible compared with that of EphB3, which likely greatly improves the specificity of the compound [165]. A highly potent covalent inhibitor of JNK family kinases also targets a conserved cysteine on the hinge-loop region (JNK 1/2 Cys 116, JNK 3 Cys 154) [166]. FGFR4 contains another unique hinge region cysteine (Cys552) that is not conserved among the three other human FGFR paralogs and is rare (>1%) among human kinases in general. Hagel *et al.*, developed BLU9931, a covalent compound with ≥50-fold greater inhibition of FGFR4 as compared with FGFR1–3, and which exhibited significant antitumor activity in hepatocellular carcinoma xenograft models [167]. Curiously, all FGFRs carry a Cys on the glycine-rich loop that is the target of the potent irreversible pan-FGFR inhibitor, FIIN-1, which was developed from the noncovalent inhibitor PD173074 [168]. To our knowledge FIIN-1 is the only current example of a covalent inhibitor that targets a cysteine at this specific position, although many kinases (and pseudokinases) including PLKs, RSKs and ZAK also have cysteines on their glycine-rich loops [157].

Cysteine amino acids situated on the 'roof region' of the ATP-binding pocket, immediately following the glycine-rich loop, can also be accessible for covalent modification. Cohen *et al.*, exploited such a Cys to target p90 ribosomal protein S6 kinase (RSK) family members with the compound FMK [169]. In the design of FMK, Cohen *et al.,* took advantage of a small gatekeeper Thr (residue 463) found in RSK to ensure selectivity over kinases with analogous Cys residues, but that contain bulky gatekeeper residues that are prohibitive to compound binding. Cys22 of human centrosomal kinase, NEK2, is located at an identical position to that of the RSK family of kinases and is targeted by oxindole propynamide 16 (JH295), which irreversibly inhibits NEK2 cellular activity [170]. Deregulation of the pseudokinase HER3 is a feature of several cancers [171] and the protein was initially thought to be 'undruggable' as it lacked detectable kinase activity despite binding ATP [24]. How-

ever, TX2–121–1-adamantane conjugates form a covalent bond with Cys721 on the roof of the HER3 ATP-binding pocket, and partially blocks HER3-dependent signaling by targeting the protein for degradation in some HER3-dependent cell lines [25].

A specific Cys residue adjacent to the DFG motif is another possible target for covalent modification, and one whose solvent accessibility might be regulated through conformational fluctuations in pseudokinase structures. However, this Cys residue is present in about 10% of all human kinases, which decreases the likelihood of obtaining acceptable levels of selectivity [133]. In agreement, the natural product cis-enone covalent inhibitor hypothemycin was shown to bind to >90% of tested kinases carrying this conserved Cys residue, including ERK1, MEK1/2, PDGFRs, FLT3 and VEGFRs [172]. Even in this extreme case, careful structure-guided design facilitated the generation of a compound which could discriminately target VEGFR-2 over proteins bearing analogous Cys residues [173]. This serves to demonstrate the influence that alternate chemical scaffolds can have on an inhibitors ability to achieve adequate selectivity, even when targeting highly conserved binding sites. Sequence analysis of the pseudokinase NOK reveals that it also contains a Cys at this position [18]. Interestingly, there are only two canonical human protein kinases containing a Cys residue at the gatekeeper position (SgK494 and MOK), neither of which have been targeted covalently, and based on our analysis no human pseudokinases.

Cys residues located outside the ATP-binding pocket on the activation loop are also potential targets for small-molecule intervention. However, the challenge of structure-guided design of such compounds is exacerbated by the inherent flexibility of this region. Interestingly, Cys covalent modification of the activation segment by biologically active factors that induce allosteric inhibition have already been described for several canonical kinases [155,156,174,175] which suggest that covalent targeting of the activation loop may be a viable prospect. Unfortunately, much like allosteric inhibitors, most non-ATP-binding site compounds have been discovered by chance, which reduces the ability to identify suitable modifiable-thiol groups. Recently however, the rationale design of an irreversible inhibitor of CDK12 and 13 that targets a Cys remote from the kinase domain has been reported [53]. THZ531 achieves CDK12 and CDK13 inhibition by covalently binding to a C-terminal cysteine residue and making additional contacts with the ATP pocket via its flexible linker region. Even more surprising was the discovery that MLKL activity could also be regulated by a covalent compound that exerted its function by binding to a region distal to the active site. Cys86 on the N-terminal coiled coil domain of MLKL was covalently modified by necrosulfonamide, an interaction that blocked necroptosis in human cells [77]. This presents the exciting opportunity to scrutinize non-ATP located Cys residues (including outside the pseudokinase domain) for covalent modification and may greatly expand the number of potential drug targets.

