The Development of Inhibitors to Target the Driving Functions of PAK1 in Cancer

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Abstract

The p21-activated kinases (PAKs) are a family of protein kinases that play major roles as downstream effectors of the small G-proteins Cdc42 and Rac1 under normal physiological conditions and disease states. Specifically, p21-activated kinase 1 (PAK1) has been shown to play a driving role in cancer development by impacting fundamental cellular functions, such as growth factor signaling and morphogenic processes. As a result, there is increased interest in developing small molecule inhibitors that modulate the activity of PAK1 as anti-cancer agents. This review evaluates the past ten years of research involving challenges related to efficacy, toxicity, and selectivity of the inhibitors that have prevented progress past pre-clinical development, informing the development of new strategies to address these challenges.

Introduction

The p21-activated kinases (PAKs) are a family of protein kinases that play major roles as downstream effectors of Cdc42 and Rac1. Cdc42 and Rac1 are small G-proteins belonging to the Rac1 and Rho family (The UniProt, 2021; Dahmene et al., 2020; Rane & Minden, 2019; Semenova & Chernoff, 2017; Yi et al., 2010). As Cdc42 and Rac1 bind PAKs, the PAKs become activated and mediate downstream signaling. These signaling functions regulate multiple aspects of cellular behavior such as cell division, actin cytoskeletal rearrangements, and cell motility. Given that dysregulation of these functions is a hallmark of cancer (Hanahan & Weinberg, 2011), it has become increasingly apparent that Cdc42 and Rac1 can influence cancer progression and metastasis through the PAKs (Maldonado et al., 2020). The PAKs' involvement in cancer has been associated with their overexpression or dysregulated expression, and the genes encoding

them are not typically found to harbor mutations. The irregular expression of PAKs often interrupts their cellular roles and contributes to unchecked cell proliferation, aberrant cell signaling, increased metastasis, drug resistance, and modulation of the immune system (Rane & Minden, 2019). The goal of this work is to consolidate and evaluate research within this field to gain a clear understanding of how to facilitate progress in the development of PAK1 inhibitors which, in turn, can then help elucidate PAK1 biology and determine candidates for therapeutics.

PAK1 is one of six PAK genes that has been categorized into Group I of the two subfamilies of PAKs (Rane & Minden, 2019). PAKs 2 and 3 belong to the Group I PAK subfamily, and PAKs 4-6 belong to the Group II PAK subfamily (Rane & Minden, 2019). In Group I, the autoinhibitory domain (AID) functions as a major regulator of PAK activation. Group I PAKs are thought to be present mostly as dimers in a trans configuration where the AID from one unit is engaged with the catalytic domain of the other unit. This configuration inhibits kinase activity and autophosphorylation of an activation loop, thus preventing kinase activation. The Group I PAKs are activated via the disruption of the binding between the dimerizing PAKs by the binding of active GTP-bound Cdc42 (Cdc42-GTP) or Rac1 (Rac1-GTP) to the Cdc42/Rac1-interactive binding (CRIB) domain (Rane & Minden, 2014). Lipids have been described as playing a role in PAK activation; however, their specific role is not well understood (Zhao & Manser, 2012). Ultimately, PAK transforms into a monomer, resulting in autophosphorylation at the activation loop and other sites (Rane & Minden, 2014).

Methods

Publications were evaluated to synthesize the research on inhibitors and PAK1's biological role in the development of cancer. The domain sequence of PAK1 was constructed using DOG software (Ren et al., 2009). Domains and phosphorylation sites of PAK1 were identified using the UniProt database (2021) and "Targeting PAK1" by Galina Semenova and Jonathan Chernoff (2017). The ribbon structure of PAK1 was developed by the RCSB Protein Data Bank (Berman et al., 2000) and molecular graphics and analyses developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco Chimera were used to color the ribbon structure (Pettersen et al., 2004; Lei et al., 2000). The structures of PAK1 inhibitors were derived from associated publications and developed using ACD/ ChemSketch (Advanced Chemistry Development, 2021).

