Molecular Pathways: Targeting the Kinase Effectors of RHO-Family GTPases

Tatiana Y. Prudnikova, Sonali J. Rawat, and Jonathan Chernoff

Abstract

RHO GTPases, members of the Ras superfamily of small GTPases, are adhesion and growth factor–activated molecular switches that play important roles in tumor development and progression. When activated, RHO-family GTPases such as RAC1, CDC42, and RHOA, transmit signals by recruiting a variety of effector proteins, including the protein kinases PAK, ACK, MLK, MRCK, and ROCK. Genetically induced loss of RHO function impedes transformation by a number of oncogenic stimuli, leading to an interest in developing small-molecule inhibitors that either target RHO GTPases directly, or that target their downstream protein kinase effectors. Although inhibitors of RHO GTPases and their downstream signaling kinases have not yet been widely adopted for clinical use, their potential value as anticancer targets continues to facilitate pharmaceutical research and development and is a promising therapeutic strategy.

Background

The RHO-family proteins RAC1, CDC42, and RHOA are small GTP-binding proteins that act as molecular switches, shifting between an inactive, GDP-bound form and an active, GTP-bound form that define functions of RHO GTPases. This process is regulated by guanine nucleotide-exchange factors, GTPase-activating proteins, and guanine nucleotide-dissociation inhibitors. There are many signaling pathways that lead to RHO activation, including those initiated by physical stimuli (mechanical stress or cell–cell and cell–substrate adhesion) and chemical factors (growth factors and cytokines; ref. 2). Upon activation, GTP-bound RHO GTPases interact with a wide spectrum of effectors to regulate various cellular pathways, including cytoskeletal dynamics, motility, cytokinesis, cell growth, apoptosis, and transcriptional activity. The three best studied members of the RHO family, RAC1, CDC42, and RHOA, are essential for transformation by activated Ras (3, 4), and, in the case of RAC1 and RAC2, themselves can be oncogenic drivers in human malignancies (5, 6).

As with RAS, the RHO GTPases have proven difficult to target directly with small-molecule inhibitors. There have been limited successes with molecules that disrupt the binding of guanine nucleotide exchange factors to RAC and CDC42 (7–10), as well as with molecules that disrupt GTPase membrane association (11). While efforts continue to develop direct small GTPase inhibitors, a promising and more conventional therapeutic approach has been to block the activities of RHO GTPase effectors. Among these effectors are several protein kinases that either are or might be amenable to small-molecule inhibition (Fig. 1). For example, RAC and CDC42 share two protein serine-threonine kinase effectors in common, PAK and MLK, and inhibitors for both these kinases have been developed. CDC42 also has distinct kinase effectors, such as MRCK and the tyrosine kinase ACK, and these kinases too might provide suitable drug targets in cancer. RHOA has a distinct set of effector kinases, including the ROCK, CITRON, and PRK1, all of which regulate cellular processes that contribute to tumorigenesis, invasion, and metastasis.

p21-activated kinases (PAK), the most extensively studied CDC42 and RAC effector proteins, consist of two subgroups containing three members each: group I (PAK1–3) and group II (PAK4–6). PAKs have been implicated in a number of cellular processes critical for oncogenic transformation, including cell proliferation, cell survival, adhesion and migration, and anchorage-independent growth. PAKs are overexpressed and/or hyperactivated in a variety of human malignancies including bladder, melanoma, breast, prostate, colorectal, and ovarian carcinoma. Importantly, compelling genetic and pharmacologic evidence shows that inhibiting group I PAKs can block transformation by oncogenic drivers such as ERBB2 (14), and K-RAS (15). Thus, this kinase is a well-validated anticancer target. Importantly, loss of Pak1 is well tolerated in mice (16), implying that Pak1 inhibitors might not have unacceptable toxicities.