The human pseudokinase cysteinome

The examples discussed in this review serve to demonstrate the surprising levels of selectivity that can be achieved by drugs that target nonconserved thiol groups, many of which are also found among the human pseudokinases. To date, most studies discussing the protein kinase 'cysteinome' have precluded atypical kinases and pseudokinases from evaluation, and only Cys721 in the pseudokinase domain of HER3 (Figure 2) and a nonpseudokinase domain cysteine of MLKL have (knowingly) been targeted by a covalent compound up to now [25,77]. For this reason, we have analyzed the frequency and distribution of Cys residues among the complete set of kinase and pseudokinase domains in the human kinome, using new comparative Kinview software [176] and PKA nomenclature to standardize amino acid positions (Figure 3). Cys residues were identified at over 100 distinct pseudokinase domain loci (some 273 Cys residues in total). Interestingly, some Cys positions were observed at high frequency in both pseudo and canonical kinases (e.g., Cys at equivalent position 273), whereas other Cys residues were less evenly distributed between the two groups. For example, Cys at position 200 (within the canonical activation segment) occurs in approximately 20% of protein kinases, but a Cys was identified in just two pseudokinases (PSKH2 and VACAMKL/CAMKV). Cys-200 has known regulatory functions among canonical kinases including PKA, partly due to its close proximity to activating sites of auto-phosphorylation [175]. We therefore predict that Cys underrepresentation at this position in pseudokinases is a likely consequence of their loss of regulatable catalytic function. In marked contrast, Cys at PKA-equivalent positions 169, 228 and 268 are found at near tenfold higher frequency in pseudokinases than among conventional kinases (Figure 3, asterisks). Of particular interest is a Cys amino acid at position 169, which is found in 14% of pseudokinases, but less than 2% of canonical kinases and lies within the extended HRD motif, which is classically involved in phosphate transfer in active kinases. This residue might therefore be a useful target for pseudokinase covalent modification. Of note, a hinge region Cys residue that has previously been targeted by a covalent inhibitor of FGFR4 is also present in the pseudokinase Sgk495 (Cys 123, PKA numbering, corresponding to Sgk495 Cys 143). SgK495 is a highly degraded 'orphan' pseudokinase most closely related to

