HHS Public Access

Author manuscript

Biochem Soc Trans. Author manuscript; available in PMC 2018 May 29.

Published in final edited form as:

Biochem Soc Trans. 2017 February 08; 45(1): 79–88. doi:10.1042/BST20160134.

Targeting PAK1

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Abstract

p21-activated kinase 1 (PAK1) has attracted much attention as a potential therapeutic target due to its central role in many oncogenic signaling pathways, its frequent dysregulation in cancers and neurological disorders, and its tractability as a target for small-molecule inhibition. To date several PAK1-targeting compounds have been developed as preclinical agents, including one that has been evaluated in a clinical trial. A series of ATP-competitive inhibitors, allosteric inhibitors, and peptide inhibitors with distinct biochemical and pharmacokinetic properties represent useful laboratory tools for studies on the role of PAK1 in biology and in disease contexts, and could lead to promising therapeutic agents. Given the central role of PAK1 in vital signaling pathways, future clinical development of PAK1 inhibitors will require careful investigation of their safety and efficacy.

Introduction

PAK1 is a founding member of the Pak (p21-activated kinases) Ser/Thr protein kinase family. Initially identified as an interactor of the Rho GTPases RAC1 and CDC42 [1], PAK1 was later shown to play diverse role in cell signaling by means of its catalytic and scaffolding activities [2]. Signal transduction cascades modulated by PAK1 include proliferation and survival pathways such as MAPK, AKT, Wnt1/β-catenin, ERα, BAD and NF-κB [2]. PAK1 is also critically involved in regulation of cell motility, transmitting variety of signals controlling cytoskeleton dynamics, cell shape and adhesion [2–4].

While PAK1 shares functions with other family members, in particular PAK2 and PAK3 (which are, with PAK1, together referred to as group I Paks) much more is known of the function of PAK1 in terms of human biology and disease than any other isoform. PAK1 expression is dysregulated in several nervous system disorders, including Alzheimer disease and Fragile X syndrome [5], indicating a role in cognition. Gain-of-function alterations of PAK1 have been observed in a wide range of human malignancies, suggesting that this kinase plays a substantial role in tumor development and progression [2, 6]. Amplification of the *PAK1* gene at 11q13, as well as elevated PAK1 protein levels, are often associated with aggressive tumor phenotypes, chemotherapy resistance, and poor outcome [2, 7–9]. Apart from gene amplification and protein overexpression, PAK1 can be hyperactivated by

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

mutations in upstream regulators such as RAC1 [10], RAS [11] and Merlin [12], linking oncogenic signaling to cancer cell phenotypic changes.

For these reasons, targeting PAK1 may represent a promising therapeutic approach in certain disease contexts, and multiple efforts in identification of potent and selective PAK1 inhibitors have been made in the past decade [2, 13]. Here we discuss the suitability of PAK1 as a drug target and recent advances in the development of PAK1 inhibitors.

PAK1 structure and regulation

PAK1 is a 545 amino acid multidomain protein that contains an N-terminal regulatory region and a C-terminal kinase (catalytic) domain (Figure 1) [14, 15]. The PAK1 catalytic domain has the characteristic two-lobe kinase structure with a single phosphorylation site (Thr423) within the activation loop. The amino terminal end of PAK1 harbors several sequence motifs responsible for interacting with partner proteins. Residues 75–90 correspond to the CDC42/RAC1 interactive-binding (CRIB) domain, which partially overlaps the auto-inhibitory domain (AID, aa 83-149). Three Pro-rich N-terminal motifs interact with SH3-domain containing adaptor proteins, including GRB2 (aa 12–18), NCK (aa 40–45), and the exchange factor PIX (aa 186–203) [15]. A positively charged basic region adjacent to CRIB domain is critical for PAK1 binding to cell membrane phosphoinositides [16]. Several phosphorylation sites located in the regulatory region play role in enabling and stabilizing the active conformation of PAK1 (Figure 1A) [17–19].