The Structure of PAK1

The 545 amino acid sequence of PAK1 (Figure 1) consists of two principal regions: a N-terminal regulatory domain and a C-terminal kinase (catalytic) domain. These regions are involved in the structural changes that alter the activity of PAK1 via their interaction with other proteins and through phosphorylation (Semenova & Chernoff, 2017). The PAK1 gene contains 23 exons, including 6 exons that are part of the 5'-untranslated region (UTR) and 17 coding exons (Kumar et al., 2017). The N-terminal regulatory domain consists of several motifs that are involved in the interaction with other proteins. The CRIB domain (aa 75-90) overlays the AID (aa 83-149) and p21 binding domain (aa 67-150) (Kim et al., 2016; Semenova & Chernoff, 2017). Adjoining the CRIB motif is a positively charged basic region that is involved in the binding to cell membrane phosphoinositides. Additional amino terminal domains include binding domains for growth factor receptor-bound protein 1 (GRB1) (aa 12-18), Nck (aa 40-45), and PAK-interacting exchange protein (PIX) (aa 186-203), which are proline rich and associate with these SH3-domain containing adaptor proteins (Semenova & Chernoff, 2017). The catalytic domain consists of a two-lobe kinase structure and activation loop, which contains a phosphorylation site at Thr423 (Semenova & Chernoff, 2017).

The General Cellular Functions of PAK1

PAK1 is implicated in the development of cancers (Khan et al., 2020; Semenova & Chernoff, 2017). This is not surprising given that expression of PAK1 has consistently been implicated in growth factor signaling networks and morphogenetic processes, such as cytoskeleton remodeling, cell proliferation, angiogenesis, and cell motility (Khan et al., 2020; Kumar et al., 2017; Ong et al., 2011). PAK1 regulates actin depolymerization and nucleation through multiple interactions (Kumar et al., 2017; Vadlamudi et al., 2004). One interaction includes the phosphorylation of actin-related protein (Arp) c1b-Thr21, resulting in the modulation of Arp2/3 actin nucleation complex. Indeed, the Arp2/3 actin nucleation complex is linked to cancer cell motility as shown by the fact that overexpression of Arpc1b promotes tumorigenic properties of cancer cells (Molli et al., 2010). Another actin binding protein, Cortactin, is phosphorylated by PAK1 and is overexpressed in some cancer types (Moshfegh et al., 2014). PAK1 is also known to interact with and phosphorylate dynein light chain 1 (DLC-Ser88) and integrin-linked kinase-1 (ILK-Thre173/Ser246), which were found to contribute to tumorigenesis (Acconcia et al., 2007). Microtubule biogenesis

is regulated via phosphorylation of Tubulin Cofactor V (TCoB-Ser65/ Ser128) by PAK1, which influences the invasiveness of cancer cells (Vadlamudi et al., 2005). The inhibitory activity of the protein Stathmin on microtubule stabilization is turned off via PAK1 phosphorylation of Stathmin (Kumar et al., 2017).

PAK1 plays a role in the nuclear compartment and impacts mitotic events and gene expression, thus regulating cell viability. For example, in breast cancer cells, activated PAK1 is dispatched to the nucleus upon growth factor stimulation (Singh et al., 2005). PAK1 modulates transcription, including phosphofructokinase-muscle isoform (PFK-M), nuclear factor of activated T-cell (NFAT1), and tissue factor (TF) genes (Sánchez-Solana et al., 2012). PAK1 may modulate the expression of PFK-M and NFAT1 genes through its interactions with chromatin (Singh et al., 2005). PAK1 stimulates the expression of TF, consequently modulating coagulation processes, embryonic development, and cancer-related events (Sánchez-Solana et al., 2012). Through the phosphorylation of microrchidia CW-type zinc finger 2 (MORC2), PAK1 signaling can regulate chromatin remodeling in cancer cells that were subjected to ionizing radiation (Li et al., 2012). In colon cancer cells, the B-cell lymphoma-6 corepressor in the nuclei is inactivated through phosphorylation by PAK1, resulting in modulation of cell-cycle progression (Kumar et al., 2017; Barros et al., 2012).

Multipolar spindle phenotypes and malfunctioning mitotic segregation of chromosomes in cancer cells are connected to the dysregulated expression and activation of PAK1. Centrosome maturation is modulated via its interaction with PAK1. Once this occurs, the activity of PAK1 is facilitated by G-protein-coupled receptor (GPCR) kinase-interacting protein 1 (GIT1), causing the phosphorylation of Aurora-A on Thr288/Ser342 (Kumar et al., 2017). Through the phosphorylation of histone H3.3A/H3-Ser10, the condensation of chromosomes during mitosis is also thought to be linked to the activity of PAK1 (Kumar et al., 2017). The polymerization and depolymerization of microtubules during mitosis is also regulated by PAK1. Polymerization occurs via the phosphorylation of Tubulin Cofactor B by PAK1 on Ser65 and Ser128 (Vadlamudi et al., 2005). Depolymerization occurs via the phosphorylation of mitotic centromere-associated kinesin by PAK1 on Ser192 and Ser111 (Pakala et al., 2012). Inadequate centrosome duplication can also occur via phosphorylation of Arpc1b-Thr21 by PAK1 on the Arp2/3 complex (Kumar et al., 2017).

