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The VEGF/Rho GTPase signalling pathway: A promising target for anti-angiogenic/anti-invasion therapy

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It has become increasingly apparent that current antiangiogenic therapy elicits modest effects in clinical settings. In addition, it remains challenging to treat cancer metastasis through antiangiogenic regimes. Rho GTPases are essential for vascular endothelial growth factor (VEGF)-mediated angiogenesis and are involved in tumour cell invasion. This review discusses novel therapeutic strategies that interfere with Rho GTPase signalling and further explores this network as a target for anticancer therapy through interference with tumour angiogenesis and invasion. Recent findings describe the development of innovative Rho GTPase inhibitors. Positive clinical effects of Rho GTPase targeting in combination with conventional anticancer therapy is of increasing interest.

Introduction

The hypothesis introduced by the late Professor Judah Folkman that chronic angiogenesis is necessary to support the growth of many tumour types is now firmly established [1]. Several antiangiogenic agents are now approved including monoclonal antibodies and small-molecule inhibitors [2].

Tumour angiogenesis is controlled by a balance in pro- and anti-angiogenic factors. During tumour growth the balance is shifted and favours proangiogenic factors, also referred to as the angiogenic switch [3]. Following the adaptation of an angiogenic phenotype, tumours produce and excrete proangiogenic factors, which activate the endothelial cells (ECs) of nearby blood vessels such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and angiopoietins. Vascular endothelial growth factor (VEGF) is arguably the most important angiogenic factor implicated in physiological and pathological angiogenesis. Different factors can increase VEGF expression in the tumour, for example inflammatory cytokines, growth factors and hypoxia [4]. VEGF signals via the binding of several receptor tyrosine kinases (RTKs) including VEGF receptor (VEGFR)-1, -2 and -3. These receptors hetero- or homo-dimerize, initiating downstream signalling, upon binding with VEGF. VEGF-A is the major mediator of tumour angiogenesis. There are several spliced variants of VEGF-A that can be produced simultaneously. The most predominant forms are VEGF-A_{121} and VEGF-A_{165}. These isoforms signal mainly through VEGFR-2, the receptor that regulates tumour angiogenesis. Binding of VEGF-A to VEGFR-2 results in autophosphorylation of the receptor, and the phosphorylated tyrosine residues function as a docking site for various signal transduction proteins that eventually activate cellular processes involved in angiogenesis [5]. One of the signalling cascades activated by VEGF is the Rho GTPase pathway. Rho GTPases are small proteins that function as molecular switches. They are a subfamily of the Ras superfamily of small GTPases and control cellular

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processes such as vesicle trafficking, cytoskeleton regulation, cell polarity, microtubule dynamics, membrane transport and transcription factor activity [6]. The Rho family of small GTPases consists of >20 members and can be divided into typical and atypical Rho GTPases. Typical Rho GTPases, which include Cdc42 and the Rac and Rho subfamily members, cycle between an inactive state in which guanine diphosphate (GDP) is bound to the signalling protein and an active state when guanine triphosphate (GTP) replaces GDP. In the active state Rho GTPases can bind downstream effector proteins, and transduce signals from various membrane receptors for cytokines, growth factors, adhesion molecules and G-protein-coupled receptors (Fig. 1).

As for many other anticancer agents, initial sensitivity of the tumour towards antiangiogenic agents gives way to the development of resistance [7,8]. In addition, antiangiogenic therapy can paradoxically select for more-invasive and metastatic tumour types [9,10]. Combinatorial strategies that target proteins involved in angiogenesis and invasion mechanisms in tumour and endothelial cells could improve therapeutic outcome and provide a useful approach toward enduring and effective anticancer responses. This review will outline that Rho GTPases are an attractive target for such an improved anticancer therapy.