PAK1 activity is regulated by a *trans*-autoinhibitory mechanism [20]. Inactive PAK1 folds into an asymmetric homodimer (Figure 1B), in which the AID of each molecule binds to the kinase domain of its counterpart. Interacting with the large lobe of kinase domain AID positions its C-terminal extension (aa 136–149) into catalytic cleft of the kinase domain (Figure 1B). This interaction prevents dimer deconstruction, opening of the catalytic site, and autophosphorylation that are essential for full PAK1 catalytic activity [17, 19]. Binding of GTP-loaded CDC42 and RAC1 to the CRIB motif causes an AID conformational change resulting in the dis-inhibition of the catalytic domain, dissociation of the dimer and phosphorylation of the regulatory region and the activation loop. Maximally active monomeric PAK1 can phosphorylate a variety of substrate proteins. PAK1 activity can also be regulated in a GTPase-independent manner. Such mechanisms of PAK1 activation include binding to phospholipids and SH3-containing proteins, as well as cross-phosphorylation by other kinases [21–25].

A detailed understanding of PAK1 regulation and catalysis has suggested two distinct strategies for pharmaceutical inhibition of PAK1: direct disabling of phosphotransfer in the active site (ATP-competitive inhibitors) and exploitation of unique PAK1 regulatory mechanisms (allosteric inhibitors).

ATP-competitive PAK1 inhibitors

ATP-competitive inhibitors are typically designed to occupy the ribose-binding pocket of the kinase active site, precluding ATP access [26]. Developing such ligands for PAK1 has proven challenging, as the PAK1 kinase domain is highly plastic and the ATP binding cleft

is particularly open and flexible (Figure 2) [14, 27]. These challenges have not, however, prevented several serious attempts to create potent and specific inhibitors, and efforts to identify ATP-competitive PAK1 inhibitors with high affinity and kinase selectivity are ongoing. As the PAK1 catalytic domain shares strong sequence similarity with those of other Pak family members (~ 95% with PAK2 and PAK3, ~ 54% with PAK4,5,6), it is not surprising that the majority of described PAK1 targeting small molecules either inhibit all Paks ("pan-Pak") or are group specific (*e.g.*, PAK1/2/3 inhibitors) [2, 13] (Figure 3).

Indolocarbazoles

Alkaloid staurosporine (and its analog K252) is a broad spectrum ATP-competitive kinase inhibitor with high affinity for STE20 family kinases, including PAK1 [28, 29]. The staurosporine structure mimics ATP and interacts with conservative catalytic domain residues, efficiently inhibiting >70% of human kinases [28–30]. Despite its high potency, the utility of staurosporine has been limited by its poor target selectivity, thus further derivatization of the indolocarbazole scaffold was needed to improve selectivity for PAK1. Attempting to exploit the capacious ATP binding pocket of PAK1, Meggers and colleagues designed a metal-containing scaffold with Pak inhibitory activities [31]. A bulky, octahedral ruthenium complex inhibitor (Λ-FL172) (Figure 2, 3) efficiently filled the large catalytic pocket (Figure 2), gaining high PAK1 inhibitory efficacy as well as reasonably high selectivity over related protein kinases (Figure 3). Among 264 kinases tested, only 15 kinases (5.7% of total) showed an inhibition of > 80%. However, no information on cell activity and pharmacokinetics has been reported for this organometal conjugate compound, therefore it is unclear whether further pursuit of this strategy will yield clinically useful inhibitors.

Pyrrolopyrazoles (Pfizer)

The Pfizer group has described and evaluated a series of pyrrolopyrazole-based Pak inhibitors [13], including compound PF-3758309, the only Pak inhibitor to date to proceed to human trials [32]. Though originally designed as a PAK4 inhibitor, PF-3758309 efficiently inactivates all Pak family kinases, as well as a number of off-targets (Figure 2, 3). This orally available compound with potent cellular activity (1.3–3.9 nM) was probed in a number of preclinical cancer models and exhibited marked antitumor efficacy [32–37]. PF-3758309 inhibited cellular proliferation in a panel of tumor cell lines [32–36], as well as tumor growth in xenograft models of melanoma ($M24^{met}$, SK-MEL23, 537MEL) [10, 32], colorectal carcinoma (Colo-205, Collo-201, HCT116, RKO, PDT xenografts)[32, 36, 37], breast (MDA-MB231, BT-474)[32, 35], prostate (PC3)[34], lung cancer (A549, H358-S, H1299)[32, 33] and a transgenic *Kras*-driven squamous cell carcinoma model [38]. PF-3758309 was also suggested for the treatment of pigmentation disorders [39] and osteoclast-related disorders[40].