PAK1 Regulation and Associated Signaling Pathways

The activation and inactivation of PAK1 affects many signal transduction cascades that influence fundamental cellular roles. The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway plays a major role in cell growth, proliferation, and survival (McCain, 2013). After CDC42/Rac1 activate PAK1, mitogen-activated protein kinase kinase (MKK) 4 and MKK7 are activated downstream. Sequentially, c-Jun N-terminal kinase (JNK) 1/2 is phosphorylated by MKK4 or MKK7 (Y. Wang et al., 2018). Furthermore, PAK1 has shown to activate MAPK/ERK kinase (MEK1) 1 and c-Raf, which are crucial effectors of the MAPK/ERK pathway (Ong et al., 2011).

Estrogen receptor alpha (ERα) is a nuclear transcription factor. ERα binds to associated DNA regulatory sequences to regulate specific target gene transcription (Paterni et al., 2014). The phosphorylation of ERα by PAK1 at Ser305 stimulates the transactivation of the receptor, consequently upregulating cyclin D1 levels and promoting movement through the cell cycle (Oladimeji et al., 2016).

Bcl-2 antagonist of cell death (BAD) is a member of the protein family Bcl-2 and has shown to stimulate apoptosis. BAD phosphorylation leads to protein binding with Bcl-2 and 14-3-3 proteins, resulting in inhibition of its apoptotic abilities. PAK1 had been identified as a kinase involved in directly phosphorylating BAD at Ser111 and indirectly at Ser112 via Raf-1. These phosphorylation sites have been linked to modulation of BAD binding to Bcl-2 (Ye et al., 2011).

The nuclear factor-κB (NF-κB) pathway regulates cellular behaviors such as inflammatory responses, cellular growth, and apoptosis (Dolcet et al., 2005; Barkett & Gilmore, 1999). How PAK1 specifically participates in NF-κB signaling remains unknown; however, its participation in the signaling pathway has shown to be supported by several observations. For example, Kaposi's sarcoma is a tumor of endothelial cells that is portrayed by exceptionally vascularized lesions on the mucosal tissue and skin (Gaglia, 2021). PAK1 was identified as a downstream signaling molecule in the cell transformation pathway of Kaposi's sarcoma-associated herpes virus encoding a GPCR to NF-κB. Additionally, a PAK1-dependent mechanism involving the upstream regulatory kinase NF-κB inducing kinase (NIK) activated NF-κB affected cyclin B1 expression via NF-κB's activity in gastric cancer and when Helicobacter pylori infected gastric epithelial cells (Zhou et al., 2019; Liu et al., 2009).

Validation of PAK1's role in Cancer

As noted before, the levels and activity of PAK1 contribute to the development of multiple cancers (Kichina et al., 2010). Expression of PAK1 is increased in 55% of breast cancers and amplification of PAK1 is further correlated with breast cancer invasiveness (Park et al., 2015). The cytoskeletal markers filamin A, LIM kinase and tubulin cofactor B are phosphorylated by PAK1, therefore influencing cytoskeletal remodeling in breast cancer (Kanumuri et al., 2020). The signaling pathways MAPK, MET, NF-kB, BAD, and ERα, which the PAKs participate in as regulators and effectors, have been shown to drive breast cancer tumorigenesis (Kanumuri et al., 2020). The phosphorylation of snail, p41-Arc, and C-terminal binding protein-1 (CtBP1) by PAK1 modulates the cell motility of breast cancer cells (Kanumuri et al., 2020). As shown, PAK1 contributes to the development of breast cancer through a multitude of ways.

PAK1 is associated with a variety of proteins that are implicated in the progression of lung adenocarcinoma (Jin et al., 2020; Yang et al., 2019; Lv et al., 2016). Apelin-13 and the APJ (a presumed receptor associated with AT1) receptor have been found to induce cell migration of human adenocarcinoma. The phosphorylation of PAK1 and cofilin is influenced by apelin, resulting in the promotion of lung adenocarcinoma cell migration (Lv et al., 2016).

