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Approaches of targeting Rho GTPases in cancer drug discovery

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Abstract

Introduction—Rho GTPases are master regulators of actomyosin structure and dynamics and play pivotal roles in a variety of cellular processes including cell morphology, gene transcription, cell cycle progression and cell adhesion. Because aberrant Rho GTPase signaling activities are widely associated with human cancer, key components of Rho GTPase signaling pathways have attracted increasing interest as potential therapeutic targets. Similar to Ras, Rho GTPases themselves were, until recently, deemed "undruggable" because of structure-function considerations. Several approaches to interfere with Rho GTPase signaling have been explored and show promise as new ways for tackling cancer cells.

Areas covered—This review focuses on the recent progress in targeting the signaling activities of three prototypical Rho GTPases, i.e. RhoA, Rac1, and Cdc42. The authors describe the involvement of these Rho GTPases, their key regulators and effectors in cancer. Furthermore, the authors discuss the current approaches for rationally targeting aberrant Rho GTPases along their signaling cascades, upstream and downstream of Rho GTPases and posttranslational modifications at a molecular level.

Expert opinion—To date, while no clinically effective drugs targeting Rho GTPase signaling for cancer treatment are available, tool compounds and lead drugs that pharmacologically inhibit Rho GTPase pathways have shown promise. Small molecule inhibitors targeting Rho GTPase signaling may add new treatment options for future precision cancer therapy, particularly in combination with other anti-cancer agents.

Keywords

Cancer; Cdc42; Rac1; RhoA; RhoGEF; Rho GTPases; Small molecule inhibitor; Targeted therapy

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1. Introduction

Rho GTPases are members of the Ras GTPase superfamily [1]. Since the first discovery in 1980s as Ras homologous small GTPases, over twenty mammalian Rho family members have been identified that can be divided into several subgroups [2]. Among Rho GTPases, RhoA, Rac1, and Cdc42 are the best characterized members, and have well-characterized roles in regulating actin cytoskeleton organization and dynamics [3]. RhoA, Rac1 and Cdc42 were first described to promote the formation of stress fibers, lamellipodia and filopodia in fibroblast respectively [4], and were subsequently appreciated for their roles in regulating signaling pathways affecting cell polarity, gene expression, cell-cycle progression, and cell survival [5].

Like Ras, classical Rho GTPases such as RhoA, Rac1, and Cdc42 are molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form. They bind to GTP or GDP and can hydrolyze bound GTP. Three main families of regulatory proteins tightly control Rho GTPase activities: guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine-nucleotide dissociation inhibitors (GDIs) (**Figure 1**). GEFs catalyze the exchange of GDP for GTP to activate Rho GTPases [6], while GAPs accelerate the intrinsic GTPase activity of Rho GTPases to inactivate them [7]. After translation, the Rho GTPases are geranylgeranylated, or less commonly farnesylated, in a C-terminal CAAX motif to promote their translocation to intracellular membranes where they are activated [8]. Rho GDIs bind to Rho GTPases in their inactive GDP bound form and sequesters them in the cytosol, thus acting as the inhibitor of Rho GTPases [9].

The actions of Rho GTPases are mediated by effector proteins [10]. When bound to GTP, Rho GTPases undergo conformational changes and associate with a large number of potential effectors, including enzymes and scaffolding proteins, to mediate diverse yet specific cell behaviors. For example, during 2-dimensional cell migration, Rac1 and Cdc42 promote the actin-driven protrusions at the cell leading edge, whereas RhoA controls the actomyosin contraction at the cell body and rear. In 3-dimensional conditions, cells adapt different modes of migration dependent on spatial and temporal activation of Rho GTPases in response to the environmental cues [11,12]. RhoA mediates stress fiber formation mainly via Rho-kinase (ROCK) and mammalian diaphanous (mDia), while Rac1 and Cdc42 regulate actin re-organization by signaling via p21-activated kinases (Pak) or IQ-domain GTPase-activating protein (IQGAP). In addition, Cdc42 further signals via the Wiskott-Aldrich syndrome proteins (WASP) and Rac1 via the WASP-related WAVE to initiate actin rearrangement. In addition to actin-dependent activities, Rac and Cdc42 also activate the JNK and p38 MAP kinase cascade [13,14] that may affect tumor cell proliferation and metastasis.

In this review, we focus largely on the prototypical Rho GTPases, RhoA, Rac1, and Cdc42, and discuss their involvement in cancer and the ongoing strategies targeting their signaling pathways for future cancer therapy.

2. Rho GTPase signaling pathways and cancer

Rho GTPases, as well their regulators and effectors, have been implicated in multiple aspects of cancer [15-17] (**Table 1**). Unlike Ras, which is mutated in approximately 20-30% of human cancers, mutations in Rho GTPases are much less frequent and have just been appreciated. While emerging data suggest that mutations in various Rho proteins may occur in multiple cancer types, the main body of experimental evidence has focused on the regulatory mechanisms by which Rho GTPase activities are controlled. These mechanisms include altered expression levels of Rho GTPases or their regulators and altered localization patterns.

2.1 Rho GTPases in cancer

In addition to the notable case of RhoH [18], activating mutations in RhoA and Rac1 have been reported in several cancer types (**Table 1**). According to Catalogue of Somatic Mutations in Cancer (COSMIC) [19], somatic mutations of RhoA, Rac1, and Cdc42 are present in a variety of tumor tissues, most at low frequency (0.5% and below). Rac1 mutations are enriched in melanoma (5-6%) and RhoA mutations in both hematopoietic/lymphoid (4.6%) and stomach (7%) tissues. Cdc42 mutations, however, show no obvious tissue enrichment. The hotspot mutations of RhoA (G17V) and Rac1 (P29S) account for 46% and 68% of all mutations identified, respectively. However, the biological and clinical significance of each hotspot mutation remains unclear. A recent study suggests that Rac1 P29S melanoma mutation may confer resistance to RAF inhibitors [20]. Although somatic mutations of Cdc42 are detected in many tumor tissues, these mutations are more sporadic. In a study to identify driver mutations for melanoma, G12V mutation of Cdc42, similar to an oncogenic Ras mutation in various cancers, was identified in one sample [21]. Whether such Cdc42 mutation can drive cancer needs to be verified.

There is ample experimental evidence that over-expression of Rho GTPases contributes directly to the proliferative and metastatic properties of cancer cells [22,23]. Most of these experiments involve manipulating the expression or activity of Rho GTPases in cancer cell lines by over-expressing constitutive active form or dominant negative form of Rho GTPases, their regulators or effectors, or using RNAi or small molecule inhibitors. However, those experiments are limited by approaches regarding specificity, dosage, and cell clonal variation. More recent genetic mouse models targeting individual Rho GTPases have provided convincing evidence for their physiological roles. Direct targeting of RhoA, Rac1, or Cdc42 is embryonic lethal, so a variety of tissue-specific conditional knockout mouse models have been generated (reviewed in [24-26]).