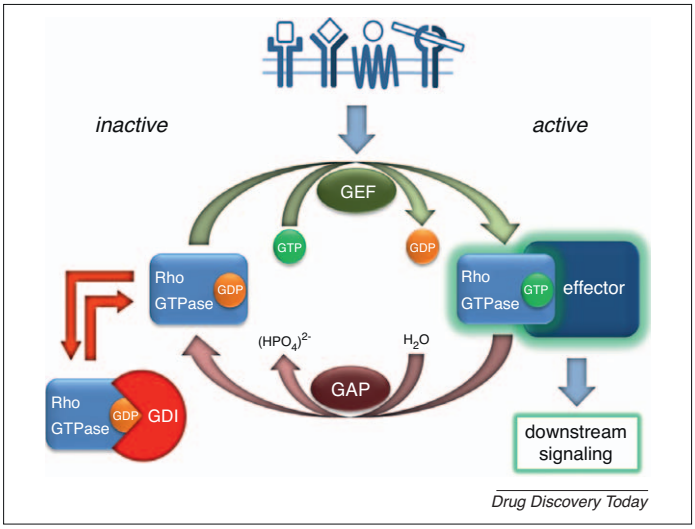

FIGURE 1

Regulation of Rho GTPases. The spatio-temporal regulation of typical Rho GTPases is controlled by >150 regulatory proteins, divided into three classes: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) [6]. Activation of typical Rho proteins is controlled by GEFs, which catalyze the exchange of guanine diposphate (GDP) for guanine triphosphate (GTP). To date, >80 GEFs have been described and are divided into two distinct families. The diffuse B-cell lymphoma-related (Dbl) family of GEFs possess a Dbl homology (DH) catalytic domain that executes GEF activity and also a pleckstrin homology (PH) domain for autoinhibition [75]. The dedicator of cytokinesis (DOCK)-related or CDM and Zizimin homology (CZH) family of proteins contain DOCK homology region (DHR) 1 and 2 catalytic domains, which are responsible for GEF activity. The action of DOCKs appears to be restricted to Cdc42 and Rac subfamily members, in contrast to Dbl GEFs [76]. GAPs inactivate typical Rho GTPases by enhancing their ability to hydrolyse bound GTP to GDP. Like GEFs, RhoGAPs outnumber the Rho GTPases they regulate and ~70 RhoGAPS have been identified [77]. GDIs function by clamping Rho GTPases in the GDP-bound state by preventing access to GEFs. GDIs can also prevent signalling by Rho GTPases by retaining them in the cytosol. GDIs can bind the C-terminal prenyl group of Rho GTPases, which is needed for association with cell membranes where activation and subsequent downstream signalling can take place. Phosphorylation can lead to dissociation of GDIs from Rho GTPases allowing translocation to the cell membrane. To date, three human GDI proteins have been described [78]. Over 70 downstream effectors of Rho proteins have been identified, with a wide variety of functions including tyrosine kinases, serine/threonine kinases, lipases, lipid kinases, phosphatase subunits and scaffolding proteins. Rho GTPases associate with multiple effector proteins, and some effector proteins can interact with multiple Rho GTPases [79].

Rho GTPases in angiogenesis

Following the discovery of Rho GTPases, studies were performed with dominant negative (dn) or constitutively active (ca) Rho proteins to gain insight into their cellular functions. In addition, bacterial toxins, RNA interference (RNAi) and knockout approaches have been used to investigate the effect of Rho GTPase inhibition *in vitro* and *in vivo*. Whereas some results in knockout mice correlate with dn approaches *in vitro*, other methods produce conflicting results. A considerable drawback of dn mutants is that they non-specifically block the action of guanine exchange factors (GEFs) that act on multiple GTPases [11]. In addition, adaptive compensation by upregulation of related Rho GTPases or isoforms after loss of a certain Rho GTPase could also be a factor that masks phenotypes in knockout mice [12]. Global knockout of Rac1 or Cdc42 in mice results in embryonic lethality, indicating that these Rho GTPases are essential for development. By contrast, global knockout of other Rho proteins (including Rac2, Rac3, RhoB, RhoC, RhoG and RhoH) does not result in severe developmental defects. Conditional knockout mouse models have also been used to study the effects of organ- or cell-specific deletion of Rho proteins [6,12].

A growing body of evidence indicates a crucial role for Rho GTPases (and their regulatory and effector proteins) in ECs during all processes involved in angiogenesis such as vascular permeability, extra cellular matrix (ECM) degradation, cellular migration, proliferation and lumen formation [13]. Moreover, several studies have shown that these processes are mediated by VEGF-induced activation of Rho GTPases through VEGFR-2 (Fig. 2) [5,14].

Vascular permeability

Paracellular vascular permeability is controlled by adherens junctions and tight junctions between ECs. Rho GTPases are involved in the regulation of actin cytoskeleton and microtubule dynamics and, thus, can interfere with these cellular junctions. In the case of angiogenesis VEGF secreted by tumours activates Rho GTPases in ECs and destabilizes endothelial barrier integrity, primarily by disruption of adherens junctions [15].

VEGF treatment induced an increase in reactive oxygen species (ROS) production and vascular permeability in human pulmonary microvessel ECs (HPMECs). Knockdown of Rac1 prevented VEGF-induced ROS production, adherens junction protein phosphorylation and vascular permeability [16].

A recent study has shown that treatment of bovine retinal ECs (BRECs) with VEGF increases cell permeability, an effect that can be reduced by simultaneous treatment with the Rho-associated coiled-coil-forming kinase (ROCK) inhibitor Y27632, indicating a role for RhoA-mediated signalling [17].

