Recent advances in the development of p21-activated kinase inhibitors

Natalia Coleman1 and Joseph Kissil2,7,*

1Department of Biological Sciences; University of the Sciences; Philadelphia, PA USA; 2Molecular and Cellular Oncogenesis Program; The Wistar Institute; Philadelphia, PA USA

*Correspondence to: Joseph Kissil; Email: jkissil@scripps.edu
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The p21-activated kinases (PAKs) are downstream effectors of the small G-proteins of the Rac and cdc42 family and have been implicated as essential for cell proliferation and survival. Recent studies have also demonstrated the promise of PAKs as therapeutic targets in various types of cancers. The PAKs are divided into two major groups (group I and II) based on sequence similarities. Although the different roles the PAK groups might play are not well understood, recent efforts have focused on the identification of kinase inhibitors that can discriminate between the two groups. In this review these efforts and newly identified inhibitors will be described and future directions discussed.

Introduction

The p21-activated kinases (PAKs) are downstream effectors of the Ras-related Rho-family GTPases Rac and Cdc42. As such, the PAKs are involved in the regulation of cell survival, proliferation and migration, making them attractive therapeutic targets for cancer. The biological roles of the PAKs under normal and pathological conditions such as cancer are covered by other reviews in this issue and we will therefore summarize the recent and ongoing research being conducted to develop inhibitors of the PAKs.

The PAK Protein Family and Structure

There are two groups of mammalian p21-activated kinases (PAKs): group I, which is comprised of PAK1, PAK2 and PAK3, and group II consisting of PAK4, PAK5 and PAK6 (Fig. 1). While the groups carry out different functions and have distinct modes of regulation, both groups share a high degree of protein similarity within the kinase domains. The group I PAKs share 93–95% sequence identity within the kinase domain and group II share 75% sequence identity within this domain.1 Comparing group I to II there is an overall 54% sequence identity within this domain.2 Previous studies have indicated that the group I PAKs exist as homodimers that are regulated by an auto-inhibitory domain (AID) from one molecule in the dimer that interacts, in trans with the kinase domain of the other molecule.3 This AID overlaps with a p21-binding domain [PBD, a.k.a. cdc42/Rac interactive binding (CRIB) domain] and upon binding of a GTP-bound form of Rac or cdc42 the auto-inhibition is alleviated (Fig. 1). Recent work from multiple groups now indicates that all PAKs harbor an AID.4 The Kung group has identified an auto-inhibitory fragment at the N-terminal of PAK5 that can inhibit kinase activity in the absence of Cdc42 binding.5 Recently published data showed that PAK5 and PAK6 contain the same related CRIB/AID and that PAK4 contains AID positioned similarly to the PAK I AID (Fig. 1).6 However, in spite of these similarities it appears the mechanisms underlying the activation of the group I and II PAKs are different.

Clearly, one of the major challenges has been subgroup-specific drug design/discovery given the similarity in structure of the PAK-family proteins. Conceptually, a number of approaches can be employed to develop such inhibitors. One possibility would be to develop allosteric inhibitors, which would target regions outside the active site that are divergent between group I and II kinases. An alternative approach would be to take advantage of the sequence differences that do exist between the two groups kinase domains to develop small molecule ATP competitive inhibitors. Such an approach requires a better understanding of the physical structure of the active site of each of the PAKs.

PAK Inhibitors

Allosteric inhibitors. The first report on the identification and characterization of a highly selective allosteric small molecule inhibitor that targets the auto-regulatory mechanism of group I PAKs was described by the Peterson group. By developing a screen for allosteric inhibitors targeting PAK1 activation they identified the inhibitor IPA-3 (p21-activated kinase inhibitor 3). IPA-3 has an IC50 of 2.5 µM and prevents Cdc42-stimulated PAK1 autophosphorylation.6 IPA-3 is highly selective toward group I PAKs. It showed limited activity against the group II PAKs and against a panel of kinases from which it significantly inhibited (> 50% inhibition at 10 µM) only 9 from 214 kinases tested (4% total). Further studies showed that IPA-3 binds covalently to the PAK1 regulatory domain and thus prevents binding to the GTPase Cdc42. The kinase inhibition by IPA-3 in vitro occurs in a temperature-dependent and irreversible manner. However, pre-activated PAK1 is not inhibited by IPA-3.7 Unfortunately, a structural isomer of IPA-3, PIR-3.5, was found to have no inhibitory activity against PAK1. The primary screen...
of 33,000 structurally diverse small molecules was performed based on catalytic activity of full-length PAK1. A secondary screen was based on identification of compounds that are non-ATP competitive. From 32 compounds shown to inhibit PAK1 activity at 1 mM ATP, IPA-3 was identified as a lead. IPA-3 is clearly an interesting lead compound and further work is required to overcome issues related to the in vivo stability and oral availability of the compound. In particular, the presence of a disulfide bond suggests that the compound might act through covalent redox modification of PAK1. Nonetheless, the approach taken to identify allosteric inhibitors of the PAKs will prove useful for the study of PAKs biology as well as identification of additional inhibitors.

In addition to the small molecule allosteric inhibitors, a number of other inhibitors have been described. These include peptide inhibitors, such as the PAK1 AID, which unfortunately exhibits PAK1-independent effects and the cell-permeable PAK1-inactivating peptide TAT-PAK18, which has been previously suggested to inhibit PAK activity by disrupting the interaction between PAK1 and PIX and to block the growth of ovarian cancer cell lines.