Given the relatively poor target selectivity of PF-3758309 it remains difficult to ascribe these desirable biological effects to Pak inhibition alone, since off-target inhibition most certainly contributes to the cellular activity of the compound. In any case, PF-3758309 was withdrawn from clinical investigation due to unexpectedly low oral bioavailability in humans, insignificant tumor responses, and adverse effects.

Aminopyrimidine-based series (Afraxis/Genentech)

Afraxis, Inc. developed a series of Pak-inhibiting compounds based on a pyrido[2,3-*d*]pyrimidine-7-one core for the treatment of neurological disorders. Subsequently FRAX compounds were utilized in various cancer preclinical studies. One such compound, FRAX597 (PDB ID: 4EQC), potently inhibits PAK1 (IC_{50} = 7.7 nM), while showing moderate selectivity against other kinases, particularly receptor tyrosine kinases [41]. This compound is orally available and was successfully used in a neurofibromatosis type 2 (NF2) orthotopic schwannoma model [41] and a meningioma model [42], as well as a transgenic $Kras^{G12D}$ squamous cell carcinoma mouse model [38].

Another compound of this chemical series, FRAX486 has been studied as a possible treatment of fragile X syndrome (FXS), a genetic disorder caused by inactivation of the fragile X mental retardation 1 (*Fmr1*) gene [43]. *Fmr1* knockout (KO) mice recapitulate human FXS symptoms, including hyperactivity, repetitive behaviors, and seizures, as well as morphological synaptic abnormalities [43, 44]. FRAX486 has excellent PAK1 potency (IC_{50} = 8.25 nM) and pharmacokinetic properties upon subcutaneous injection, including effective blood–brain barrier penetration, allowed its exploitation in an *Fmr1* KO model. Strikingly, single administration of FRAX486 was sufficient to ameliorate the FXS phenotype at both cellular and behavioral levels, in line with previous studies on genetic inactivation of Pak in this *Fmr1* KO mouse model [45].

An advanced member of this series, FRAX1036 (PDB ID:5DFP), exhibits high PAK1 potency (PAK1 Ki = 23 nM), refined kinome selectivity [42, 46, 47], and represents a useful tool compound for single and combinatorial experimental therapeutics [42, 46–48]. However, all of these early FRAX compounds were found to have strong adverse inhibition of hERG potassium channels. Also, the compound permeability was far from ideal [49]. Addressing these concerns, Genentech designed a further compound based on FRAX1036, termed G-5555 (PDB ID: 5DEY, Figure 2) [49], with favorable cellular activity and permeability, as well as low hERG channel activity. To our knowledge, G-5555 is the most selective ATP-competitive inhibitor of PAK1 reported to date (Figure 3). Only 8 out of 235 kinases tested (other than PAK1) showed an inhibition of >70%. Additionally G-5555 showed desirable *in vivo* pharmacokinetic properties, suggesting a potential for good human exposure.

G-5555 was tested on a panel of 23 breast cancer cell lines and has shown greater growth inhibition in Pak-amplified cell lines [50]. In a non-small lung cancer xenograft model and in a *PAK1*-amplified breast cancer xenograft model tumor growth was significantly impaired when exposed to 25 mg/kg BID G-5555, however greater doses were not tolerated. Mouse tolerability studies revealed acute cardiovascular toxicity of aminopyrimidine-based inhibitors, including G-5555, which makes it unsuitable for clinical development, and also raised questions about the feasibility of targeting group I Paks in the clinical setting [50].

Bis-anilino pyrimidines (AstraZeneca)

AstraZeneca has recently disclosed ATP-competitive kinase inhibitors with exceptional potency against PAK1 [51]. Overlay of two chemotypes (bis-anilino pyrimidine and

previously described 7-azaindole Pak inhibitor [52]) bound to PAK1 allowed the visualization and design small molecules with improved binding mode. One of the resulting *in vitro* probe compounds AZ13705339 inhibits PAK1 at IC_{50} <1 nM with only 8 off-target kinases giving >80% inhibition, mainly Src family kinases (Figure 3). Another compound of the series AZ13711265 displayed better pharmacokinetic properties and was proposed for *in vivo* studies.