RhoA is highly over-expressed in a variety of cancer types (**Table 1**). In the case of breast cancer, CD44 interacts with a RhoA-specific GEF to induce RhoA and ROCK activation, which promotes cell growth and metastasis [27]. Inhibition of RhoA signaling by over-expression of a dominant negative form of ROCK blocks the metastatic activity by CD44. RhoA is also important for the metastasis of hepatocellular carcinoma cells. Inhibition of ROCK by expressing dominant negative ROCK or using small molecule inhibitor Y-27632 reduces the intrahepatic metastasis of those cells in SCID mice [28]. Similarly, knockdown of RhoA or RhoC in aggressive non-inflammatory or inflammatory breast cancer cells reduces

invasion, motility and growth rate [29]. In colonic adenocarcinoma cells, leptin-induced invasion is potentiated by over-expression of the active form of RhoA and abrogated by the dominant negative form of RhoA or Rac1 [30]. In lung carcinoma A549 cells, ROCK inhibitor Y-27632 enhances the cisplatin-induced cytotoxicity through suppression of focal adhesion kinase (FAK)-independent mechanism [31]. These observations suggest that inhibition of RhoA signaling enhances the effect of anti-cancer agents. The role of RhoA in cancer is beginning to be revealed by genetic studies. RhoC deletion of RhoC shows no effect on tumor growth but decreases tumor metastasis in a breast cancer model [32]. Gene targeting of RhoA does not appear to affect K-Ras driven lung adenoma formation; rather, a combined RhoA and RhoC gene deletion is required to suppress lung cancer initiation [33].

Increased expression of Rac1 is reported for a variety of cancers (**Table 1**). Rac1 signaling is important for leptin-induced invasion in colonic adenocarcinoma cells [30]. Rac1 and Cdc42 are necessary for autotaxin-induced tumor motility in A2058 melanoma cells by a mechanism that may involve PAK and FAK [34]. In line with the observations obtained using dominant negative form of Rac1, RNA interference-mediated depletion of Rac1 strongly inhibits lamellipodia formation, cell migration and invasion of SNB19 glioblastoma cells and BT549 breast carcinoma cells [35]. Similarly, in human PC-3 prostate cancer cells, shRNA knockdown of Rac1 inhibits tumor cell diapedesis [36]. Inhibition of Rac1 by dominant-negative mutant inhibits aberrant cell proliferation of NF2-deficient cells [37]. Rac1 conditional knockout mouse studies have shown that Rac1 is required for K-Ras driven tumorigenesis in lung and skin cancer [38,39]. Such a Rac1 requirement may be cancer-type dependent as endothelial-specific Rac1 deletion shows no effect on tumor growth or angiogenesis [40].

Over-expression of Cdc42 has also been detected in some cancer types (**Table 1**). Rat mammary adenocarcinoma cells MTLn3 with over-expression of dominant negative form of RhoA or Cdc42 shown reduces number of focal contacts, inhibits colony formation in soft agar and affects cell growth *in vivo* [41]. In anaplastic large cell lymphoma (ALCL) driven by oncogenic fusion proteins, knockdown of Cdc42 by shRNA results in a cell cycle arrest and apoptosis of ALCL cells. In addition, Cdc42 is necessary for the growth and maintenance of established lymphomas *in vivo* [42]. Genetic deletion of Cdc42 in Ras-transformed cells inhibits proliferation, cell cycle progression and tumorigenicity [43]. Loss of Cdc42 also attenuats the tumorigenicity of mutant intestinal tumor cells [44]. Notably, the role of Cdc42 in cancer may be double-sided, as Cdc42 knockout in hematopoietic stem cells results in myeloproliferative disorder [45].

2.2 Rho regulators in cancer

The fact that Rho GTPases are often over-expressed or hyper-activated but less frequently mutated in many cancers suggests that regulatory proteins likely play a crucial role in dysregulating signaling pathways that promote cancer initiation and progression. Up-regulation of RhoGEFs or inhibition of RhoGAP and RhoGDI can lead to increased activation of Rho GTPases and promote aberrant signaling cascades. Mutations or aberrant expression of RhoGEFs, RhoGAPs, and RhoGDIs have all been detected in human

cancer [16,17]. Because RhoGEFs are positive regulators, they represent good candidates for aberrant Rho GTPases activation in cancer.

There are two classes of RhoGEFs: the classical Dbl family RhoGEFs and the DOCK family RhoGEFs. The Dbl family of RhoGEFs includes over 70 members, many of which are conserved from yeast to human [46]. Dbl RhoGEFs share conserved tandem Dbl homology (DH)-pleckstrin homology (PH) domains for GEF activity and diverge significantly elsewhere [6]. The DOCK family of RhoGEFs includes 11 members and acts as GEFs for Rac and/or Cdc42, but not RhoA, and is structurally and mechanistically distinct from Dbl RhoGEFs [47]. Several RhoGEFs are mutated or up-regulated in human cancer. Among them, Dbl, Vav1/2/3, Ect2, Tiam1/2, P-Rex1/2 are best validated and extensively reviewed [46,48]. Compared to up-regulation of GEF activity in human cancer, gain-of-function mutations in RhoGEFs are less common. One example of activating mutation is the leukemia-associated RhoGEF (LARG), which was isolated as a fusion partner to the mixed lineage leukemia (MLL) gene in a patient with acute myeloid leukemia (AML) [49]. Later, it was shown to specifically activate RhoA *in vivo*, suggesting a role of LARG/RhoA signaling axis in leukemia [50]. Mutations in LARG are identified at low frequency in a variety of tumor tissues (COSMIC). LARG belongs to the subfamily of the regulator of G-protein signaling (RGS) domain-containing RhoGEFs and couples the signaling from G-protein coupled receptors through $G\alpha_{12/13}$ to RhoA signaling. In childhood AML driven by the fusion gene AML1-ETO (AE), LARG is identified as a direct target for AE. As a transcription factor, AE significantly increases the expression level of LARG, resulting in hyper-activation of RhoA. Remarkably, LARG-specific shRNA targeting in human AE cells leads to increased apoptosis and inhibits the growth of AE+ leukemia without affecting control human blood progenitor cells or MLL-AF9 expressing leukemia cells. These findings, together with the ubiquitous expression of LARG, make this RhoGEF an exciting target for pharmaceutical intervention.

The negative Rho GTPase regulators, RhoGAPs and RhoGDIs, are relatively less well understood in the context of cancer. RhoGAPs constitute a large family with more than 70 members in eukaryotes [7]. All RhoGAPs contain a conserved ~150-amino-acid RhoGAP catalytic domain but share little other sequence homology. In both RhoGEFs and RhoGAPs, the diversity outside the catalytic domain allow them to be controlled in a spatial and temporal manner and serve as critical nodes to specifically mediate different signaling events. RhoGDIs, including RhoGDI1, 2 and 3, contain two domains: the C-terminal domain that contains the geranylgeranyl-binding pockets and binds the Rho GTPases, and the N-terminal regulatory domain that locks-in the Rho GTPases and prevents their nucleotide exchange and hydrolysis. They associate with Rho GTPases in their GDP-bound form and maintain a stable, soluble pool of inactivated Rho GTPases in the cytosol [8].