PAK1 overexpression modulates gastric cancer progression, metastasis, and prognosis (Zhou et al., 2019; Liu et al., 2015; Liu et al., 2009). Knockdown of PAK1 expression hindered gastric cancer anchorage-dependent and anchorage-independent growth. Furthermore, PAK1 modulates cyclin B1 transcription in gastric cancer through the regulation of NF-kB (Liu et al., 2009). Understanding PAK1's role in the previous signaling pathways provides an explanation for its role in the development of breast, lung, and gastric cancers.

Development and Use of PAK1 Inhibitors

Based on the involvement of PAK1 in the progression of cancer, a considerable effort has been put into the development of novel inhibitors. These types of inhibitors include ATP-competitive and non-ATP-competitive (allosteric) inhibitors. The ATP-competitive inhibitors prevent the transfer of a phosphate group to the active site. The development of ATP-competitive inhibitors has proven to be quite challenging due to the high plasticity of the PAK1 kinase domain and the flexibility of the ATP binding cleft (Semenova & Chernoff, 2017). As noted, the

strong sequence identity of the PAKs impairs the specificity of the ATP-competitive inhibitors, leading to inhibition of all the PAKs or a group of the PAKs (Semenova & Chernoff, 2017; Rudolph et al., 2015). The non-ATP-competitive inhibitors bind to other sites on PAK1, thus exploiting the regulation of PAK1-specific mechanisms, resulting in more specificity but also decreased potency (Semenova & Chernoff, 2017). The issues with PAK1 and its inhibitors can provide insight on the biology of PAK1.

ATP-Competitive Inhibitors

The ATP-competitive group of indolocarbazoles (Figure 2) has shown promising effects in inhibiting PAK1 biological activities (C. Wang et al., 2018). The alkaloid staurosporine (Figure 2A), a kinase inhibitor, was obtained from a Streptomyces strain and demonstrates high toxicity and low selectivity (Karaman et al., 2008; Rüegg & Burgess, 1989; C. Wang et al., 2018). Staurosporine inhibits more than 70% of human kinases by interacting with conserved catalytic domain residues (Semenova & Chernoff, 2017). Staurosporine has influenced the direction of kinase inhibitor development by providing a scaffold for the analog Λ-FL172 (Figure 2B) (C. Wang et al., 2018; Semenova & Chernoff, 2017). To fill the large ATP binding site of PAK1, the inhibitor Λ-FL172 was selected due to its rigid and bulky octahedral ruthenium complex. Λ-FL172 decreases the binding of ATP at the active site by binding to the adenine pocket (Semenova & Chernoff, 2017; Rudolph et al., 2015; Blanck et al., 2012; Maksimoska et al., 2008). To determine whether metallo-organic inhibitors are suitable, a simple ruthenium complex (R)-1, based on a simple pyridylphthalimide scaffold, was developed that utilizes different coordinate ligands within the PAK1 ATP-binding site. When compared to Λ-FL172, the simple ruthenium complex (R)-1 (Figure 2C) demonstrated improved affinity and potency (Blanck et al., 2012). It can be shown that metal inhibitors involving specific coordinate ligands can potentially play a vital role in the inhibition of PAK1. The potential for further development of members of this class of inhibitors as therapeutics to treat cancer is unclear, and additional research on the toxicity and pharmacokinetics of this class is needed (Semenova & Chernoff, 2017).

The inhibitor PF-3758309 (Figure 2D), was derived from pyrrolopyrazoles and was identified through a high-throughput screen for PAK4 inhibition and has further demonstrated activity against PAK1 (Senapedis et al., 2016). PF-3758309 prevents PAK1 autophosphorylation at Ser144 (Chang et al., 2017). PF-3758309

was selected for phase I clinical trials with patients that had advanced/ metastatic solid tumors and was tolerated by those with advanced malignancies (Mileshkin et al., 2011; Ndubaku et al., 2015; Rudolph et al., 2015; Semenova & Chernoff, 2017; Senapedis et al., 2016). PF-3758309 inhibited the activity of PAK1 and the Group II PAKs, likely due to similarities in catalytic domain (Murray et al., 2010; Eswaran et al., 2007). PF-3758309, however, showed off-target activities and limited selectivity, causing it to be withdrawn from clinical trials (Semenova & Chernoff, 2017). Outside of clinical trials, PF-3758309 showed promising effects in the regulation of various cancers. For example in pancreatic cancer, PF-3758309 enhanced the effects of various chemotherapeutic reagents, which included 5-FU, gemcitabine, and abraxane (Wang et al., 2019). It is worth further investigation into the prevention of autophosphorylation at Ser144 of PAK1.