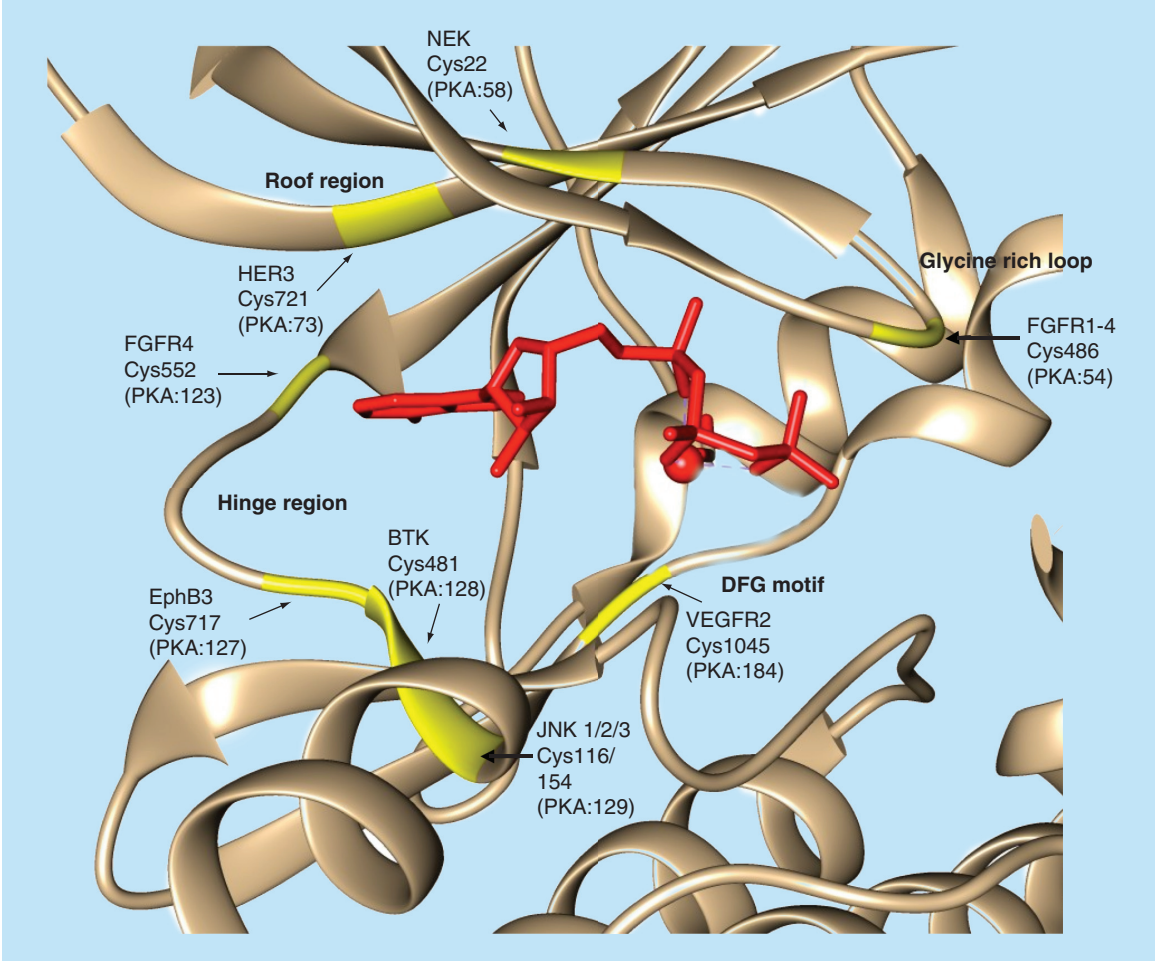


Figure 2. Schematic representation of positional distribution at key positions among the targeted protein human kinome 'cysteinome'. Yellow residues indicate positions of Cys residues that have been targeted for covalent modification in protein kinases and pseudokinases, and modelled using the HER3 tyrosine kinase structure (PDB ID: 3LMG). The actual site of covalent attachment, and the PKA equivalent residue, is also shown. AMP–PNP ligand is shown in red.

the Tribbles subfamily of pseudokinases [22]. Although our simple analysis does not take in to account the thiol side-chain solvent accessibility of Cys residues, it clearly demonstrates that these residues are present, potentially biologically relevant, and potentially targetable by appropriate chemical reagents in human pseudokinases. They could therefore be evaluated much more closely as new molecular targets for small molecules related to classical kinase inhibitors. One obvious avenue for drug screening, perhaps based on simple biophysical measurements or cell-based coupling, might include focused libraries comprised known inhibitors, including those with designed covalent bond forming capabilities (Figure 1).

Future perspective

Pseudokinases evolved unique signaling mechanisms to regulate a broad range of cellular processes, many of which go awry in disease. It might therefore make sense to develop novel medicinal chemistry approaches to treat pathophysiological conditions by interfering with pseudokinase-mediated signaling. The past decade has seen a huge expansion in our understanding of various aspects of pseudokinase structure and function, which has presented new opportunities to interrogate their function with small-molecule inhibitors. Although this review has primarily focused on inhibitors that directly (or indirectly) interfere with potential pseudokinase transitional states, there is tremendous scope for inhibitors with alternative modes of action, such as those that might sterically interfere with pseudokinase-driven protein–protein interactions. In this regard, stapled peptides might be considered to disrupt pseudokinase interactions, as illustrated by the development of stapled peptides that impair AKAP-mediated localization of the PKA complex in an isoform-selective manner [177,178]. Indeed, although pseudokinases frequently possess zero or vestigial catalytic activity, they can still form functional complexes with active kinases, often facilitating a

crucial layer of allosteric regulation. By interfering with this process, pseudokinase function might indirectly be modulated with compounds that preferentially target the induced active-state of binding partners, as exemplified by inhibitors directed against the HER2–HER3 oncogenic pairing [51,61]. A final, potentially significant, challenge if pseudokinase therapeutics are to succeed is the likely development of drug resistance, a scourge associated with the structurally dynamic kinase domain fold. Whether the innate dynamic conformational plas-