Mutations or dysregulation of RhoGAPs or RhoGDIs has been detected in human cancer [16,17], although less evident than that for RhoGEFs. One of the best characterized RhoGAPs, Deleted in liver cancer-1 (DLC-1), was originally discovered as a gene deleted in liver cancer [51]. Subsequently, it has been found to lose expression in many human cancers (**Table 1**). Reintroduce of DLC-1 into liver, lung or breast cancer cell lines results in reduced growth *in vitro* and *in vivo* [52-54]. These observations suggest an important role of DLC-1 in

cancer as a tumor suppressor [55]. Another RhoGAP implicated in cancer is p190RhoGAP. p190-A is encoded on chromosome 19q13.3 that is often deleted in oligodendrogliomas [56]. There is evidence that p190-A can act to inhibit PDGF-induced gliomas in mice and serves as tumor suppressor [57]. Whereas P190-B, located on chromosome 14q12, is likely a target for amplification in hepatocellular carcinoma [58]. RhoGDIs have been associated with many cancers [9,16] (**Table 1**), but the specificity of each RhoGDI for each Rho GTPase is less understood. Since there are only three RhoGDIs that may act on more than twenty Rho GTPases, each RhoGDI may interact with multiple Rho GTPases and thus be subject to complex regulation. The involvement of RhoGDIs in cancer is likely complicated [9]. RhoGDI expression patterns in a single type of cancer, expression of a given RhoGDI among cancer types, and their activities toward particular Rho GTPases, vary significantly. For example, RhoGDI1 expression is upregulated in colorectal and ovarian cancer, but reduced in brain cancers. In breast cancers, both RhoGDI1 and GDI2 expression levels are reported to be increased or decreased in different studies.

2.3 Downstream Rho effectors and signaling in cancer

Rho GTPases utilize a wide variety of downstream effector proteins to regulate diverse cellular functions in response to various intracellular and extracellular stimuli [10]. There is a long-standing interest in understanding how each Rho GTPase, when hyper-activated by mutations or aberrant expression of upstream regulators, contributes to tumorigenesis through a unique set of effectors and signaling pathways.

Two of the best-characterized effector proteins downstream of RhoA are the Rho-associated protein kinases, ROCK1 and ROCK2 (also known as Rho kinases). They are serine/threonine kinases that phosphorylate multiple targets including myosin light chain (MLC), the myosin binding subunit (MYPT1) of the MLC phosphatase, and LIM kinases 1 and 2 (LIMK1 and LMK2). Activated ROCKs lead to increased myosin-driven contraction through MLC phosphorylation and promote actin filament stabilization through LIMK-mediated phosphorylation and inactivation of cofilin. ROCKs regulate cancer cell invasion and metastasis and have been studied extensively [59]. Recent studies have revealed additional functions of ROCK to their classic roles in cell motility, which include gene transcription, proliferation, differentiation, apoptosis and oncogenic transformation [60]. Notably, somatic ROCK1 activating mutations have been identified in human cancers [61], and large-scale sequencing has revealed mutations in both ROCK1 and ROCK2 that are associated with human cancers [62,63].

Another well known effector protein serine/threonine kinase family implicated in cancer is the P21-associated kinase (Pak) family effectors of active Rac1 and Cdc42 [64-66]. There are six mammalian members that can be divided into two subgroups based on their homology: group I (PAK1-3) and group II (PAK4-6). In mice, PAK1 is highly expressed in brain, muscle, and spleen, PAK2 and PAK4 is ubiquitously expressed, whereas PAK3, PAK5, and PAK6 are enriched in neuronal tissues. Genetic deletion of each Pak showed that they have both overlapping and distinct functions. Group I Paks are autoinhibited homodimers when inactive and binding of Rac/Cdc42 results in the dissociation of the dimer and subsequent activation, but the activation mechanism of group II Paks is less clear. In addition to

dysregulated Rac/Cdc42 activities, over-expression or mutational activation of Pak isoforms contributes to the increased Pak activity found in various human cancers. Amplification of *PAK1* on chromosome 11q13 has been reported in breast [67], ovarian cancer [68], and melanoma [69]. Similarly, amplification of *PAK4* on chromosome 19q13 is commonly observed in pancreatic cancer [70,71] and oral squamous-cell carcinoma [72]. Recently, activating mutations in the *PAK4* and *PAK5* gene are associated with colon and lung cancers [73,74].

Activated Paks drive several oncogenic signaling pathways to impact tumor cell motility, survival and proliferation [66]. As the major effectors of Rac1 and Cdc42, Paks promote cell motility via several mechanisms. PAK1 facilitates actin stabilization through phosphorylation of MLC, LIMK, filamin A and dynein light chain 1 (DLC1) [75]. The PAK1/LIMK pathway is required for Rac1-induced actin reorganization at the cell leading edge during migration [76]. PAK1 also functions to induce rapid turnover of focal contacts at the cell leading edge via phosphorylation of paxillin [77]. Expression of dominant negative PAK1 in invasive breast carcinoma cells reduces invasion and metastasis [78]. Group II Paks seem to utilize different mechanisms to participate in cytoskeleton reorganization. Cdc42 recruits PAK4 to the Golgi and induces the formation of filopodia. Activated PAK4 leads to dissolution of stress fibers and loss of focal adhesions [79]. In addition to their role in tumor invasion and metastasis, most Paks promote cell cycle progression when over-expressed. Paks activate the Erk, PI3K/Akt, and Wnt signaling pathways that are tightly associated with cell proliferation. In the Erk pathway, PAK1 phosphorylates both MEK1 and Raf1 for efficient Erk activation. It has been shown that PAK1 drives anchorage-independent growth in human mammary epithelial cells through MAPK and MET signaling [80]. PAK1 and PAK4 also induce proliferation independent of RAF/MEK/ERK or PI3K/Akt pathways in mutant K-RAS or BRAF colon cancer cells by an unknown mechanism [81]. In the Wnt pathway, PAK1 and PAK4 directly interact and phosphorylate β-catenin, a key component of Wnt signaling [82,83]. Paks are also linked with the NF-κB signaling pathway, although a direct target in this pathway has yet to be identified. Other targets of Paks include nuclear hormone receptors such as estrogen receptor (ER) [84], androgen receptor (AR) [85], apoptosis signaling molecules such as BAD [86], and the E-cadherin repressor Snail [87].

There are many other Rho effectors in addition to ROCKs and Paks. Rac1 regulates components of the MAPK pathways, especially JNK and p38. Rac1 and Cdc42 both regulate cell polarity via PAR6. Rac1 also constitutes part of the phagocyte NADPH oxidase complex NOX2 that generates reactive oxygen species (ROS). This enzyme complex consists of at least six components: two membrane-bound subunits $p22^{phox}$ and $gp91^{phox}$, and four cytosolic regulatory subunits Rac1/2, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. Activated Rac1/2 binds to $p67^{phox}$, leading to the translocation of the regulatory complex from cytosol to plasma membrane for full assembly and activation of the NOX2 complex. In addition as a host defense, ROS also activates various transcription factors such as NF-κB, AP-1, HIF-1α and STAT3 and plays critical roles in many signaling pathways including cell-cycle progression, apoptosis, and inflammation.