Non-ATP-competitive PAK1 inhibitors

An alternative to ATP-competitive kinase inhibitors are allosteric inhibitors that bind outside of the ATP-binding cleft. PAK1 allosteric inhibitors achieve greater selectivity across the kinome relative to ATP-competitive inhibitors as they target less conserved regions. However such inhibitors have reduced potency compared to ATP-competitive inhibitors since protein pockets they target are not as deep and rich in inhibitor binding residues.

Naphthtols

IPA-3 (inhibitor p21-activated kinase-3), a sulfhydryl-containing compound that targets the N-terminal regulatory domain of PAK1, was discovered in a deliberate attempt to find non-competitive PAK1 inhibitors [53]. Reversible covalent binding of IPA-3 to the PAK1 regulatory domain prevents GTPase docking and the subsequent switch to a catalytically active state [54]. This unique mechanism of action accounts for the exceptional target specificity of IPA-3 (Figure 3), making it a useful tool compound for *in vitro* research. Unfortunately, cellular redox effects caused by the reduction of IPA-3 sulfhydryl moiety greatly limits its usefulness in cellular and animal models. This can be partially managed using structural inactive isomer of IPA-3 termed PIR-3.5 (PAK1 inhibitor-related 3.5) - a negative-control compound with similar redox effects [53]. Despite these limitations IPA-3 is widely used in cell culture and several attempts to evaluate IPA-3 potential *in vivo* have been made. In one such study encapsulation in sterically stabilized liposomes (SSL) has been used to improve IPA-3 metabolic stability and efficacy in inhibiting prostate cancer growth in xenograft model [55].

Overall IPA-3 represents a distinctive proof-of-concept compound with unique PAK1 binding mode and selectivity, and discovery of next generation IPA-3-like molecules with more suitable drug properties is desirable. In this regard, it is interesting to note that a second series of compounds have recently been identified that allosterically inhibit PAK1 by interacting with its regulatory domain [56]. Unlike IPA-3, these 1,4-naphthohydroquinone (1,4-NHQ)-based compounds do not appear to form covalent adducts with PAK1, and also display selectivity for PAK1 and PAK3 over PAK2 [56]. These features may be useful in limiting potential toxicities, as discussed in more detail below. As with IPA- 3, IC_{50} values for the 1,4-NHQ inhibitors are in the low micromolar range.

Dibenzodiazepines (Novartis)

Allosteric dibenzodiazepine PAK1 inhibitors emerged as hits in fragment-based screening performed by Novartis. These compounds bind to a novel allosteric site adjacent to ATP-binding pocket and interfere with ATP binding [57]. Optimized compound NVS-PAK1-1

(*a.k.a.* compound 3 [57]) showed exceptional selectivity across the kinome, including Pak intragroup isoform selectivity (Figure 3). Remarkably NVS-PAK1-1 demonstrates higher inhibitory activity on PAK1 than PAK2 as measured by *in vitro* kinase assays, K_d measurement, and immunoblot estimation of intracellular phospho-PAK1/2 levels. Thus NVS-PAK1-1 represents the first example of "PAK1 only" small molecule inhibitor. Despite high biochemical activity, NVS-PAK1-1 was not sufficiently potent to inhibit phosphorylation of the downstream substrate MEK1 Ser289 or cell proliferation. This phenomenon might hypothetically be explained by Pak isoforms functional redundancy [2]. In addition inhibition of PAK2 and/or PAK3 might be required for the functional activity in cells expressing these Pak isoforms. PAK1 amplified tumors that are highly dependent on PAK1 signaling might be chosen for further NVS-PAK1-1 validation studies.