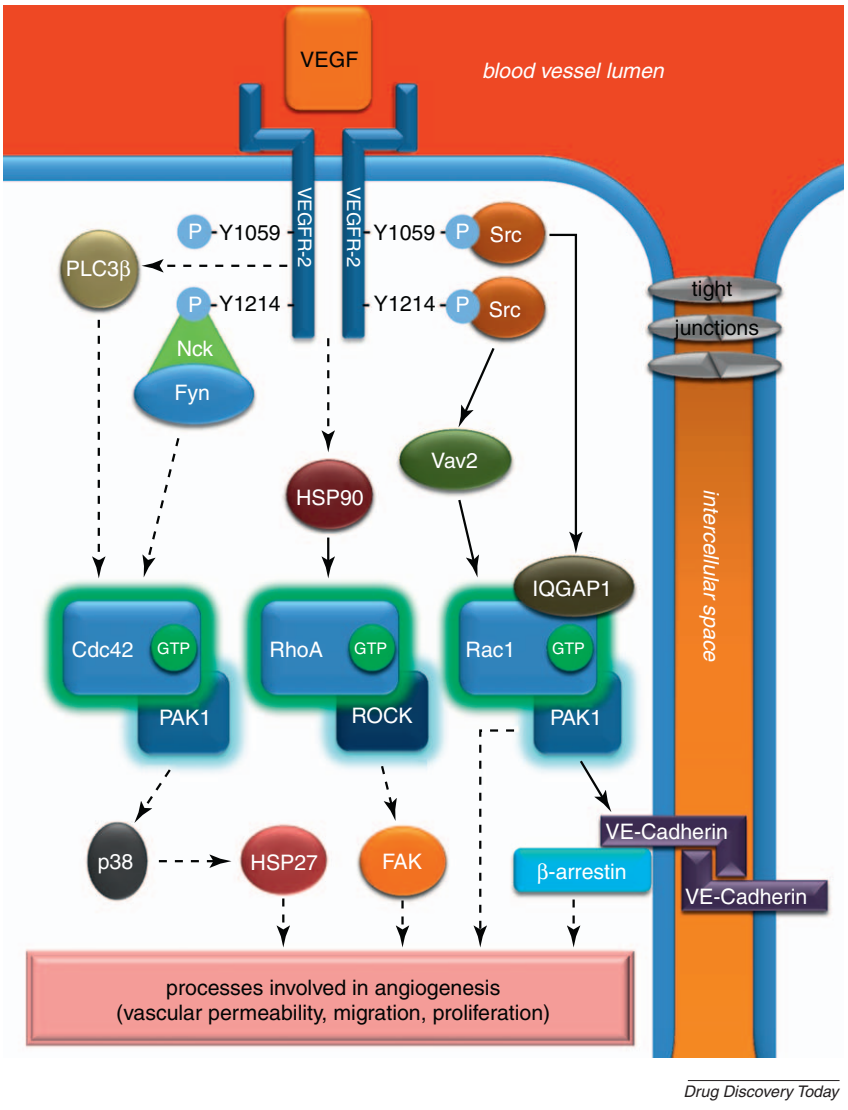

FIGURE 2

VEGF-mediated VEGFR-2 activation of Rho GTPases. VEGF-driven VEGFR-2 activation induces recruitment of proteins to the phosphorylated tyrosine residues such as c-Src [80]. Rho GTPase-dependent cellular processes activated in this way include Vav2-mediated migration and vascular permeability [60,81] and IQ motif containing GTPase activating protein (IQGAP)-1-induced migration, proliferation and vascular permeability [82–84]. Other signalling pathways involved in VEGF-mediated cellular processes include RhoA-dependent activation of focal adhesion kinase (FAK) [85,86] and activation of Cdc42 in a phospholipase β3 (PLCβ3)-dependent [87] or Nck/Fyn-dependent manner [85,86]. (Solid arrows display direct interactions and dashed arrows display indirect interactions.)

Degradation of the basement membrane

Basement membrane degradation by matrix metalloproteinases (MMPs) allows ECs to migrate and invade the ECM.

One study describes antagonistic roles for RhoA and Cdc42 in regulating MMP-2 activation by increasing Cdc42 activity and decreasing RhoA activity [18]. More recently, it has been demonstrated that Rho/ROCK signalling was not required for VEGF-mediated MMP-1 and MMP-9 expression because treatment of BRECs with Y27632 did not suppress VEGF-stimulated MMP expression [17]. Human microvascular ECs (HMECs) expressing a ca form of RhoA displayed greater invasive ability when compared to control cells, probably caused by the induction of MMP-9 expression in the RhoA-overexpressing cells. Addition of tissue inhibitor of metalloproteinases (TIMP)-1, an endogenous MMP inhibitor, reduced the invasion of RhoA-expressing cells [19].