Another common effector of CDC42 and RAC, the mixed-lineage kinases (MLK), are a family of serine-threonine kinases that translate signals from cell surface receptors to MAPKs. MLKs can function as MAP kinase kinase kinases. Because of their ability to activate multiple MAPK pathways, MLKs mediate a variety of biologic processes. For example, overexpression of MLK3 induces transformation and anchorage-independent growth of NIH-3T3 fibroblasts (17), and MLK3 is required for proliferation and survival of various cancer cell lines, including colon, ovarian (18), and breast cells (19), and for the migration/invasion of ovarian, triple-negative/basal breast (20), and gastric carcinoma cells (21).
Activated CDC42 kinase (ACK or TNK2) is a ubiquitously expressed nonreceptor tyrosine kinase that binds to and is activated by CDC42 (22). ACK1 has been reported to regulate the receptor tyrosine kinase AXL, to promote activation of AKT, androgen receptor, and negatively regulate the tumor suppressor WWOX (23). Recent findings implicate ACK1 in carcinogenesis. Amplification or activating mutations of ACK1 have been identified in prostate, lung, ovarian, and pancreatic cancers, and ACK1 expression positively correlates with increased tumor invasiveness (24). Inhibition of ACK causes cell-cycle arrest, sensitizes cells to ionizing radiation, and induces apoptosis (22). Recently, one miRNA, miR-7, was identified as a negative regulator of ACK1 expression in human schwannoma (25). Overexpression of miR-7 inhibited NF2-null schwannoma cell growth both in culture and in the xenograft tumor models in vivo, suggesting that ACK1 inhibitors could be potential therapeutic molecules for targeting malignant schwannoma (25).

Myotonic dystrophy kinase-related CDC42-binding kinase (MRCK) acts as a downstream effector of CDC42, affecting actin/myosin reorganization by phosphorylating myosin-II light chain (Mlc2) and the myosin-binding subunit (MYPT1, also known as MBS) of myosin light-chain phosphatase (MLCP; ref. 26). MRCKα was also found to phosphorylate LIM kinases 1 and 2 (LJMK1 and LJMK2), resulting in increased...
phosphorylation and inactivation of the filamentous actin severing protein coflin (27), which would contribute to actin–myosin contractility. In addition, MRCK α-induced phosphorylation of moesin in vitro (28), which links integral membrane proteins to filamentous actin, suggesting that actin–myosin contractility may be further promoted by MRCK through enhanced coupling of the cytoskeleton to the membrane. Elevated MRCK expression has been found in various cancer cells, such as lymphoma, breast cancer, lung cancer, and pancreatic adenocarcinoma (29).

RHO-associated protein kinases (ROCK I and II) are key regulators of the actin cytoskeleton downstream of the small GTPase RHO. The main function of ROCK signaling is regulation of the cytoskeleton through the phosphorylation of downstream substrates, leading to increased actin filament stabilization and generation of actin–myosin contractility (30). Upon activation, ROCK promotes actin filament stabilization through LIMK-mediated phosphorylation and also through MLC phosphorylation, leading to increased actin filament bundling and myosin-driven contraction.

ROCK signaling is required for many cytoskeleton-dependent processes, including cell adhesion, motility, phagocytosis, and cell–cell, and cell–matrix adhesion. As regulators of such cellular processes, ROCKs play critical roles in a range of human diseases, including cancer. ROCK activation is associated with cancer progression; its expression is elevated in several types of cancers, and ROCK inhibition has been shown to block ROCK tumor growth and reduced metastasis in vivo (31).

The LIMKs (LIMK1 and LIMK2) can be phosphorylated by PAKs, ROCK, and MRCK. The major LIMK substrate is the actin depolymerizing and severing protein coflin (32). LIMK-dependent phosphorylation of coflin leads to its inactivation and promotes stabilization of actin cytoskeleton (32). LIMKs also translocate from the cortical cytoplasm and focal adhesions, where they modulate cell morphology and motility, and to the centrosome and nucleus, where they regulate mitosis and cytokinesis (33). LIMKs are overexpressed and implicated in various cancers, such as breast, lung, skin, liver, and prostate, and several tumor cell lines (34, 35). Downregulation of LIMK1 activity reduces the invasiveness in several cancer cells, such as breast cancer and hepatoma cells (36).