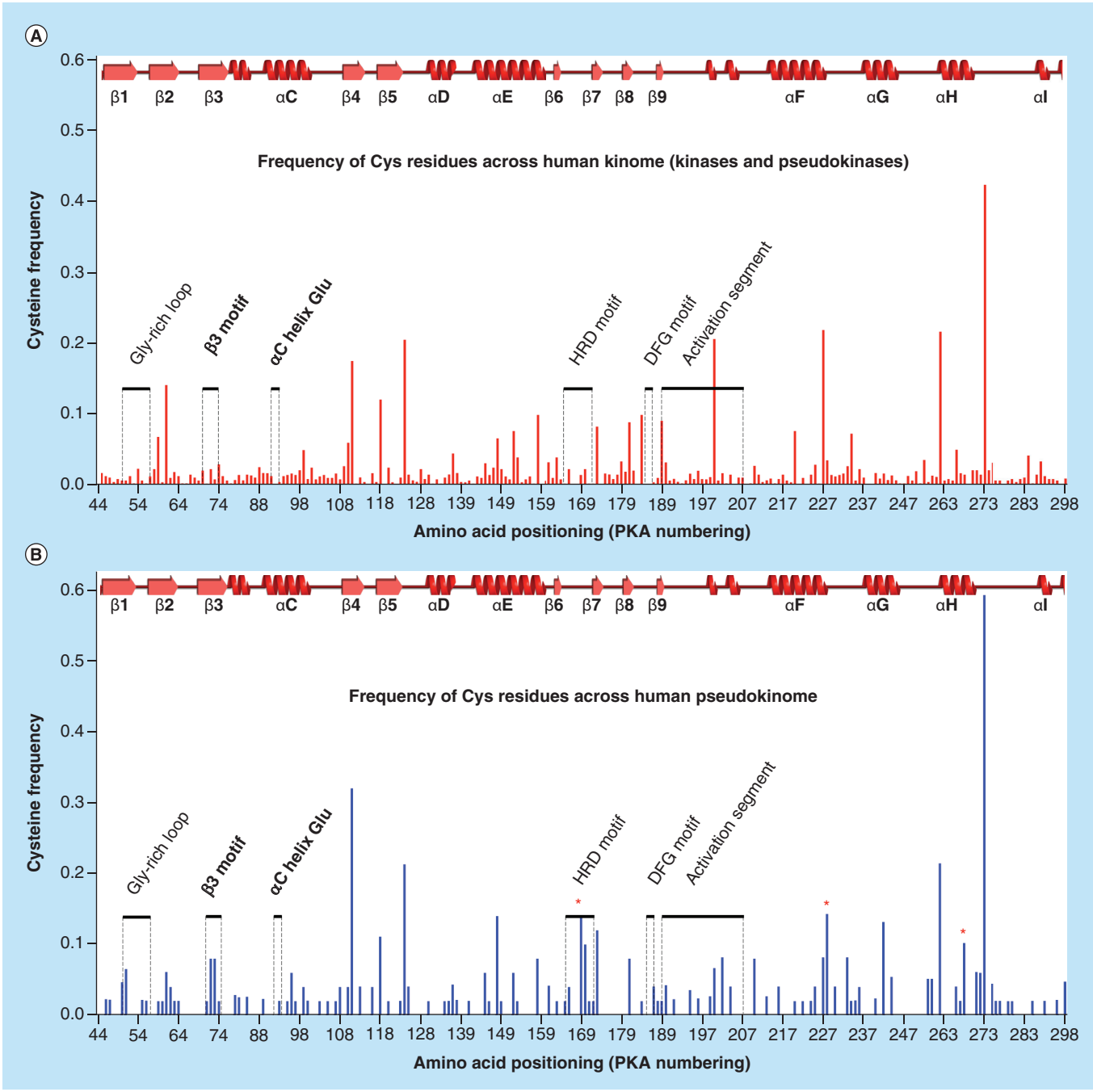


Figure 3. Frequency and distribution of cysteine amino acids found within the human kinome. The protein kinase ontology (ProKinO) browser was used to perform an integrative analysis across all human kinase and pseudokinase sequences (the kinome) in order to reveal Cys-residue frequencies and their linear positions relative to the PKA kinase domain. **(A)** Frequency and distribution of cysteine residues in all human kinases. **(B)** Frequency and distribution of cysteine residues in the human pseudokinases. The relative position of secondary structure elements for the canonical PKA kinase domain is shown in both panels, with canonical catalytic and regulatory motifs highlighted. Asterisks distinguish positions where a particularly high frequency of cysteine residues are present in pseudokinases, which if chemically labile, represent potential targets for chemical covalent modification.

ticity of pseudokinases, which has only recently been appreciated, underpins the majority of their regulatory output is an important factor for consideration in compound design. In this context, it will be important to observe whether, as seems possible, pseudokinases (like kinases) possess the inherent flexibility to succumb to mutations that interfere with drug binding while preserving their signaling function.

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Executive summary

- Pseudokinases are ubiquitously found in eukaryote kinomes, and are involved in a myriad of cellular processes, where they often serve as signaling modulators.
- The disruption of pseudokinase signaling function is strongly associated with a wide range of human diseases, including cancer.
- Regulated pseudokinase-dependent signaling is achieved by ligand or protein induced conformational transitions, with signal transduction closely linked to adoption of an appropriate pseudo-active state.
- Small-molecule inhibitors with the ability to target and (de)stabilize different pseudokinase structural states are promising candidates for the development of new pseudokinase-targeted therapeutics.

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