3. Approaches to target Rho GTPase signaling

Key components in the Rho GTPase pathways are attractive targets for therapeutic interventions in cancer. Rho GTPases themselves are difficult to target by small-molecule modulations. Given the micromolar GTP concentration in cells and the sub-nanomolar binding affinity of Rho GTPases for GTP or GDP, it is difficult to drug Rho GTPases by nucleotide analogs like that of protein kinases. Besides the nucleotide-binding pocket, there are few stable, tractable cavities present on Rho GTPases. Thus, the family of small GTPases, including Ras and Rho, are generally deemed "undruggable" in cancer research. A variety of bacterial toxins can modify the activity of Rho GTPases [88]. For example, the exoenzyme C3 transferase, an ADP-ribosyltransferase from *Clostridium botulinum*, inactivates RhoA/RhoB/RhoC, while *Clostridium difficile* toxin A and B glucosylate and inactivate multiple Rho GTPase subfamilies. These bacterial toxins have been widely used to dissect the biological functions of Rho GTPases. However, they are large enzymes that introduce covalent modifications to the substrates and are non-specific, therefore cannot be used clinically. Based on the biochemical mechanisms of Rho GTPase regulation and function, significant effort has been dedicated to developing small molecule inhibitors that act on various aspects of Rho GTPase signaling mechanisms (**Figure 2**). In this section, we discuss these strategies and representative inhibitors (**Table 2**).

3.1 Inhibition of Rho GTPase activation by RhoGEFs or prevention of Rho-GTP formation

To activate Rho, Rac or Cdc42, RhoGEFs catalyze the GDP/GTP nucleotide exchange on the Rho GTPases. As Rho GTPases are activated by different RhoGEFs that response to different signals in a temporally and spatially manner, targeting specific RhoGEFs provide better selectivity compared to targeting Rho GTPases directly. To date, however, only limited success in targeting RhoGEF activity directly has been achieved. The diversity of the sequences and domain structures beyond the shared DH-PH domains indicates the existence of diverse mechanisms for the regulation and functions of RhoGEFs.

Attempts have been made to block the formation of Rho-GTP by interrupting the interactions between RhoGEFs and Rho. There are several small molecule inhibitors developed to bind Rho GTPases at the GEF-binding surface and inhibit RhoGEF function. For example, Rac1 inhibitor NSC23766 was discovered through a structure-based virtual screening of compounds to fit into a surface groove of Rac1 that interacts with GEFs [89] (**Figure 3A**). NSC23766 specifically inhibits Rac1 activation by the Rac-specific GEFs Trio or Tiam1 in a dose-dependent manner, but not Cdc42 or RhoA activation by their GEFs. Another small molecule inhibitor, CASIN, specifically inhibits Cdc42 by a similar mechanism. CASIN inhibits Cdc42 activation by its GEF intersectin in a specific, reversible and dose-dependent manner without affecting other Rho GTPases [90]. Since these Rho inhibitors target the surface of Rho GTPases that is required for activation by various GEFs, they are unlikely to be GEF-specific.

Another way to target Rho GTPases activity is through targeting specific RhoGEFs and preventing their binding to Rho GTPases. Conceivably, this strategy could lead to better selectivity conferred by individual GEFs. One example is the development of inhibitors for the RhoA-specific GEF, LARG. With different screening methods, two groups

independently identified several structurally distinctive inhibitors for LARG. Using a fluorescence polarization guanine nucleotide-binding assay, five compounds were identified to selectively inhibit LARG-stimulated RhoA-GTP binding [91]. Subsequently, Y16 was identified to bind to the hinge region of LARG DH/PH domain through virtual screening coupled with high throughput screen [92] (**Figure 3B**). Y16 specifically inhibits LARG and other RGS-containing RhoGEFs by binding to RhoA without detectable effect on other Dbl family of RhoGEFs, Rho effectors, or RhoGAPs. Y16 selectively inhibits serum-induced RhoA signaling and mammary sphere formation in MCF7 breast cancer cells. Since this discovery, the inhibitors of the RhoGEF-Rho interaction are proven as useful research tools to provide experimental evidence that targeting RhoGEF to suppress Rho GTPase activation is feasible. Whether this approach will result in lead compounds with suitable potency and selectivity for clinical trials remains to be seen.

In addition to the discussed competitive inhibitors, non-competitive inhibitors have been developed to inhibit Rho signaling by promoting the loss of bound nucleotide and inhibiting nucleotide re-association. These include EHT 1864 for Rac subfamily [93] and ML141 (CID2950007) for Cdc42 [94]. Those compounds may function as conformation-dependent allosteric inhibitors that bind to guanine nucleotide-bound Rho GTPase, thus inducing dissociation of the bound nucleotide and locking the respective GTPase in an inactive conformation. ETH 1864 does not affect the interaction between Rac1 and its GEF Tiam1 *in vitro*, but inhibits nucleotide exchange induced by Tiam1. Similarly, ML141 acts independently of effects on GEF activity or interaction with Cdc42.

3.2 Stimulation of GTP-hydrolysis activity of Rho GTPases by RhoGAP

RhoGAPs stimulate the intrinsic GTPase activity of RhoA, Rac and Cdc42 by up to 10^5-fold. Our current knowledge suggests that involvement of RhoGAPs in cancer is often associated with loss-of-function mutations, and they often exhibit properties consistent with tumor suppressors. RhoGAPs are less attractive to researchers as potential targets for cancer therapy since it is difficult to develop small molecule agonists than antagonists. Further understanding of the mechanisms by which RhoGAPs are regulated in specific cellular contexts may reveal more possible approaches to target this important family of Rho GTPases regulators. Like RhoGEFs, RhoGAPs exist in large numbers and show tremendous diversity in their sequences beyond the conserved GAP catalytic domain. RhoGAPs typically contain multiple additional protein-protein and protein-lipid interacting domains, as well as many phosphorylation sites. Coordinately, RhoGEFs and RhoGAPs control Rho GTPase on/off status in a highly regulated temporal and spatial manner. It remains a challenge in the Rho GTPase field to uncover the signaling pathways by which RhoGAPs regulate specific aspects of Rho GTPase function. In particular, better understanding of which and how RhoGAPs are involved in cancer would promote the development of small molecule modulators of RhoGAPs.

There is limited but promising evidence that small molecules can be used to enhance the ability of RhoGAPs to suppress Rho GTPases. One class of RhoGAPs, the Rac-specific Chimaerins (CHN), has a C1-zinc finger domain that binds the lipid second messenger diacyglycerol, a cofactor for its activity [95]. Chimaerins are implicated in development,

axon guidance, metabolism, cell migration, and T-cell activation, and deregulation of *CHN* genes is associated with cancer and many other diseases [96]. It is speculated that small molecules that bind C1 domains may activate the GAP activities which in turn down-regulate Rac signaling. Although there are other proteins with C1 domains [97] and off-target effect could be a potential problem, C1 binding molecules may still have some degree of selectivity for a subset of C1-containing proteins and would be a therapeutic option for Rac hyperactivation dependent cancers.