Migration

During EC migration activation of Rho GTPases is required to regulate the actin cytoskeleton resulting in a forward force at the front of the cell; while, simultaneously, adhesion forces at the rear of the cell are disrupted. Cdc42 controls the formation of filopodia, which are important for cell–cell contact and sensing the environment. Cdc42 also controls directional migration. Rac1 is responsible for the formation of lamellopodial membrane protrusions at the front of the cell, and RhoA regulates the formation of stress fibres at the rear of the cell and the cell body. These stress fibres mediate cell contractility and thereby enable forward movement [6,20].

Although the role of RhoA in EC migration has been debated, treatment of HMECs with Y27632 blocked VEGF-mediated migration [21]. Recently, it was reported that VEGF-mediated BREC wound closure could be blocked by the ROCK inhibitor Y27632

[17]. Deletion of Rac1 in primary ECs derived from mice by the Cre/Flox approach inhibited VEGF-stimulated cell migration [22]. Knockout mouse studies and morpholino approaches in zebrafish identified the Rho GEF Syx (PLEKHG5) as essential for angiogenesis [23]. Knockdown of Syx in rat ECs reduced basal and VEGF-induced migration and tube formation but no effect of Syx inhibition on EC invasion was shown, indicating that Syx does not play a part in EC invasion [23]. RNA levels of Cdc42GAP, a GTPase-activating protein (GAP), were reduced in tubule forming HMECs [24]. HMECs transfected to overexpress Cdc42GAP had reduced tubule-forming capacity compared with control cells, whereas small interfering RNA (siRNA)-mediated knockdown of Cdc42GAP increased tube formation [24]. Similar effects were demonstrated in EC migration, indicating that the effect on tube formation was predominantly caused by reduced cell migration [24].

Proliferation

Local cellular proliferation is required for the formation of vascular branches. Rho GTPases are required for the G1/S transition phase in cell cycle progression [25]. RNAi-mediated inhibition of RhoA and RhoC in HMECs decreased cell proliferation by 80% [26]. However, Y27632 treatment did not interfere with VEGF-stimulated BREC proliferation, indicating that Rho signalling is not required [17].

Lumen formation

The final step in the angiogenesis process consists of lumen formation and stabilization of the newly formed vessel. Studies performed in zebrafish and in mice suggest that ECs can form lumens by two distinct mechanisms termed cell hollowing and cord hollowing [27]. Cell hollowing involves intracellular vacuole formation and fusion of these vacuoles to form lumens. Kamei *et al.* showed that lumen formation in the intersomitic vessels of zebrafish is mediated by formation and fusion of pinocytic intracellular vacuoles that co-localize with Cdc42 [28]. Although, traditionally, the cell-hollowing mechanism has been regarded as the method by which ECs form lumens, recent evidence derived from studies in zebrafish and mice has shown that lumen formation occurs through the cord-hollowing mechanism [29]. Strilic *et al.* demonstrated that VEGF-mediated ROCK activation results in EC shape changes that ultimately lead to extracellular lumen formation [29].

The role of RhoA/ROCK I/II in angiogenesis signalling is controversial and studies investigating the effects of pharmacological inhibition of RhoA/ROCK report conflicting results. Kroll *et al.* have utilized the oxygen-induced retinopathy mouse model to demonstrate effects of ROCK I/II inhibition by H1152P [30]. In this assay, inhibition of ROCK increased neovascularization. Treatment with the VEGF receptor antagonist PTK787/ZK222584 (vatalanib) inhibited neovascularization, and a combination of H1152P and vatalanib did not reverse the effect of vatalanib treatment alone, suggesting that ROCK I/II act downstream of VEGF. Interestingly, in the presence of H1152P and VEGF, human umbilical vein ECs (HUVECs) failed to induce stress fibres. Stress fibres are involved in cell contractility, which is required for migration. In the same assay, siRNA-mediated knockdown of ROCK I/II enhanced sprouting angiogenesis and could be inhibited by treatment with vatalanib. The results of the experiments taken together suggest that ROCK I/II act as negative regulators of VEGF-mediated angiogenesis [30].

In another study, Bryan *et al.* provide results suggesting that blocking Rho inhibits VEGF-stimulated angiogenesis [17]. Murine retinal explants show VEGF-induced sprouting in a collagen gel, which could be inhibited by ROCK I/II inhibitor Y27632. BRECs plated between collagen layers formed vessels after VEGF treatment. Interestingly, a combination of VEGF and Y27632 enhanced cord formation when compared to VEGF alone, but the formed network appeared morphologically distinguishable, indicating immature vessel formation. Furthermore, in a Matrigel™ tube formation assay with ROCK I/II siRNA-transfected mouse pancreatic ECs, it was shown that ROCK II largely mediates VEGF-driven angiogenesis. This effect was confirmed *in vivo* showing lower vascular density in lungs from heterozygote knockout ROCK1 and ROCK2 mice than is seen with wild type (wt) mice [17].