Levels of LIMK1/2 are elevated in human vestibular schwannomas, and its inhibition arrests cells in early mitosis (37). Knockdown of either LIMK1 and LIMK2 has also been shown to decrease invasiveness, metastasis, and cell-induced angiogenesis in pancreatic cancer cells in zebrafish xenografts; double knockdown completely blocked invasion and formation of micrometastasis in vivo (38).

Clinical–Translational Advances

Despite the challenge presented by small GTPases, some small-molecule inhibitors of RHO GTPases have shown promising results in in vitro and in vivo studies. For example, NSC23766, a first-generation RAC-specific inhibitor, has been shown to inhibit RAC-CGEF, TIAM1, and oncogenic RAS-induced transformation as well as to suppress prostate cancer cell proliferation and invasion (39, 40). Recently, more potent NSC23766 derivatives have been developed for possible clinical application (41). One of these, AZA1, a specific small-molecule inhibitor of CDC42/RAC1 GTPase, was shown to suppress the growth of prostate cancer in vivo and also improve survival in mice (42). Another related CDC42 inhibitor, AZA197, reduced colon cancer growth and prolonged survival to 50% in a preclinical mouse xenograft model (43). Finally, FRAX-597, a RAC1 inhibitor, and RHOsin, a RHOA-specific inhibitor, was shown to inhibit proliferation and invasion of breast cancer cells, suggesting therapeutic potential of these inhibitors in breast cancer therapy (43, 44). Despite these promising results, no RHO GTPase inhibitors are currently in clinical use. However, due to the potential value of these inhibitors as cancer therapeutics, there remains substantial interest in the development of RHO GTPase inhibitors for use in patients with cancer.

Because downstream effectors of RHO GTPases play important roles in regulating oncogenic processes, and are generally amenable to inhibition by small molecules, the best understood effectors, PAK, LIMK, ROCK, MRCK, and ACK, have become attractive targets for anticancer drug development. For example, because mounting evidence suggests that PAKs act as oncogenes in number of human cancers, the search for specific PAK inhibitors has intensified. The best studied of these compounds, and the only compound to be used so far in a human clinical trial is the pan-PAK inhibitor PF3758309 (45). Despite its potency and promising effects in preclinical models, the phase I trial of PF3758309 was discontinued due to undesirable pharmacologic properties, primarily drug efflux. Another PAK inhibitor, the group I PAK-specific FRAX-597, was recently shown to reduce cancer cells proliferation in vitro and tumor progression in animal models. FRAX597 inhibits the proliferation of NF2-deficient schwannoma cells in culture and displayed potent antitumor activity in a K-RAS mouse model; tumor volume was reduced by 89% (15, 46). PAK inhibitors have also shown promise in a number of in vivo studies in melanoma, breast, and prostate cancer, and in NF2-deficient schwannoma, indicating the potential benefit of clinical PAK pathway inhibition (13).

Initial functional analyses of MLKs suggested their role in promoting cell death in neuronal cells, and therefore, the inhibitor of MLKs, CEP-1347, an ATP analogue that selectively inhibits the MLK family of kinases, was used in clinical trials for Parkinson disease, where it lacked efficacy in delaying disease progression (47). The fact that MLKs promote cell proliferation, survival, and migration makes them potential targets for targeted cancer therapy. Recent studies have shown that CEP-1347 induces G1/M arrest, metaphase entry block, and apoptosis in three different ER+ breast cancer cell lines, with only minor effects on nontumorigenic mammary epithelial cell lines (48). Whereas CEP-1347 displayed minimum toxicity in clinical trials, it has a potential in breast cancer therapeutics.

ACK1 activation and/or gene amplification occurs in multiple types of cancer. Recently, the ACK inhibitor AIM-100 was shown to suppress ACK1-mediated phosphorylation and to inhibit the growth of prostate cells by causing cell-cycle arrest in G0–G1 phase (49). The ability of AIM-100 to inhibit autoactivated ACK1 (E346K mutant) further shows that it is effective in repressing both oncogene-induced and ligand-modulated ACK, indicating the potential therapeutic importance of this inhibitor (23).