3.3 Inhibition of Rho-effector interaction or effector activity

Approaches to target upstream Rho signaling by either blocking Rho activation through RhoGEFs or stimulating Rho intrinsic GTPase activity through RhoGAPs, are still in the early stage of development with relatively limited success. The task to modulate RhoGEFs or GAPs is a particular challenge in part due to the diversity and complexity of those upstream regulators signaling and regulation, which are still largely unclear. Another approach to block Rho signaling is by interfering the activation of effectors by Rho GTPases or inhibiting specific effector activities directly. Similar to targeting upstream signaling of Rho, higher selectivity may be achieved targeting Rho effectors. In contrast to upstream signaling pathways, downstream effector signaling pathways are much better understood. As a result, development of small molecules inhibitors against effector signaling has been more successful.

Some downstream effectors are classical kinases and, therefore, are more readily targeted by traditional pharmacologic approaches. In fact, inhibitors of ROCKs, the major effector family downstream of RhoA, are most promising among all therapeutic interventions of Rho signaling for cancer therapy. ROCK inhibitors are reviewed extensively elsewhere [98,99] and will only be briefly discussed here. One widely used ROCK inhibitor, Y-27632, is a pyridine-analog that competes with ATP for binding to ROCKs [100] (**Figure 4A**). Y-27632 treatment decreases invasion and alters cell survival of melanoma cells *in vitro* resulting in a reduction in melanoma tumor volume *in vivo* [101]. Another extensively studied inhibitor of ROCKs is Fasudil (also known as HA-1077), which is also ATP-competitive and inhibits a wide spectrum of kinases [102] (**Figure 4A**). Fasudil is the only clinically approved ROCK inhibitor, initially approved for the treatment of cerebral vasospasms and pulmonary hypertension. Various animal models of cancer have revealed that Fasudil can inhibit tumor invasion and metastasis [103]. Recently developed analogs of Fasudil and Y-27632, with improved selectivity and potency, such as WF-536 (Y-27632 derivative) [104], H1152 (Fasudil derivative) [105], and RKI-1447 [106], have been shown to reduce tumor progression. Despite the great interest in ROCK for cancer therapy, no ROCK inhibitor has been approved for cancer treatment. Newer generations of ROCK inhibitors have proven more potent and selective and several are currently under clinical trials for glaucoma and ocular hypertension [107-109] and erectile dysfunction [110]. The multi-AGC kinase inhibitor AT13148, which also inhibits ROCK, shows potent antitumor and anti-metastatic activities [111,112] and is in phase I clinical trial for advanced solid tumors.

Another family of effectors that are attractive targets for drug discovery is the Pak family, downstream of both Rac and Cdc42. Several ATP-competitive inhibitors have been

identified for Paks. Earlier generations of Pak inhibitors include K252 [113], and K252 derivative KT-D606 and CEP-1347 [114]. Although they demonstrate potent Pak inhibition, these inhibitors are less selective and have limited utility. Later, more selective ATP-competitive Pak inhibitors were developed, such as OSU-03012 [115], Λ-FL172 [116], PF-3758309 [117], LCH-7749944 [118], and FRAX597 [119]. Of them, Λ-FL172 is of particular interest (**Figure 4B**). Λ-FL172 and related molecules were generated by modifying other compounds with bulky and rigid octahedral ruthenium scaffolds to selectively target the large ATP-binding site of PAK1. Among 264 kinases tested, only fifteen showed an inhibition similar to that of PAK1 [116]. However, such compounds usually have poor solubility and relatively high toxicity, and their potential as clinical drugs remains to be determined. FRAX597 was a potent inhibitor of group I Paks identified from high-throughput screening and shows beneficial effects on inhibiting cancer in animal models (**Figure 4B**). Non-ATP-competitive inhibitors have also been described. For example, IPA-3 binds to the regulatory domain of group I Paks, locking the kinases in their auto-inhibited conformation [120]. Thereby, IPA-3 is exceptionally selective. A few studies have shown that IPA-3 induces apoptosis in a number of cancer cell lines [121] and decreases cell spreading and adhesion in Schwannoma cell lines [122]. However, IPA-3 is unstable under physiological condition and unsuitable for further clinical development.

In addition to directly targeting effector activities, blocking Rho GTPase-effector interactions prevents effector activation and allows for greater selectivity through targeting unique Rho GTPase interactive sites. One example is the development of small molecule inhibitor phox-I1 for $p67^{phox}$, a subunit of the NOX2 superoxide producing enzyme that is required for Rac1-GTP binding [123] (**Figure 4C**). Phox-I1 and derivatives recognize a surface pocket on $p67^{phox}$, disrupt Rac1 binding and effectively inhibit NOX2-mediated superoxide production dose-dependently in human and mouse neutrophils. Although phox-I1 might not have sufficient efficacy to be used clinically, this study provides a proof of principle that inhibition of downstream effector activation by a specific pathway via rational targeting of a Rho GTPase-effector interface is a viable approach for drug development.

3.4 Inhibition of Rho posttranslational modification

To be effective as on/off switches, Rho GTPases require proper subcellular localization to function at defined intracellular sites. The recruitment of Rho GTPase to cell membranes is mediated by post-translational modification processes as well as signaling events that release Rho-GDP from bound RhoGDI. The first and crucial step is the progressive post-translational modification of a Rho GTPase at the C-terminal “CAAX box”. A geranylgeranyl, or less frequently, a farnesyl lipid group is attached to the cysteine residue of the CAAX sequence in the cytoplasm, catalyzed by geranylgeranyl or farnesyl transferases. This allows the Rho GTPase to translocate to the ER, where the –AAX tail is cleaved off and the newly exposed α-carboxyl group of the C-terminal cysteine residue is methylesterified. Incorporation of geranylgeranyl or farnesyl group is essential for the proper localization and biological activity for most small GTPases. In contrast, the latter methylesterification is only essential for farnesylated Ras, but not geranylgenranylated Rho [124]. However, this methylesterification step may have additional functions for Rho GTPases as

isoprenylcysteine carboxyl methyltransferase (ICMT) knockout resulted in greatly reduced level of RhoA [125].

Inhibitors of prenyltransferases have long been proposed as potential therapeutic drugs. There are three related enzymes of prenyltransferases: farnesyltransferase (Ftase), geranylgeranyl transferase I (GGTase I) and GGTase II. Ftase and GGTase I recognize the CAAX motif and catalyze the lipidation of their protein substrates. GGTase II is specific for Rabs and its working mechanism is different from that of Ftase and GGTase I. Since Ras is well established as the most frequently mutated oncogene in human cancer and both normal and oncogenic Ras require farnesylation, Ftase has been one of the most attractive targets for anti-cancer drug discovery. A large number of Ftase inhibitors (FTIs) have been developed and tested in more than seventy clinical trials [126]. The results were largely disappointing, however, with the surprising finding that H-Ras and K-Ras can still be geranylgeranyled when Ftase is inhibited. Comparing to FTIs, less work has been done on GGTase I-specific inhibitors (GGTIs). To date, several GGTIs have been developed, including inhibitors that mimic the CAAL peptide such as GGTI-286 [127], GGTI-298 [128], GGTI-2154 [129], and GGTI-2166 [130], as well as inhibitors that are non-peptidomimetic such as GGTI-Du40 [131], P61A6 [132], P61-E7 [133]. P61A6 significantly suppresses tumor growth and cell proliferation in a human pancreatic cancer xenograft [134] and non-small cell lung cancer xenograft in nude mice [135]. However, PPTIs have failed to show clinical efficacy thus far [136].