FRAX1036 (Figure 2E), FRAX486 (Figure 2F), FRAX597 (Figure 2G), and G-5555 (Figure 2H) belong to a class of small molecules based on an aminopyrimidine-scaffold that had significant activity against PAK1 (Semenova & Chernoff, 2017; Rudolph et al., 2015). The inhibitor FRAX597 was developed through a high-throughput screen partnered with a structure-activity relationship approach (Yeo et al., 2016; Licciulli et al., 2013). FRAX597 bypasses the Met344 gatekeeper residue of PAK1 by placing its thiazole ring in the back cavity of the ATP binding site (Licciulli et al., 2013). By binding at the back cavity of the ATP binding site, FRAX597 demonstrated increased selectivity and potent antitumor activity in vivo (Rudolph et al., 2015; Licciulli et al., 2013). FRAX597 shows promise as a therapeutic due to its ability to target specifically Group I PAKs and suppress cancerogenic activity (Yeo et al., 2016; Licciulli et al., 2013). FRAX597, however, has shown to inhibit off-target kinases, including RET, YES1, TEK, and CSF1R but has minimal inhibitory effects towards Group II PAKs (Licciulli et al., 2013). Similar to FRAX597 off-target activity, FRAX1036 is highly selective for PAK2 and PAK3 (Korobeynikov et al., 2019). Another inhibitor, FRAX486 targets PAK2 and modulates the scaffolding function of off-targeting upstream receptor tyrosine kinases (Siekmann et al., 2018). Although, FRAX486 suppresses GPCR-induced PAK1 autophosphorylation at Ser144, rendering it inactive (Chang et al., 2017). Progress on the inhibition of PAK1 requires investigation into bypassing the characteristic gate keeper Met344 and the inhibition of autophosphorylation at Ser144 due to potentially increasing selectivity and inactivity. The high selectivity of group I PAKs FRAX597, FRAX486,

and FRAX1036 indicate suitability for validating in vitro experiments as a tool compound due to its moderate kinase selectivity and high potency (Korobeynikov et al., 2019).

G-5555 shows high selectivity as an ATP-competitive inhibitor for PAK1. G-5555 showed an inhibition >70% for 9 out of 235 kinases, including PAK1, PAK2, and PAK3 (Semenova & Chernoff, 2017; Ndubaku et al., 2015). The G-5555 inhibitor was derived from FRAX1036 and demonstrates an increase in stability and potency (Knippler et al., 2019; Ndubaku et al., 2015). The methyl pyridine of G-5555 binds to the ATP-competitive pocket, which is bordered by a catalytic Lys299, gatekeeper Met344, and αC-helix (Ndubaku et al., 2015). G-5555 inhibited the proliferation of PAK-amplified breast cancer cell lines (Rudolph et al., 2016). In animal models of non-small lung cancer and a PAK1-amplified breast cancer, G-5555, however, demonstrated that oral delivery at effective doses resulted in cardiovascular toxicity and death. As a result, G-5555 does not appear to be suitable for clinical development (Rudolph et al., 2016). G-5555, nevertheless, can be used a validation tool for group I PAKs activity.

Two additional ATP-competitive inhibitors, developed from bis-anilino pyrimidines, include AZ13705339 (Figure 2I) and the analogue AZ13711265 (Figure 2J). The inhibitor AZ13705339 displayed good selectivity, with over 80% inhibition in 8 kinases out of a 125 panel (McCoull et al., 2016). Although AZ13705339 performed well in vitro, it did not in vivo, due to the lack of drug-like properties presumably from high lipophilicity (McCoull et al., 2016). A better choice could be the inhibitor AZ13711265, which has lower lipophilicity, resulting in better pharmacokinetic properties and low toxicity (McCoull et al., 2016).