Peptide inhibitors

Peptide inhibitors of PAK1 have been widely used as laboratory tools. For example, the PAK1 autoinhibitory domain (AID, aa 83–149) (Figure 1) effectively inhibits PAK1 activity. Interestingly, the induction of cell cycle arrest by the PAK1 AID can occur independent of inhibiting PAK1 kinase activity [58], most likely due to AID binding to the fragile-X proteins FMR1 and FRX1, which modulate the stability of the cyclin-dependent kinase inhibitor 1 $p21^{waf1}$ [59, 60]. Contrariwise, the AID derived from PAK2 lacks FMR1/FXR1 binding and presumably exerts its biological effects mostly through Pak inhibition. Two other peptide inhibitors, comprising the cell permeant TAT peptide fused to the PIX-interacting motif (TAT-PAK18) or the NCK binding motif of PAK1, have also been described. These peptides are thought to prevent proper cellular localization (and activation) of PAK1 through disruption of PAK1-NCK or PAK1-PIX interactions. The Pak-mediated growth suppression effect of TAT-PAK18 has been shown on PAK1-dependent ovarian cancer cell lines [61], while a Pak-NCK inhibitory peptide affects endothelial cell migration and contractility[62, 63]. Issues regarding the delivery of peptide-based inhibitors into cells, however, present a challenge in terms of therapeutic use.

Conclusions and prospects

PAK1 represents a promising druggable therapeutic target for conditions with elevated PAK1 levels or deregulated Pak upstream/downstream signaling [2, 5, 6]. Such "signalopathies" include several tumorigenic disorders, primarily cancers associated with *PAK1* amplification [8, 47]. Positioned downstream of difficult-to-target oncogenes such as RAS, PAK1 might open a new window for disruption of RAS-driven tumorigenesis [11, 38, 64]. PAK1 inhibition could also be beneficial for tumor growth targeting in neurofibromatoses type 1 (NF1) and type 2 (NF2) patients [41, 42, 64], as well as for management of NF1-associated social learning deficit [65]. Likewise, cognitive dysfunction in the context of FXS might be rescued upon PAK1 inactivation [43, 45].

Most described PAK1 inhibitors also bind related group I Pak family members PAK2 and PAK3. In our view, the major impediment to clinical development of Pak inhibitors relates to balancing efficacy and toxicity. To address this question, we need to better understand the functions of the three group I Paks in adult tissues. Genetic models have already established

an essential role for *PAK2* in cardiovascular function on adult mice, though the molecular underpinnings for this role are unclear [66]. In contrast, total *PAK1* loss is well-tolerated in mice, with few obvious phenotypic consequences, though α*MHC-Cre;Pak1*$^{f/f}$ mice, which delete *PAK1* in cardiac tissues, are prone to heart failure when subjected to pressure overload [67]. Whether this phenotype represents a developmental defect or a functional defect has not been established. *PAK3* loss-of-function mutations in man are associated with mental retardation, but, as with *PAK1*, whether this represents a developmental or functional defect is not known. These genetic studies, coupled with the observed acute toxicities associated with several distinct classes of Pak inhibitors, necessarily introduce a note of caution into Pak inhibitor development.

Assuming it is safe to target PAK1, one path forward might be to develop isoform-specific inhibitors. In that regard, it is encouraging that the Novartis compound NVS-PAK1-1 displayed reasonable selectivity for PAK1 over PAK2. However, given that this compound had little effect on the growth of a panel of cancer cells, it is possible that blockade of PAK1 alone will be insufficient in many instances. To address this issue, better selection of tumor types may be required. Recent work from our group and others [46, 47, 68] suggests that using *PAK1* gene amplification as a biomarker might select for tumor cells that are sensitive to PAK1 inhibition, and it will be instructive to test NVS-PAK1-1 and similar isoform-selective Pak inhibitors in this setting. Similarly, tumors bearing activating mutations in the small GTPase RAC1 (*e.g.*, ~5% of melanoma) [69, 70], which encodes a direct activator of group I Paks, might be good candidates for consideration for future anti-Pak agents.

Acknowledgments

We thank Peter J. Huwe for the help Figure 2.