Two independent screens identified dasatinib as a potent inhibitor of ACK1 [Kd = 6 nmol/L; IC50 = 1 nmol/L (refs. 50, 51)]. Dasatinib was originally identified as an inhibitor of the tyrosine protein kinases SRC and ABL (52). It is commercially available as an oral multifamily tyrosine kinase inhibitor for patients with chronic myelogenous leukemia and is in clinical translation. Despite these promising results, no RHO GTPase inhibitors are currently in clinical use. However, due to the potential value of these inhibitors as cancer therapeutics, there remains substantial interest in the development of RHO GTPase inhibitors for use in patients with cancer.

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trials for other malignancies. Interestingly, ACK1 failed to rescue H292 lung cancer cells from dasatinib-induced cell death, while other dasatinib target kinases such as SRC, FYN, Lyn, and LCK were able to do so (50). These results call into question the role of ACK1 as an oncogenic driver in these lung cancer cells.

Several inhibitors of MRCK have been described, including the natural compounds chelerythrine, cycloartane-3,24,25-triol, and staurosporine. Chelerythrine, originally identified as a PKC inhibitor with in vitro IC50 of 660 nmol/L (53), was subsequently shown to inhibit MRCKα kinase activity with an in vitro IC50 of 1.77 μmol/L [54]. Other off-target effects have also been found for chelerythrine, such as reactive oxygen species generation, DNA intercalation, and inhibition of acetylcholinesterases, making this compound difficult to use for identifying MRCK functions (29). Another compound, the natural product cycloartane 3,24,25-triol, was discovered as a potential MRCKα inhibitor (55) due to its ability to compete with an immobilized ligand for binding to the ATP-binding site. Cycloartane-3,24,25-triol reduced the viability of PC-3 and DU145 cell lines with IC50 values of 2.226 ± 0.28 μmol/L and 1.67 ± 0.18 μmol/L, respectively (56). The nonselective kinase inhibitor staurosporine, the ROCK inhibitors, fasudil, H89 and IC50 of 1.77 μmol/L (53), was subsequently shown to inhibit MRCKα kinase activity with an in vitro IC50 of 1.77 μmol/L (54). Other off-target effects have also been found for chelerythrine, such as reactive oxygen species generation, DNA intercalation, and inhibition of acetylcholinesterases, making this compound difficult to use for identifying MRCK functions (29). Another compound, the natural product cycloartane 3,24,25-triol, was discovered as a potential MRCKα inhibitor (55) due to its ability to compete with an immobilized ligand for binding to the ATP-binding site. Cycloartane-3,24,25-triol reduced the viability of PC-3 and DU145 cell lines with IC50 values of 2.226 ± 0.28 μmol/L and 1.67 ± 0.18 μmol/L, respectively (56). The nonselective kinase inhibitor staurosporine, the ROCK inhibitors, fasudil, H89 and Y27632, and the pKB kinase inhibitor TCP-A1-1 have also been shown to inhibit MRCKβ kinase domain activity in vitro (57). However, at this time, there are no sufficiently selective MRCK inhibitors to use as adequate tools to study MRCK activity and functions. Despite this paucity, given the differences in structure between the catalytic domains of MRCK and its close relative ROCK, it seems likely that such selective inhibitors could be identified.

Because of its effects on cell motility, invasion, and angiogenesis (58), as well as effects on tumor stromal cells (59), ROCK has long been considered as a potential target in oncology. The ROCK inhibitors Y-27632 and fasudil have been used in several cancer studies (31). Y27632 is effective in vitro; however, the existence of multiple off-target effects makes it less than ideal for use in in vivo studies. Fasudil has been shown to inhibit tumor progression in vivo; moreover, this inhibitor has been tested extensively in humans for the treatment of cerebral vasospasm. The recently discovered ROCKI/II inhibitors, RKI-18 and RKI-1447, have been shown to prevent migration, invasion, and anchorage-independent growth of human breast cancer cells (60, 61). Furthermore, RKI-1447 also demonstrated antitumor activity in a transgenic mouse model of breast cancer, reducing mammary tumor growth by 87% (60). However, despite the potential value of ROCK inhibitors for cancer treatment, to date such compounds have not found a therapeutic niche.