In contrast to studies claiming an essential role for Rac1 in angiogenesis, D'Amico *et al.* recently showed that Rac1 depletion in the tumour endothelium of adult wt mice had no effect on tumour growth, tumour angiogenesis and VEGF-mediated angiogenesis [31]. However, in β3-integrin-null mice these processes were dependent on Rac1 expression. Furthermore, Rac1 depletion inhibited VEGF-mediated tube formation and cell migration in 2D scratch wound healing assays in wt and β3-null ECs. Interestingly, knockdown of Rac1 in wt ECs had no effect on VEGF-induced cell migration in 3D Boyden chamber assays but did so in β3-null ECs. This correlates with normal tumour angiogenesis found in wt mice with Rac1-depleted ECs [31]. The αvβ3 integrin, a receptor for fibronectin and vitronectin, expressed on vascular ECs is involved in angiogenesis; and αvβ3-induced activation of Rac1 has been suggested by previous studies [14]. D'Amico *et al.* suggest that increased levels of active Rac1 might compensate for β3-integrin loss because β3-integrin expression rescue in β3-null ECs resulted in active Rac1 levels comparable to wt ECs. The authors conclude that Rac1 might be an attractive target for antiangiogenesis therapy in tumours that display low levels of β3-integrin in the vasculature, or in combination with anti-β3-integrin therapy [31]. In contrast to the results obtained by D'Amico *et al.* with a Tie1-driven endothelium-specific knockout of Rac1 [31], conditional knockout of Rac1 in a Tie2-driven model results in embryonic lethality owing to severe defects in the development of major blood vessels [22]. There are several possible explanations for the variation in outcome between the different models. Deletion of Rac1 in the Tie2/Cre-driven model occurs in early embryogenesis, whereas the Tie1-driven model used by D'Amico *et al.* induced Rac1 deletion mostly in adult tumours or endothelial cells [31]. Moreover, Cre activity in the Tie2 model also induces Rac1 depletion in hematopoietic cells, although it is probable that the lentiviral Tie1/Cre-lox-regulated model does not affect these cells [22,31].

Rho GTPases in tumour invasion

In addition to the requirement for Rho GTPase signalling in ECs mediating angiogenesis, numerous studies have highlighted Rho GTPase involvement in tumourigenesis *in vitro*, more recently in knockout mouse models *in vivo* and, most importantly, in patients [32]. Interestingly, in contrast to RAS proteins, no mutations of Rho GTPases causing ca forms of the protein in tumour cells have been reported. However, overexpression and reduced expression have frequently been documented. Furthermore, mutations in

Rho GTPase regulatory proteins that might eliminate or hyperactivate Rho GTPase signalling have also been reported [32].

Of particular interest is Rho GTPase control of tumour cell movement, as migration properties of tumour cells form the basis of tumour cell invasion into the surrounding tissue and subsequent metastasis. Tumour cell migration can occur in two different ways: amoeboid and mesenchymal movements. Amoeboid migration is characterized by rounded cell morphology, the active formation of blebbing protrusions and high cortical tension. In mesenchymal migration cells have an elongated morphology and move through cellular protrusions that attach and drive the cell forward which requires local extracellular proteolysis of the ECM. There seems little difference in the rate of invasion of both these types, because the amoeboid type of migration allows cells to squeeze through the matrix [33].

Sanz-Moreno *et al.* have shown that Rho proteins are not only required for tumour cell migration but even control whether tumour cells migrate in an amoeboid or mesenchymal manner. A systematic siRNA screen of all known human GEFs and GAPs in melanoma cells was performed to identify Rho GTPase regulators of the mode of tumour cell invasion. A mechanism was proposed in which mesenchymal migration is dependent on Rac, whereas amoeboid movement is RhoA-dependent. The GEF dedicator of cytokinesis (DOCK)-3 acts together with the adaptor protein neural precursor cell expressed, developmentally down-regulated 9 (NEDD9) to activate Rac. Rac induces mesenchymal migration through Wiskott–Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE)-2, which simultaneously suppresses amoeboid movement through negative regulation of myosin light chain (MLC)-2 phosphorylation and subsequent inhibition of actomyosin contractility. RhoA/ROCK signalling promotes amoeboid migration and simultaneously activates the Rac GAP ARHGAP22 leading to suppression of mesenchymal tumour cell movement [34]. Furthermore, the same group has demonstrated that Cdc42 is activated by DOCK10 resulting in amoeboid movement. Assays performed with cells expressing a dn form or knocked down Cdc42 revealed that Cdc42 is also required for mesenchymal migration, indicating that there are specific Cdc42-activating pathways involved in both types of tumour cell motility [35].