Abbreviations

1,4-NHQ	1,4-naphthohydroquinone
AID	autoinhibitory domain
CRIB	CDC42/RAC1 interactive-binding
FMR1	fragile X mental retardation 1
FXS	Fragile X syndrome
IPA-3	inhibitor p21-activated kinase-3
KO	knockout
NF1	neurofibromatosis type 1
NF2	neurofibromatosis type 2
PAK1	p21-activated kinase 1
MAPK	mitogen-activated protein kinase

ERα	estrogen receptor alpha
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
BAD	Bcl-2-associated death promoter
GRB2	growth factor receptor-bound protein 2
hERG	human *ether-à-go-go*-related gene

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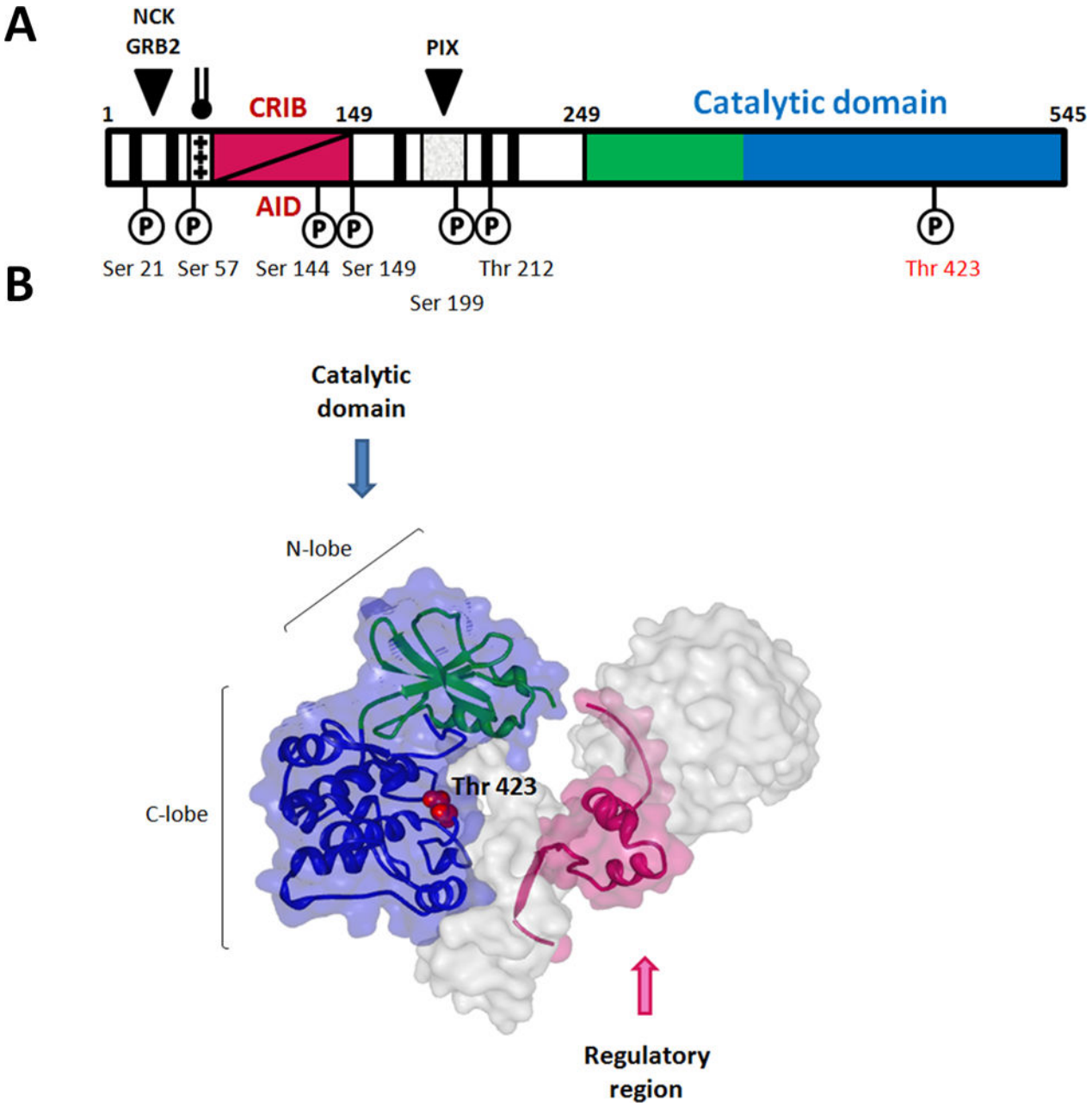


Figure 1. PAK1 structure

Organization of the PAK1 polypeptide chain highlighting sites of kinase phosphorylation. Numerals indicate residue numbers. PAK1 auto-regulatory region is in magenta, N-lobe of the catalytic domain is in green, and C-lobe is in blue. Proline-rich SH3-binding sites are shown as black bars. Phosphoinositide binding region enriched with basic residues is shown as srossed bar.