The novel ATP-competitive inhibitor CP734 (Figure 2K) is highly selective for and inhibits the ATPase activity of PAK1 by targeting residue V342 (Wang et al., 2020). When comparing cell growth inhibition, CP734 demonstrated a stronger effect than G-5555 but a weaker effect than FRAX597. By reducing the activity of PAK1's downstream signaling pathways, such as Ras-Raf, CP734 suppresses pancreatic tumor growth (Wang et al., 2020). A limitation of CP734 is cytotoxicity toward normal human pancreas duct cell hTERT-HPNE and normal human lung bronchus epithelial cell (Wang et al., 2020). Negligible toxicity toward other main organs, however, was observed in vivo (Wang et al., 2020). CP734 exhibited strong synergistic effects with gemcitabine or 5-fluorouracil towards pancreatic cancer cells (Wang et al., 2020). CP734 is a candidate for specifically targeting PAK1 and for synergistic effects with gemcitabine or 5-fluorouracil for treating pancreatic cancer.

The analysis of ATP-competitive inhibitors indicates recurrent problems when developing a PAK1 inhibitor. Met344, for example, is a gatekeeper residue that when bypassed may increase the specificity for PAK1, as seen with G-5555 and FRAX597. If the small molecule inhibitors do not contain a moiety to traverse the residue, this can limit the binding of the inhibitor to PAK1. Other issues are associated with inhibitors FRAX486, FRAX597, FRAX1036, PF-3758309, and G-5555, which had off-target activities that inadvertently interfered with other signaling pathways. Furthermore, high toxicity, as seen with CP734, can be deleterious to healthy cells, causing an escalation of problems. CP734, however, was selective for PAK1 and can be used as a biological tool to validate the activity of PAK1.

Non-ATP-Competitive Inhibitors

The non-ATP-competitive inhibitor IPA-3 (Figure 3A) is a naphthol found through a high-throughput screen to identify inhibitors that targeted PAK1 activation (Deacon et al., 2008). IPA-3 covalently binds to the PAK1 regulatory domain, impairing the interaction with active Rac1/Cdc42 and autophosphorylation at Thr423 (Chen et al., 2019; Viaud & Peterson, 2009; Deacon et al., 2008) . In melanoma and colon carcinoma cell lines with NRAS and KRAS gene mutations, the administration of IPA-3 resulted in increased cytotoxicity to the cells (Singhal & Kandel, 2012). Moreover, the combination of IPA-3 with auranofin in adenocarcinoma and squamous cell carcinoma cells lines that carry EGFR or KRAS mutations synergistically suppressed tumor growth in vivo (Ito et al., 2019). While a potent inhibitor, IPA-3 displayed problems in in vitro and in vivo experiments due to its disulfide moiety, which is quickly reduced, thus rendering the compound inactive (Rudolph et al., 2015).

NVS-PAK1-1 (Figure 3B) is a dibenzodiazepine and was discovered as a PAK1 inhibitor through a fragment-based screen (Semenova & Chernoff, 2017). NVS-PAK1-1 displayed about 100 times more selectivity for PAK1 than PAK2 and has excellent physicochemical properties (Hawley et al., 2021; Karpov et al., 2015). NVS-PAK1-1 inhibits the autophosphorylation of PAK1 (Hawley et al., 2021). Compared to FRAX1036, NVS-PAK1-1 was observed to be more cytostatic and not cytotoxic, resulting in insignificant cell death (Hawley et al., 2021). Another limitation of NVS-PAK1-1 is its short half-life *in vivo* and in vitro (Hawley et al., 2021). Further studies are needed to determine the prolonged effects with the administration of the inhibitor NVS-PAK1-1 (Karpov et al., 2015).

Peptides that mimic the AID can inhibit PAK1. Two such inhibitors include fusions with sequences of the cell permeant TAT motif along with the PIX-interacting motif (TAT-PAK18) or the Nck binding motif of PAK1 (PAK-Nck). The TAT-PAK18 inhibitor impedes the PAK1-PIX interaction, resulting in an inhibition of ovarian cancer cell line growth associated with amplification of PAK1 (Zhang et al., 2017; Hashimoto et al., 2010). The PAK-Nck inhibitor prevented the interaction of PAK1 and Nck, which disrupted endothelial cell migration and contractility in vitro and inhibited angiogenesis in vivo (Shin et al., 2013; Kiosses et al., 2002). The delivery of peptide inhibitors to tumor cells, however, remains an obstacle for therapeutic use (Semenova & Chernoff, 2017).