The involvement of Rho proteins in mechanisms regulating invasive and metastasizing cancer types has been extensively documented. For example, studies performed with RhoC knockout mice and mice that develop mammary carcinomas and lung metastases demonstrated that loss of RhoC decreased the metastatic potential of tumour cells *in vivo* and reduced migration and invasion of these cells *in vitro* [36]. The role of Rac1 in tumour invasion has been shown in two types of brain tumours: glioma and medulloblastoma. Rac1-transfected cells demonstrated reduced tumour cell migration and invasion [11]. A similar approach was utilized to study the invasive behaviour of medulloblastoma cells [37]. Importantly, immunohistochemical (IHC) analysis demonstrated overexpression of Rac1 in glioblastoma and medulloblastoma tissue versus non-neoplastic brain, and marked plasma membrane staining of Rac1 indicated hyperactivation of this GTPase in these tumours [37,38].

Besides Rho GTPases, GEFs and GAPs have also been shown to be deregulated in tumour invasion and metastasis. Melanoma cells stably transfected to express a ca GTPase-deficient mutant of the G-protein $G\alpha_{13}$ displayed reduced chemokine-induced invasion compared with cells expressing the wt protein. Additionally, cells expressing mutant $G\alpha_{13}$ displayed higher p190RhoGAP chemokine-mediated phosphorylation when compared with wt-expressing cells. Knockdown of p190RhoGAP reversed the reduced invasion of the mutant-expressing cells and recovered RhoA activation, indicating that p190RhoGAP activation reduces RhoA-mediated invasion of melanoma cells [39]. The GEFs Ect2, triple functional domain protein (Trio) or Vav3 are overexpressed in glioblastoma when compared with low-grade glioma or normal brain tissue. Silencing of the GEFs in SNB19 and U-87 glioma cell lines inhibited migration *in vitro* and invasion in an *ex vivo* rat brain slice model [38]. The involvement of Rho proteins in tumour invasion and metastasis is confirmed by frequently reported correlation between Rho protein expression and clinical outcome [32]. For example, levels of RhoC expression are elevated in head and neck squamous cell carcinoma cell lines, and IHC analysis on a tissue microarray demonstrated the correlation of RhoC expression *in vivo* with advanced clinical stage and lymph node metastasis [32,40]. Analysis of matched tumour, non-tumour and metastatic lymph node tissue from patients with urothelial carcinoma of the upper urinary tract revealed that levels of active GTP-bound Rac1 and p21-activated kinase (PAK)-1 protein expression were increased in tumour tissue and metastatic tissue when compared with non-tumour tissue. Furthermore, high levels of Rac1 activity and PAK1 expression correlated to muscle invasion, lymphovascular invasion and lymph node metastasis [41,42].

Targeting Rho GTPases

Currently, several strategies targeting Rho GTPases have been explored including specific Rho protein inhibitors, RNAi and lipid modification inhibitors (Fig. 3).

Small-molecule inhibitors

The emerging role of Rho GTPases, regulatory and effector proteins in angiogenesis has led to an increasing interest in the development of specific small-molecule inhibitors. The large number of cellular functions that are known to be controlled by RhoA, Rac1 and Cdc42 strongly suggests that systemic inhibition of these GTPases would lead to significant toxicity, implying that targeting GEFs and/or GTPase effectors might provide better therapeutic opportunities at present [43]. Indeed, most therapeutic molecules are kinase inhibitors that compete with ATP and block kinase activity of Rho GTPase effector proteins such as ROCK and PAK. Other inhibitors are aimed at blocking Rho GTPase activation by preventing binding of GTP or GEFs. Specific inhibitors are required to dissect Rho GTPase pathways further in preclinical settings and can be useful clinically to improve antiangiogenic and antitumour therapy. Established and novel small-molecule inhibitors targeting Rho GTPase signalling pathways in angiogenesis are highlighted (Table 1).

The most commonly used pharmacological inhibitors to interfere with angiogenesis by targeting Rho signalling are the ROCK inhibitors Y27632, Y32885 (Wf536), HA1077 (Fasudil) and H1152P.

Fasudil has been approved in Japan since 1995 for the treatment of cerebral vasospasm and is currently under clinical investigation for beneficial effects in cardiovascular patients [44]. A growing number of studies now describe its effects on tumour growth and angiogenesis. Fasudil has been shown to inhibit VEGF-stimulated HUVEC migration, viability and tube formation. Furthermore,