4. Conclusions

Interference of signaling from upstream regulators like RhoGEFs and RhoGAPs can impact GDP/GTP exchange or GTP hydrolysis to prevent Rho GTPases activation. Several currently available inhibitors block upstream signaling by disrupting protein-protein interactions between Rho GTPases and RhoGEFs. While RhoGAP domains have been engineered to target specific members of Rho GTPases, it remains a challenge to design chemicals that may mimic or enhance RhoGAP activity to manipulate Rho-GTP level in cells. Although RhoGDIs are proposed as potential targets in drug discovery, it is not clear if the promiscuous nature of RhoGDIs might allow for specific Rho GTPase signaling modulation. Similarly, whether targeting Rho GTPase localization through a manipulation of FTase or GGTase may satisfy selectivity and toxicity considerations.

The approach of targeting Rho GTPase effectors, in particular kinases like ROCKs and PAKs, has been extensively investigated. A number of ROCK inhibitors have been developed and significant structure-activity relationship data for improving potency and selectivity are available [137,138]. ROCK inhibitors are currently in several clinical trials, although only AT13148 is developed for cancer therapy. The development of PAK inhibitors, however, are still in their early stage and only one has entered clinical trials [139]. Owing to their large and flexible ATP binding pocket, Paks, particular group I Paks, are difficult to target. Few Pak inhibitors with satisfactory selectivity and drug-like properties have been reported to date. More studies are needed to decipher the role of each Pak in cancer before pan-Pak or Pak isoform selective inhibitors can move to clinical trials. An alternative to inhibiting effector activity is to block effector activation by disrupting Rho/effector

interaction. Proof-of-principle studies have suggested a few such inhibitors. It remains to be seen if these inhibitors meet the selectivity and efficacy requirements in animal models.

5. Expert's Opinion

Relative to Ras, Rho GTPases are considered emerging anti-cancer targets. One reason is that, while oncogenic Ras is indisputably a driver for many cancers, mutations in Rho GTPases are relatively rare in human cancers. Partly due to setbacks in strategies directly drugging Ras and the increasing understanding of Rho GTPase involvement in cancer over the last two decades, Rho GTPases have gained considerable interests as potential cancer targets. Since aberrant Rho activities associated with cancer are often caused by over-expression or dysregulation of regulators or effectors, strategies targeting Rho GTPases either directly or indirectly through other signaling pathway components have been proposed. Parallel to the continuing effort to drug Ras signaling [140], approaches in advancing drug discovery of Rho GTPase signaling axes will enrich future cancer therapy portfolio.

One of the challenges remaining is to better understand the detailed picture of Rho GTPase signaling and regulation, particularly in the context of specific cancer types. With the wide application of genome-wide analyses of cancer cells, additional evidence for aberrant Rho, GEF, GAP, and effector expression and function is expected to grow at a rapid rate. Experimental validation of their functional significance in primary cancer cells and animal models will be a rate-limiting step. Careful target validation will be key for further development of Rho pathway inhibitors for therapy. In particular because RhoGEFs and RhoGAPs are multi-domain and multi-functional, caution must be taken to more rigorously validate their roles in cancer in the context of Rho GTPase signaling.

Another challenge is druggability. To date, there is no clinically available drug that targets Rho GTPase signaling for cancer treatment. Aside from a few kinase effectors which constitutes a small portion of effector portfolios, pharmacological intervention of Rho signaling by targeting Rho, Rac, Cdc42, GEFs, Rho GTPase isoprenylation and other non-kinase effectors is still in an early stage of development. Much effort so far is proof-of-concept in nature and most academic drug leads remain far from clinical applications. Nevertheless, advances in drug design technologies, particularly those related to the design of protein-protein interaction inhibitors and high-throughput experimental assays, will accelerate the drug development process that target Rho GTPases.

Rho GTPase signaling crosstalks with other cancer-associated pathways such as EGFR, PDGFR, VEGFR, G-protein coupled receptors, integrins, p53, NF1/NF2, FAK, Src kinases, and Ras, constitute a complex and delicate network with multiple feedback and compensatory mechanisms. It is now generally accepted that specific targeting of a particular signaling pathway may show less efficacy and encounter rapid resistance, but drug combinations that target multiple nodal points within the network are more likely to achieve substantial clinical benefit. Even if Rho signaling inhibitors by themselves are not sufficient for effective cancer cell suppression, they may provide an option in combinatory therapy to achieve the desired efficacy. For example, the group I Pak inhibitor IPA-3 can overcome

resistance to PI3K inhibitors in lymphomas [141]. Thus, small molecule inhibitors targeting Rho GTPase signaling may add new treatment regimens in future precision cancer therapy, particularly in combination with other anti-cancer agents.

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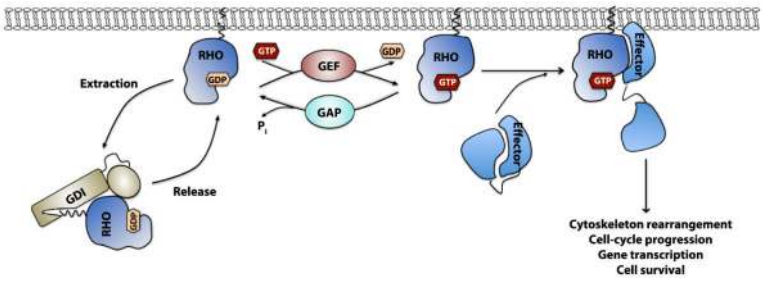

Figure 1. Biochemical model of the Rho GTPase regulatory mechanism

Rho, Rac and Cdc42 cycle between an inactive GDP-bound and an active GTP-bound state. GEFs catalyze the GDP/GTP nucleotide exchange and activate the Rho GTPases, whereas GAPs enhance the intrinsic GTP-hydrolysis activity and inactivate them. GDIs can sequester Rho GTPases in the cytosol and prevent their activation. Activated Rho GTPases can interact with a variety of effector molecules to trigger downstream signaling events leading to diverse cellular responses.