The emerging allosteric inhibitor AK963/40708899 (Figure 3C) inhibits phosphorylation of PAK1 by forming a hydrogen bond with Thr406 and through six hydrophobic interactions with Arg299, Leu396, Tyr346, Ala297, Ile276, and Met244 on PAK1. The inhibition downregulates the PAK1–NF–κB–cyclinB1 pathway and arrests the cell at the G2 phase (Zhou et al., 2019). Moreover, AK963/40708899 downregulates the PAK1/LIM domain kinase 1/cofilin and PAK1/ERK/focal adhesion kinase pathways suppressing gastric cancer cell invasion and filopodia formation while simultaneously inducing cell adhesion. Compared to IPA-3, AK963/40708899 has a higher potency and in addition has the ability to be structurally modified and used as a precursor compound for drug development (Zhou et al., 2019). These findings, after additional in vivo studies, promote AK963/40708899 as a potential therapeutic for development against gastric cancer (Zhou et al., 2019).

The existing allosteric inhibitors IPA-3, NVS-PAK1-1, TAT-PAK18, and PAK-Nck have some limitations. IPA-3 is easily reduced, rendering it inactive. NVS-PAK1-1 has limited cytotoxic activity and a short half-life. As they are peptides, TAT-PAK18 and PAK-Nck are difficult to administer to tumor cells. In contrast to these existing inhibitors, the novel inhibitor AK963/40708899 did not exhibit limitations and displayed increased potency when compared to IPA-3.

Future Directions

PAK1 plays a driving role in the progression of cancer (Kanumuri et al., 2020; Prudnikova et al., 2016; Park et al., 2015; Siu et al., 2010). Critical clinical development is needed to identify a PAK1 specific inhibitor that is potent and displays minimal toxicity in vitro and in vivo. The investigation of the selectivity of such inhibitors has provided additional insights into the biology of PAK1, bringing forth new tools to understand more of its cellular functions. Based

on the research collected in the past ten years, a variety of inhibitors have been selected that fulfill the selectivity and inhibition criteria but retain challenges including cellular toxicity in vitro and/or adverse symptoms in vivo. The emerging inhibitor CP734 has contributed to the group of ATP-competitive inhibitors by being significantly selective for PAK1. ATP-competitive inhibitors, on the other hand, have shown consistent problems with the inhibition of off-target kinases, leading to toxicity. This clearly illustrates the need to focus more on non-ATP-competitive inhibitors. AK963/40708899 has demonstrated a high potency and potential as a scaffold for future non-ATP-competitive inhibitor development. The future development of non-ATP-competitive inhibitors may be focused on identification of new inhibitors, which will require novel screening approaches. Another approach may be through the modification of known scaffolds. Promising therapeutic inhibitors of PAK1 will need to be specific, demonstrate high potency, and have minimal adverse side effects.

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Figures

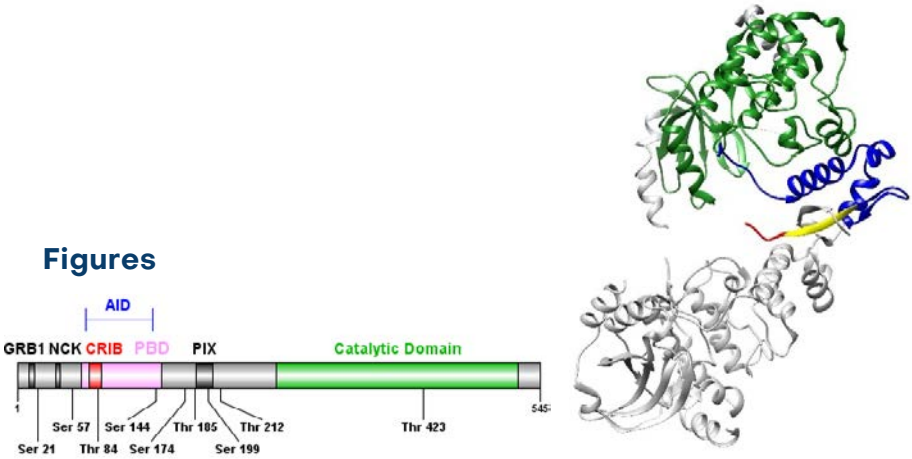

Figure 1. Domain sequence and crystal structure of PAK1

The left image is the domain sequence of PAK1, displaying the serine and threonine phosphorylation sites. The right image is the proposed 3D structure of PAK1. Blue displays the AID, yellow is the overlap of CRIB and AID, red is the CRIB, and green is the catalytic domain.

Domains and phosphorylation sites of PAK1 (Semenova & Chernoff, 2017; The UniProt, 2021). Crystal structure of PAK1 (PDB 1F3M) (Berman et al., 2000; Lei et al., 2000; Pettersen et al., 2004). Amino acids 1-77, 148-248, 417-422, and 543-545 are excluded.