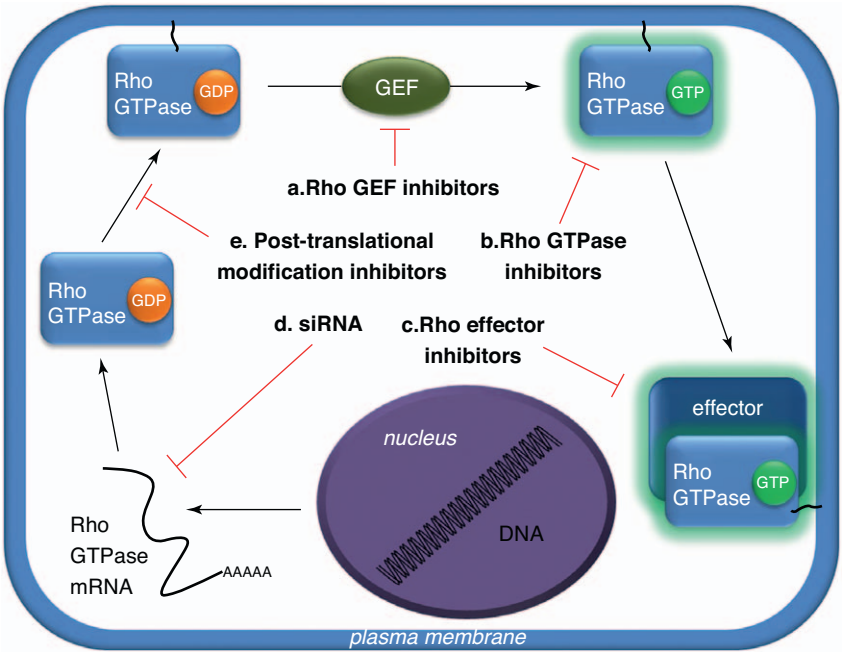

FIGURE 3

Strategies to interfere with the Rho signalling pathway. Several strategies are exploited to target proteins of the Rho signalling network. (a) Specific inhibition of Rho GEFs blocks activation of Rho GTPases. (b) Rho GTPase activation can be inhibited by prevention of GEF or GTP binding. (c) Activation of downstream Rho effector proteins such as serine/threonine kinase activation is prevented with ATP-competitive or non-ATP-competitive kinase inhibitors. (d) RNAi is a highly specific strategy used to prevent Rho protein translation. (e) Inhibition of post-translational modifications is a broadly acting strategy to interfere with the Rho signalling network.

fasudil reduced VEGF-mediated vessel formation in a directed *in vivo* angiogenesis assay [45]. Treatment of breast cancer and fibrosarcoma cells with 50 μmol/l fasudil and its metabolite fasudil–OH inhibited cell migration by 26% and 50%, respectively. Fasudil and fasudil–OH inhibited anchorage-dependent growth of breast cancer cells. Additionally, fasudil treatment significantly reduced the amount of tumours in a rat peritoneal tumour model and a murine experimental lung metastasis xenograft model when compared with control treatment groups. Interestingly, in an orthotopic xenograft mouse model fasudil treatment did not reduce tumour size. However, the number of tumour-bearing animals in the treatment group was considerably lower when compared with the control group [46].

In a recent study (as mentioned above) by Bryan *et al.* [17], inhibitory effects of Y27632 on *in vitro* and *ex vivo* angiogenesis have been demonstrated [17]. The effects of Wf536 on angiogenesis *in vitro* and tumour growth *in vivo* in combination with the MMP inhibitor marimastat have also been investigated.

TABLE 1

Small-molecule inhibitors targeting Rho GTPase signalling

Class	Target	Mode of action	Ref.
Rho GEF inhibitor			
ITX3	Trio	Inhibition of N-terminal GEF domain activity	[59]
Rho GTPase inhibitor			
NSC23766	Rac	Competitive inhibitor of GEF activation	[50]
EHT1864	Rac	Blocking Rac downstream signalling by guanine nucleotide displacement	[54]
MLS000532223	Rho GTPases	Prevention of GTP binding	[58]
Rho effector inhibitor			
Y27632	ROCK	Kinase inhibitor	[17,48]
Y32885 (Wf536)	ROCK	Kinase inhibitor	[47]
Fasudil (HA1077)	ROCK	Kinase inhibitor	[45,48]
H1152P	ROCK	Kinase inhibitor	[30,48]
PF3758309	PAK4	Kinase inhibitor	[53]
IPA-3	PAK1	Non-ATP-competitive inhibitor	[55]
Wiskostatin	N-WASP	Non-ATP-competitive inhibitor	[56]

Wf536 inhibited sphingosine-1-phosphate (S1P)-induced HUVEC vacuole formation, one of the earliest events in angiogenesis. Wf536 interrupted S1P-mediated HUVEC tube formation in Matrigel™ assays and the inhibiting effect could be increased by combination treatment. Similar effects of combination treatments on sprout formation were obtained in rabbit thoracic aortic explants embedded in collagen gels supplemented with VEGF and hepatocyte growth factor (HGF). In addition, Wf536, the MMP inhibitor marimastat or the combination treatment of both agents inhibited S1P- and EGF-mediated HUVEC migration. Finally, combination treatment inhibited tumour growth when compared with control treatment in mice bearing human prostate cancer xenografts. Moreover, the combination treatment plus paclitaxel (Taxol®) was significantly more effective than paclitaxel alone in the same tumour model [47]. H1152P, a dimethylated analogue of fasudil, is the most potent ROCK inhibitor available. H1152P inhibits ROCK activity in an ATP-competitive manner with a K_i value of 1.6 nM. By comparison, fasudil and Y27632 have K_i values of 0.33 μM and 0.14 μM, respectively [48]. Interestingly, H1152P treatment enhanced VEGF-induced in-gel sprouting angiogenesis and increased preretinal vessel formation in the oxygen-induced retinopathy (OIR) model in a study by Kroll *et al*. discussed earlier [30].