Because members of the LIMK family are key regulators of the actin cytoskeleton and are involved in cell motility and invasion, LIMK is considered as a potential therapeutic target for metastatic disease. A small-molecule inhibitor of LIMK, Pyr1 (apelin-13), has been shown to stabilize microtubules, inhibit cell motility, and increase survival in a mouse model of leukemia (62). Currently, Pyr1 is in clinical trials for a noncancer indication and idiopathic pulmonary arterial hypertension.

TS6-LIMKi, a novel LIMKi inhibitor, has been shown to inhibit neurofibromin-deficient cell growth, migration, and colony formation. Furthermore, this LIMKi/2 inhibitor was found to synergize with salirasib, a RAS inhibitor, to inhibit tumor cell growth and destabilization of actin cytoskeleton; together, these findings suggest that this drug combination could be considered to treat neurofibromatosis-1 (63).

In another study, two effective LIMK inhibitors, damnacanthal and MO-26 (a pyrazolopyrimidine derivative), were identified. These compounds had previously been shown to inhibit LCK, a SRC family tyrosine kinase. However, in vitro kinase assays revealed that damnacanthal inhibited LIMKi more effectively than LCK. Damnacanthal suppressed LIMK1-induced coflin phosphorylation and inhibited migration and invasion of the MDA-MB-231 cell line. Topical application of damnacanthal also suppressed hapten-induced migration of epidermal Langerhans cells in mouse ears (64). These inhibitors provide useful tools for investigating the cellular and physiologic functions of LIMKs and have a great potential for the development of agents against LIMK-related diseases.

Summary

Given the increasing recognition of the importance of RHO GTPase-activated kinases in cancer, and the proven suitability of protein kinases as clinical drug targets, we can expect increasing efforts in development of cancer drugs that target these enzymes. As most of these kinases have multiple cellular functions, inhibitors of these enzymes may have potent anticancer effects, but may also be associated with limiting toxicities. However, the same conceptual issues are also true for other recent successful targeted agents, and thus, while caution is warranted, this should not dampen enthusiasm for the development of inhibitors of RHO-activated kinases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T.Y. Prudnikova, J. Chernoff

Development of methodology: T.Y. Prudnikova

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.Y. Prudnikova, J. Rawat, J. Chernoff

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.Y. Prudnikova, J. Chernoff

Writing, review, and/or revision of the manuscript: T.Y. Prudnikova, S.I. Chernoff

Study supervision: J. Chernoff

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References


Correction: Molecular Pathways: Targeting the Kinase Effectors of RHO-Family GTPases

In this article (Clin Cancer Res 2015;21:24–9), which was published in the January 1, 2015, issue of Clinical Cancer Research (1), a small-molecule inhibitor of LIMK, Pyr1, was confused with an unrelated small peptide termed Pyr1(Apelin-13). The LIMK1 inhibitor Pyr1 is not involved in a clinical trial. Thus, the second sentence of the third paragraph on page 27, which currently reads, "A small-molecule inhibitor of LIMK, Pyr1 (apelin-13), has been shown to stabilize microtubules, inhibit cell motility, and increase survival in a mouse model of leukemia (62)," should read as follows: "A small-molecule inhibitor of LIMK, Pyr1, has been shown to stabilize microtubules, inhibit cell motility, and increase survival in a mouse model of leukemia (62)." The sentence that follows (i.e., "Currently, Pyr1 is in clinical trials for a noncancer indication and idiopathic pulmonary arterial hypertension") should be removed.

The authors regret this error.

Reference


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