A potential new therapeutic approach relies on the use of RNA interference, delivered by different approaches. The Kissil group has previously shown that shRNA-mediated depletion of PAK1, 2 and 3 inhibits the proliferation and tumorigenicity of NIH3T3/NF2<sup>100</sup> cells. These data suggest that using RNAi-based inhibitors
against the PAKs might be a potential tool in future applications for cancer treatment. Nevertheless, questions regarding the delivery approaches for RNAi based therapeutics as well as stability of gene silencing remain to be addressed.

**ATP competitive inhibitors.** The challenge in the developing of ATP-competitive inhibitors is the high degree of structural similarity between the ATP binding pocket of the PAKs and other kinases. That is exemplified by compounds such as the natural product staurosporine, a broad-range kinase inhibitor, and its derivative K252a which shows potent, but not selective, inhibition of PAK1. A subsequent synthetic derivative of K252a, CEP-1347, proved to be a more potent inhibitor of PAK1 but later was shown to be about 100-fold more selective toward MLK3 (mixed lineage kinase 3). Another example of a kinase inhibitor exhibiting significant PAK inhibitory capacity was provided by the Heerding group. From a series of novel AKT inhibitors containing 2,3,5-trisubstituted pyridines, one compound showed significant potency against PAK1 (IC\textsubscript{50} 31 nM). However, it was also observed to be a potent inhibitor of many other kinases in the AGC superfamily, such as p70S6K, PKD1, PKA and RSK. Work from the Ringel group reported that OSU-03012 is a direct inhibitor of PAK1. OSU-03012 is a derivative of celecoxib that competitively inhibits PDK1 in vitro. OSU-03012 unexpectedly reduced levels of phosphorylated PAK with an IC\textsubscript{50} value of ~1 μM which is lower than required to block PDK1-mediated AKT phosphorylation.

An interesting approach was taken by the Meggers group to combine organoruthenium chemistry, small-molecule screening and structure-based design to identify a PAK1 inhibitor (FL172) with an IC\textsubscript{50} value of about 100 nM. The screen of FL172 (at 3 μM) against a panel of 264 kinases revealed that only 15 kinases (5.7% of total) were significantly inhibited by this compound. FL172 also showed isoform selectivity by exhibiting poor inhibitory potency against the group II PAKs and displayed dose-dependent PAK1 inhibitor activity when tested in mammalian cells. Recently, the same group has reported the development of octahedral metal-based kinase inhibitors, including a highly specific PAK1 inhibitor (OS-2). This study demonstrated that octahedral metal complexes are sophisticated molecules providing versatile scaffolds for developing highly selective and potent kinase inhibitors.

Recent work from the Pfizer Oncology Group has identified, through high-throughput screening and structure-based design, the compound PF-3758309, a potent (K\textsubscript{d} = 2.7 nM), reversible ATP-competitive, pyrrolopyrazole inhibitor of PAK4. PF-3758309 binds the ATP binding site and makes multiple contacts with the hinge region through hydrogen-bond interactions with the pyrrolopyrazole core and the amine linker to the thiienopyrimidine ring. PF-3758309 shows similar activity across the group I and II PAKs and displays growth inhibitory activity against a broad range of tumor cell lines. In order to test selectivity, PF-3758309 was screened against a panel of 146 of the 518 known human kinases and was shown to have cellular activity against Src family kinases AMPK, RSK, CHK2 and others. PF-3758309 has been also shown to be a potent anti-tumor agent in human xenograft tumor models, with plasma EC\textsubscript{50} value of 0.4 nM. PF-3758309 demonstrated oral availability and additional steps have been taken to reduce the efflux of the inhibitor by medicinal chemistry strategies, specifically by lowering the molecular charge. These strategies ultimately lead to the discovery of a number of pyrroloaminopyrazoles as orally bio-available PAK4 inhibitors. Two compounds showed good anti-tumor growth activity (52–87%) through oral administration in a xenograft tumor model.

Recently a novel PAK4 inhibitor, LCH-7749944, was developed by the Le group through structure-informed design. This ATP-competitive inhibitor was shown to inhibit a number of PAK4 signaling pathways including the PAK4/LIMK1/cofilin and PAK4/MEK-1/ERK1/2/MMP2 pathways as well as EGFR activity.

### Future Directions

There is now ample evidence to implicate both PAK families as targets in different types of cancers. However, a number of questions remain to be answered. First, is there a need for inhibitors that would specifically target the group I or II PAK independently? Second, how difficult would it be to attain this goal?

In regards to the first question, given the different tissue and cell type distributions and substrate specificities of the different PAKs, it is clear that the identification and development of inhibitors that can distinguish between the group I and II PAKs will be instrumental to our understanding of the basic functions of these two groups of proteins, under normal physiological and disease conditions. Moreover, in the context of diseases where evidence exists implicating aberrant activation of PAKs from either of the groups, a major open question is whether inhibiting both groups of PAKs has a confounding effect. In other words, if inhibiting PAK from both groups results in undesired effects, such as reduced tolerability, then clearly inhibitors targeting one group or the other are likely to be beneficial. Further studies using highly selective tool compounds and experimental approaches, such as RNAi-based technologies to knockdown specific PAKs, will no doubt contribute to resolving these questions.

In regards to the second question, the accumulated data on structure and regulation of the two groups of PAKs now provides tools toward the development of selective inhibitors. Indeed such efforts have already resulted in competitive inhibitors that display specificity toward one group or the other, as illustrated by a number of examples discussed in this review. Efforts over the next few years using structure-informed design and novel chemistries will likely lead to even more selective and potent inhibitors. In addition, approaches to identify allosteric inhibitors are still in early phases, but will likely prove fruitful, as illustrated by the identification of IPA-3.
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