Diagram of dimeric PAK1 (PDB ID: 1F3M). One PAK1 complex is colored as in (A), Thr 423 is labeled. The other one is presented as surface diagram. Residues 1–77 and 148–248 are omitted.

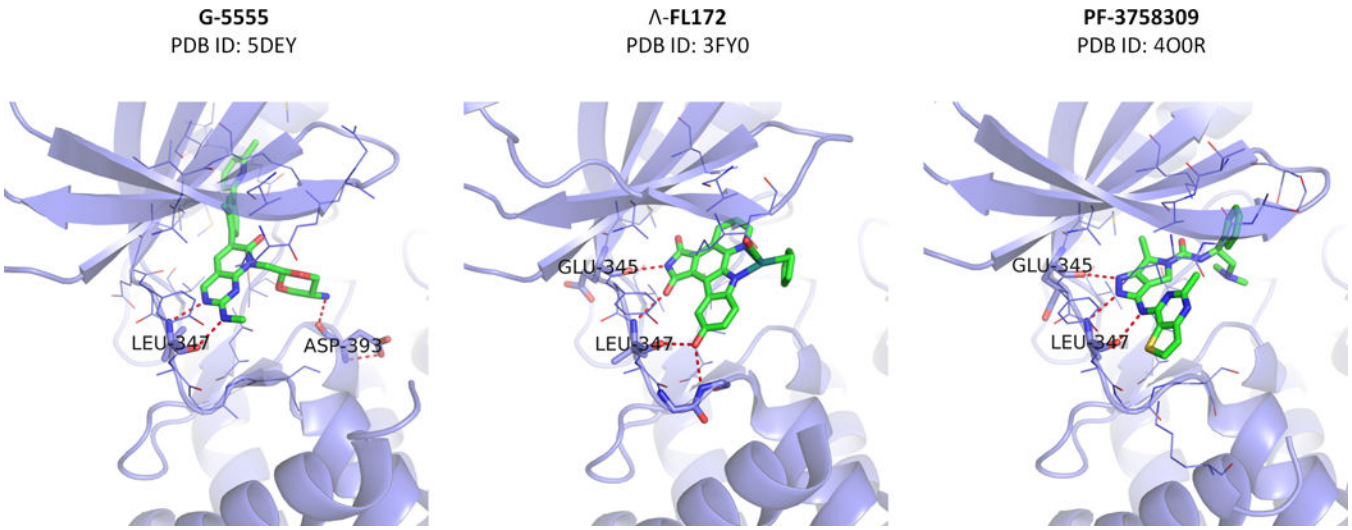
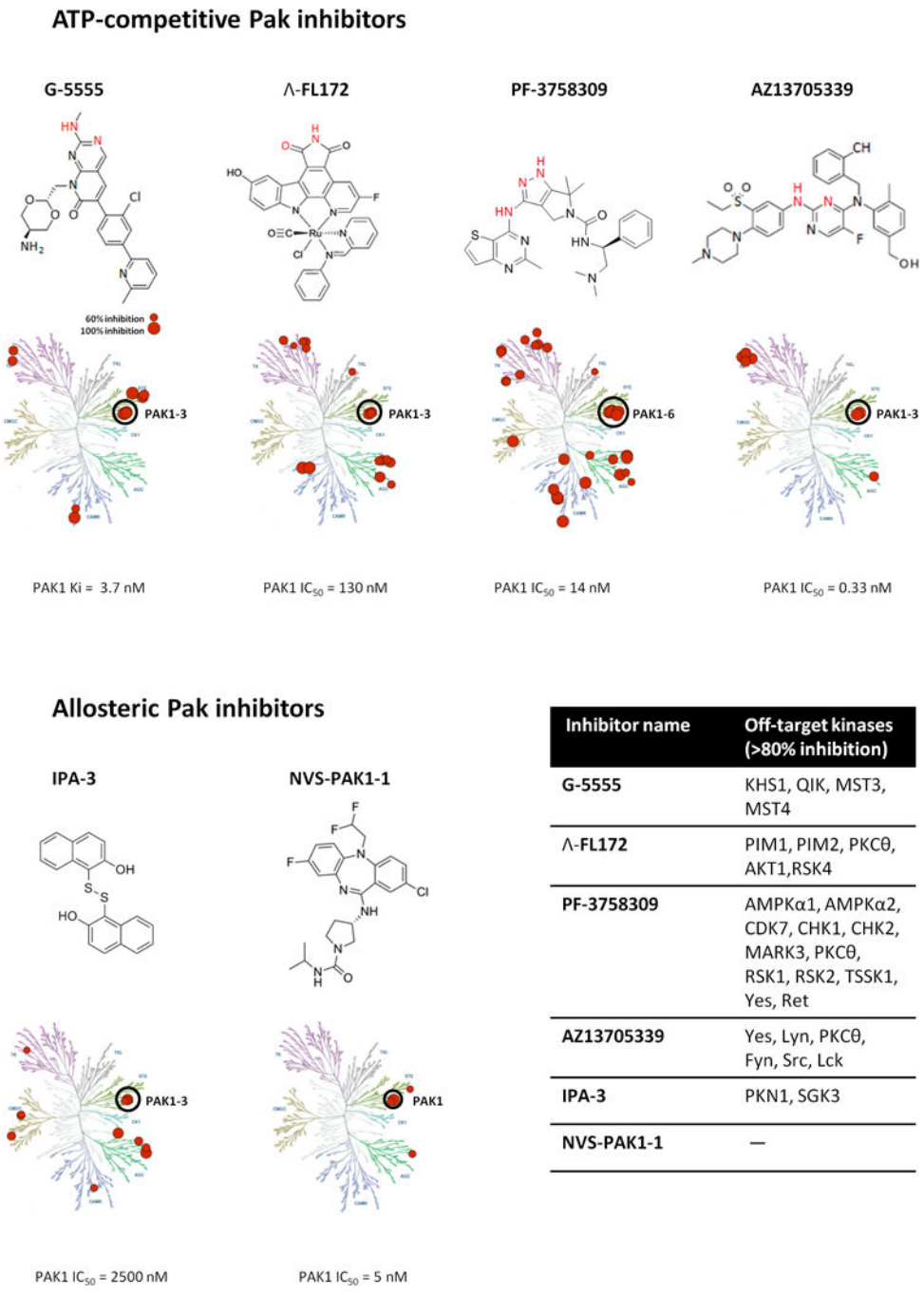