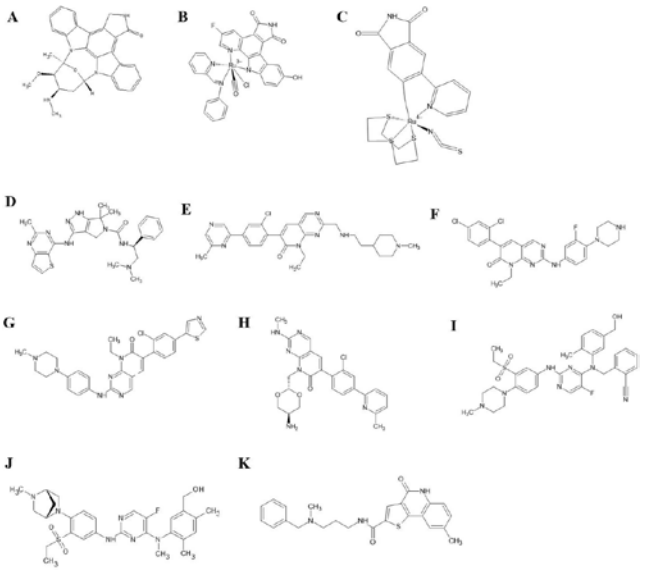

Figure 2. ATP-Competitive Inhibitors of PAK1

Competitive inhibitor identities: staurospine (**A**) (Rüegg & Burgess, 1989), Λ-FL172 (**B**) (Semenova & Chernoff, 2017), simple ruthenium complex (R)-1 (**C**) (Blanck et al., 2012), PF-3758309 (**D**) (Semenova & Chernoff, 2017), FRAX1036 (**E**) (Ndubaku et al., 2015), FRAX486 (**F**) (Dolan et al., 2013), FRAX597 (**G**) (Licciulli et al., 2013), G-5555 (**H**) (Rudolph et al., 2016), AZ13705539 (**I**) (McCoull et al., 2016), AZ13711265 (**J**) (McCoull et al., 2016), and CP734 (**K**) (Wang et al., 2020). ACD/ChemSketch was used to generate the molecular structures (Advanced Chemistry Development, 2021).

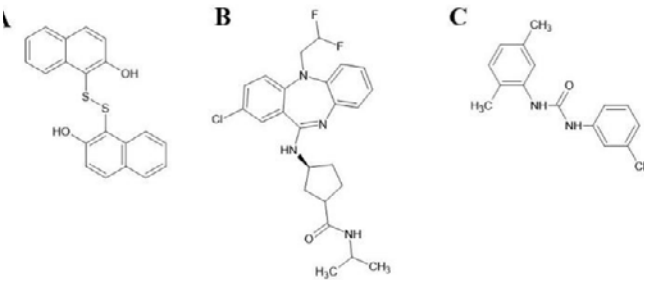

Figure 3. Non-ATP-Competitive Inhibitors of PAK1

Allosteric inhibitor identities: IPA-3 (A) (Viaud & Peterson, 2009), NVS-PAK1-1 (B) (Semenova & Chernoff, 2017), and AK963/40708899 (C) (Zhou et al., 2019). ACD/ChemSketch was used to generate molecular structures (Advanced Chemistry Development, 2021).

List of Acronyms

aa	amino acid
AID	autoinhibitory domain
Arp	actin-related protein
BAD	Bcl-2 antagonist of cell death
CRIB	Cdc42/Rac1-interactive binding
CtBP1	C-terminal binding protein-1
DLC	dynein light chain 1
ERK	extracellular signal-regulated kinase
ERα	Estrogen receptor alpha
GIT1	GPCR kinase-interacting protein 1
GPCR	G-protein-coupled receptor
GRB1	growth factor receptor-bound protein 1
ILK	integrin-linked kinase-1
JNK	c-Juna N-terminal kinase
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MKK	mitogen-activated protein kinase kinase
MORC2	microrchidia CW-type zinc finger 2
NFAT1	nuclear factor of activated T-cell
NF-κB	nuclear factor-κB
NIK	NF-κB inducing kinase
PAK	p21-activated kinase
PAK1	p21-activated kinase 1
PFK-M	phosphofructokinase-muscle isoform
PIX	PAK-interacting exchange protein
TCoB	Tubulin Cofactor V
TF	tissue factor

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