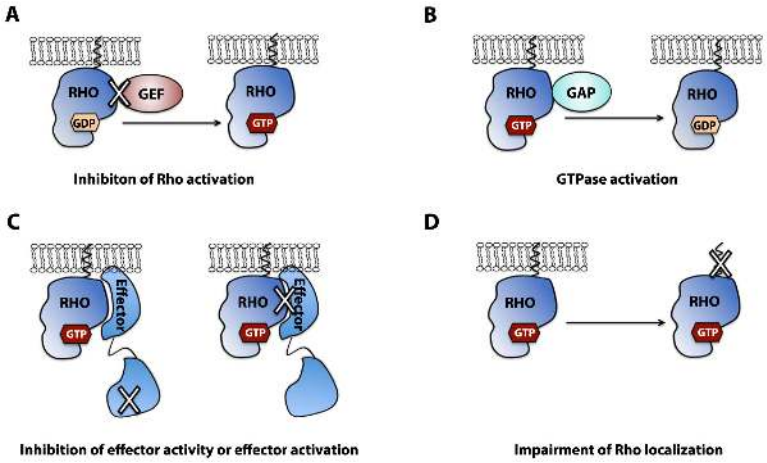

Figure 2. Approaches for rational targeting the Rho GTPase signaling module

A: Inhibition of Rho GTPase activation by GEFs via disrupting Rho-GEF interactions. B: Enhancing the intrinsic GTPase activity or Rho-GAP interaction. There has been limited experimental evidence for this approach to date. C: Inhibition of effector activity or disrupting Rho-effector interactions. D: Impairment of a Rho GTPase intracellular localization by altering its post-translational lipid modifications.

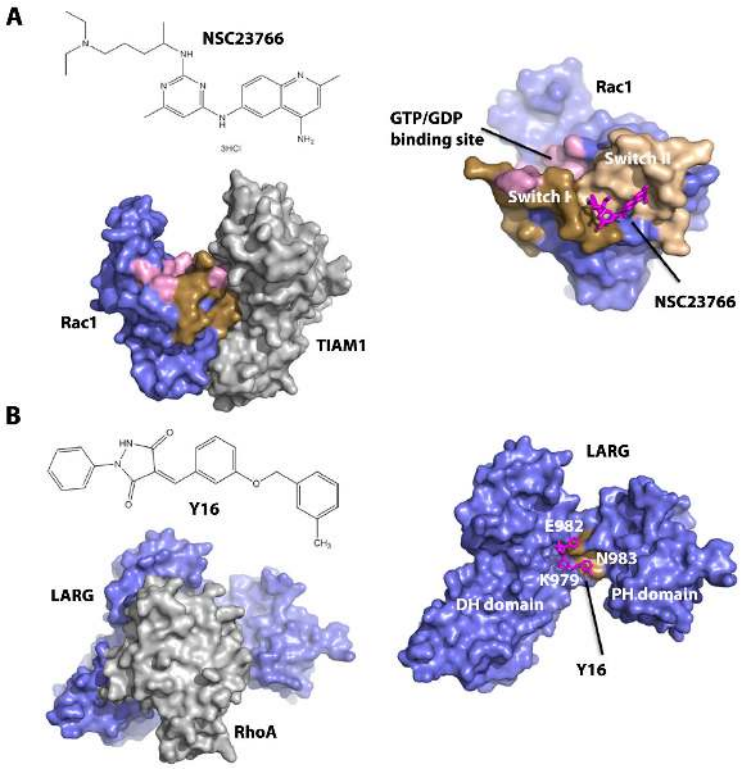

Figure 3. Structural basis for inhibition of Rac1-GEF and RhoA-GEF interactions

A: chemical structure of the lead Rac inhibitor, NSC23766 (upper left), crystal structure of Rac1-TIAM1 complex (PDB code 1FOE, lower left), and a docking model of NSC23766 onto Rac1 surface (right). B: chemical structure of Y16, a lead Rho GEF inhibitor that binds to LARG (upper left), crystal structure of RhoA-LARG complex (PDB code 1X86, lower left) and a docking model of Y16 onto the LARG DH-PH domains hinge region (right). In both A and B, the docking model was generated by AutoDock Vina [142] and visualized by PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC). Crystal structures used for docking were Rac1-TIAM1 complex and RhoA-LARG complex, respectively.

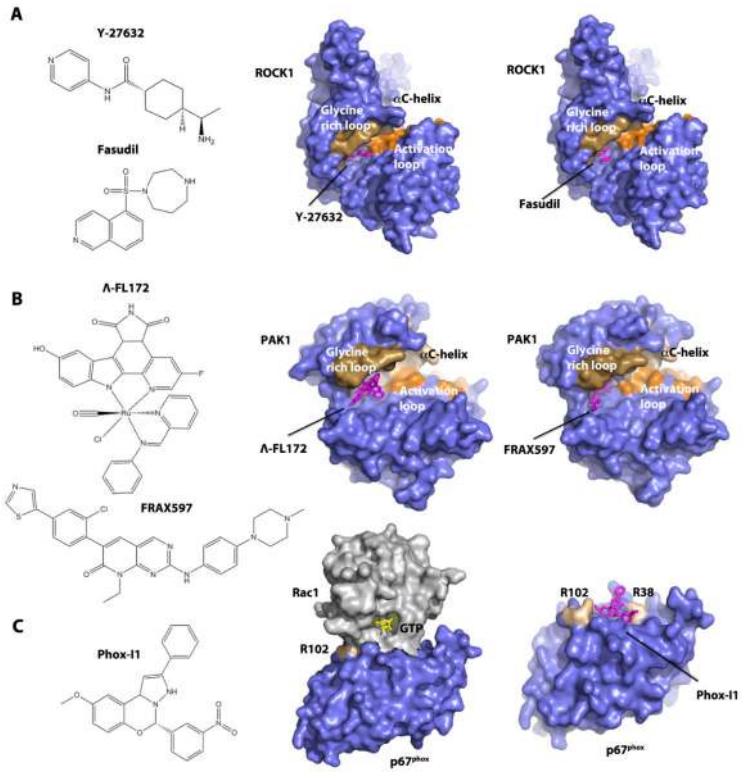

Figure 4. Structural basis for the inhibition of a Rho GTPase effector activity and a Rho-effector interaction

A: Chemical structures of the ROCK inhibitor Y-27632 and Fasudil (left), and crystal structures of ROCK1 with bound Y-27632 (PDB code 2ETR, middle) or Fasudil (PDB code 2ESM, right). B: Chemical structures of lead inhibitors of PAK, Λ-FL172 and FRAX597 (left), and crystal structures of PAK1 with bound Λ-FL172 (PDB code 3FXZ, middle) or FRAX597 (PDB code 4EQC, right). C: Chemical structure of the Rac-p67phox binding inhibitor of NOX2 enzyme, Phox-I1 (left), crystal structure of Rac1-$p67^{phox}$ complex (PDB code 1E96, middle), and the docking model of Phox-I1 on $p67^{phox}$ surface (right). This model was generated by AutoDock Vina [142] using the crystal structure of Rac1-$p67^{phox}$ complex. All crystal structures and models were visualized by PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Table 1

Selected Rho GTPases and regulators relevant to human cancer:

	Involvement in cancer	Reference
Rho GTPase		
RhoA	Mutated in gastric carcinoma, Burkitt lymphoma, peripheral and angioimmunoblastic T-cell lymphoma	[21,143-152]
	Over-expressed in gastric carcinoma, testicular germ cell tumor, head and neck squamous cell carcinoma, bladder, colon, breast and lung cancer	[147-152]
Rac1	Mutated in melanoma, head and neck, and cutaneous squamous cell carcinoma	[153-156]
	Over-expressed in oral squamous cell carcinoma, gastric cell carcinoma, breast, lung, testicular and prostate cancer	[147,148,151,152,157,158]
Cdc42	Over-expressed in breast cancer, melanoma, head and neck squamous cell carcinoma, colorectal, non-small cell lung and testicular cancer	[148,149,151,152,159-161]
RhoGEFs		
P-Rex1	Over-expressed in metastatic prostate tumor, breast cancer, and melanoma	[162-164]
P-Rex2	Over-expressed in PTEN wild type breast cancer	[165]
ECT2	Over-expressed in lung, esophageal squamous cell carcinoma, glioblastoma, colorectal carcinoma, pancreatic, non-small cell lung cancer	[166-170]
Trio	Over-expressed in glioblastoma, breast cancer	[171,172]
	Activated in adult T-cell leukemia by alternative splicing	[173]
Vav1	Over-expressed in neuroblastoma, pancreatic carcinoma, metastatic melanoma and B-cell chronic lymphocytic leukemia	[174-177]
Vav2	Over-expressed in ovarian and breast cancer; Hyper-activated in head and neck squamous cell carcinoma	[178-180]
Vav3	Over-expressed in prostate, breast cancer and glioblastoma	[171,181]
Tiam1	Over-expressed in melanoma, breast, colon, prostate and renal cell carcinoma	[182-186]
LARG	Truncated and fusion to MLL in acute myelogenous leukemia	[49]
BCR-Abl1	Fusion protein resulted from chromosome translocation associated most CML. BCR contains a GEF and a GAP domain	[187]
DOCK1	Over-expressed in glioblastoma	[188]
RhoGAPs		
DLC-1	Two missense mutations in pancreatic cancer	[189]
	Loss-of-expression or down-regulation in lung, breast, liver, colon, pancreatic, ovarian, uterine, gastric, prostate, renal cancer, lymphoma, and ALL	[52,53,190-192]
p190-B	Gene amplification and protein overexpression in hepatocellular carcinoma cells	[58]
β2-chimaerin	Down-regulated in breast cancer	[96]
BCR-Abl1	Fusion protein resulted from chromosome translocation associated with most CML. BCR contain a GEF and a GAP domain	[187]
RhoGDIs		
RhoGDI1	Over-expressed in colorectal and ovarian cancers	[193,194]
	Down-regulated in brain cancer and hepatocellular carcinoma	[195-197]

	Involvement in cancer	Reference
	Over-expressed or down-regulated in breast cancer in different studies	[152,198]
RhoGDI2	Increased expression in pancreatic and ovarian cancer	[199,200]
	Down-regulated in bladder cancer	[201,202]
	Over-expressed or down-regulated in breast cancer in different studies	[203]

Table 2

Selected small molecule inhibitors for Rho GTPase signaling:

Pathway	Target	Name of compound	Mechanism	Reference
Inhibition of Rho activation				
Rho	RhoA	Rhosin	Block GEF binding	[204]
	LARG	Y16	Block RhoA binding	[92]
	MKL1	CCG-1423	Block RhoA-dependent gene transcription	[205,206]
	PLD1	CHS-111	Block RhoA membrane recruitment	[207]
Rac	Rac1	NSC23766	Block GEF binding	[89]
	Rac1	EHop-016	Derivative of NSC23766	[208]
	Rac1/Rac2	EHT-1864	Lock in inactive state	[93]
Cdc42	Cdc42	CASIN	Block GEF binding	[90]
	Cdc42	ZCL278	Block GEF binding	[209]
	Cdc42	AZA197	Block GEF binding	[210]
	Cdc42/Rac1	AZA1	Block GEF binding	[211]
	Cdc42	Secramine	Enhance interaction with RhoGDI	[212]
	Cdc42	ML141 (CID2950007)	Block nucleotide binding	[94]
Inhibition of effector activation				
Rac	$p67^{phox}$	Phox-I1	Block Rac1-GTP binding	[123]
Inhibition of effector activity				
Rho	ROCK	Fasudil	Compete with ATP	[102]
		Dimethylfasudil	Also known as H-1152, derivative of Fasudil	[105]
		Y-27632	Compete with ATP	[100]
		WF-536	Derivative of Y-27632	[104]
		Y-39983	Also known as RKI-983, derivative of Y-27632	[213]
		SNJ-1656	Ophthalmic solution of RKI-983. In Phase II trials for glaucoma	[107]
		SB-772077-B	Compete with ATP	[214]
		GSK269962A	Compete with ATP	[214]
		K-115	Compete with ATP; in Phase II trial for glaucoma and ocular hypertension	[108,215]
		SR-3677	Compete with ATP	[216]
		AR-12286	Compete with ATP, in Phase II trial for glaucoma and ocular hypertension	[109]
		SAR407899	Compete with ATP, in Phase II trial for erectile dysfunction	[110]
		PT-262	Compete with ATP	[217]
		Azaindole 1	Compete with ATP	[218]

Pathway	Target	Name of compound	Mechanism	Reference
		Rhodblock 6	unknown	[219]
		RKI-18	Compete with ATP	[138,220]
		RKI-1447	Compete with ATP	[106]
		AT13148	Compete with ATP, multi-AGC kinase inhibitor, in Phase I trial for advanced solid tumors	[111,112]
Rac/Cdc42	Pak	K252	Compete with ATP	[113]
		KT-D606	Derivative of K252	[114]
		CEP-1347	Derivative of K252	[114]
		OSU-03012	Compete with ATP	[115]
		IPA-3	Lock Paks in auto-inhibited conformation	[141]
		Λ-FL172/Λ-FL411	With bulk scaffolds, compete with ATP	[116]
		PF-3758309	Compete with ATP	[117]
		LCH-7749944	Compete with ATP	[118]
		FRAX597	Compete with ATP	[119]
	MLK	CEP-1347	Compete with ATP; pan-MLK inhibitor	[221]
		CEP-5104	Derivative of CEP-1347, more selective for MLK1	[222]
		CEP-6331	Derivative of CEP-1347, more selective for MLK3	[222]
Cdc42	MRCK	Chelerythrine	Not compete with ATP	[223,224]
		Cycloartane-3,24,25-triol	Compete with ATP	[225]
		BDP5290	Compete with ATP	[224]
	N-Wasp	187-1	Lock N-Wasp in auto-inhibited conformation	[226]
		Wiskostatin	Stabilize N-Wasp in its inactive state	[227]
Inhibition of geranylgeranyltransferase I				
Rho/Rac/Cdc42	GGTI	GGTI-286	CAAL mimetic	[127]
		GGTI-298	CAAL mimetic	[128]
		GGTI-2154	CAAL mimetic	[129]
		GGTI-2166	CAAL mimetic	[130]
		GGTI-DU40	Nonpeptidomimetic, compete with protein substrate	[131]
		P61A6	Nonpeptidomimetic, compete with protein substrate	[132]
		P61-E7	Nonpeptidomimetic, compete with protein substrate	[133]