Figure 2. Co-crystal structures of ATP-competitive Pak inhibitors in complex with PAK1
Hydrogen-bonding interactions are shown as dotted red lines.



Inhibitor name	Off-target kinases (>80% inhibition)
G-5555	KHS1, QIK, MST3, MST4
Λ-FL172	PIM1, PIM2, PKCθ, AKT1,RSK4
PF-3758309	AMPKα1, AMPKα2, CDK7, CHK1, CHK2, MARK3, PKCθ, RSK1, RSK2, TSSK1, Yes, Ret
AZ13705339	Yes, Lyn, PKCθ, Fyn, Src, Lck
IPA-3	PKN1, SGK3
NVS-PAK1-1	—

Figure 3. Pak inhibitors

Biochemical structures and selectivity profiles (inhibition ≥ 50% shown) of select Pak inhibitors. The molecule portions of ATP-competitive inhibitors rendered in red indicate the atoms participating in kinase hinge hydrogen bonding contacts.

G-5555 (0.1 uM) screened aginst 235 kinases (Invitrogen) [49]

FL172 (3 uM) screened against 246 kinases (Millipore) [31]

PF-3758309 (1 uM) screened against 146 kinases at Pfizer, Invitrogen, and the University of Dundee.[32]

AZ13705339 (0.1 uM) screened against 125 kinases (Invitrogen), PAK3 activity was not tested in this assay.[51]
IPA-3 (10uM) screened against 214 kinases (Invitrogen) [53]
NVS-PAK1-1 (10 uM) tested against 442 kinases (DiscoverX).[57]
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