The selective small-molecule Rac1 inhibitor NSC23766 fits in a surface groove essential for GEF binding and has been shown to prevent activation by GEFs Trio and T-cell lymphoma invasion and metastasis (Tiam) [49]. Treatment of prostate cancer cells with NSC23766 in a dose-dependent manner inhibited cell proliferation and anchorage-independent cell growth. Furthermore, the invasion of tumour cells treated with 25 μM NSC23766 was inhibited by 85% [49]. Inhibition of Rac1 by NSC23766 (again in a dose-dependent manner) reduced basal HUVEC proliferation as well as proliferation induced by VEGF, HGF or a combination of the two [50].

Development of several novel Rho protein inhibitors has been reported which might be useful in targeting Rho proteins in tumour invasion and angiogenesis.

Evelyn *et al*. have shown the small-molecule inhibitor CCG1423 to have antiproliferative effects on various cancer cells and anti-invasion effects in prostate cancer cells [51].

PAK4, a downstream effector protein of Rac1 and Cdc42, is involved in EC lumen formation, and knockdown of PAK4 inhibited tube formation and invasion of HUVECs in 3D collagen gel matrices [52]. More recently, a small-molecule inhibitor of PAK4 was identified by structure-based design and high throughput screening. PF3758309 showed inhibiting effects on a broad selection of tumour cell lines in anchorage-independent and -dependent proliferation assays. Furthermore, PE3758309 inhibited tumour growth in a panel of human xenograft tumour models [53].

The novel Rac inhibitor EHT1864 interferes with the guanine nucleotide exchange process. In assays performed in mouse fibroblasts, EHT1864 inhibited Rac downstream signalling and cell growth transformation [54].

The PAK1 inhibitor IPA-3 was identified in a high throughput screen that utilized ATP hydrolysis as an indication of PAK1 activation. PAK1 is autoinhibited by the formation of inactive homodimers where an autoregulatory region of one monomer binds the catalytic domain of its partner. Upon activation of PAK1 by Rac or Cdc42, monomer dissociation and displacement of the autoinhibitory domain occurs, followed by autophosphorylation to stabilize the active monomer. IPA-3 targets this autoregulatory mechanism, possibly altering the conformation of PAK1, making it catalytically inactive [55]. A similar mechanism of action has been demonstrated for the neural-WASP (N-WASP) inhibitor wiskostatin. N-WASP can be activated by Cdc42 by the same mechanism as PAK1. Wiskostatin interacts with the regulatory GTPase-binding domain of N-WASP, thereby promoting this domain to fold into the autoinhibitory conformation and preventing N-WASP from functioning normally [56]. Targeting autoregulatory domains of kinases may provide more-specific inhibition than ATP-competitive approaches, reducing the risk of off-target effects and toxicity [57]. Utilizing a high-throughput bead-based flow cytometric fluorescent GTP-binding assay for screening compounds that target GTP binding to Rho GTPases, MLS000532223 was identified. MLS000532223 is a general inhibitor of Rho GTPases that prevents binding of GTP in a dose-dependent manner. It was shown that MLS000532223 inhibited EGF-stimulated Rac1 activation and EGF-stimulated stress fibre formation in mouse embryonic fibroblasts. In addition, MLS000532223 inhibited IgE-induced cell morphology changes in rat mast cells [58].

In a yeast-based system, the chemical ITX3 was identified. It inhibits the activity of the N-terminal GEF domain of Trio, which acts on Rac1 and RhoG. ITX3 blocked Trio N-terminal domain (TrioN)-mediated cell structures in a dose-dependent manner in rat fibroblasts, nerve growth factor (NGF)-induced nerve outgrowth in rat phaeochromocytoma cells and myotube formation in mouse myoblasts [59].

RNAi

RNAi is now used extensively *in vitro* as an efficient tool to inhibit Rho proteins specifically. One study has shown that siRNA-mediated knockdown of the Vav2 GEF in ECs prevents VEGF-induced Rac1 activation and subsequent cell migration [60].

Pille *et al*. [26] have reported on the antiangiogenic effects of RhoA siRNA treatments *in vitro* and *in vivo*. Human ECs were transiently transfected with siRNA against RhoA or RhoC utilizing the commercially available cationic lipid carrier Cytofectin™. Transfected ECs showed decreased proliferation and bFGF-stimulated tube formation was perturbed when compared with control cells. Intratumoural injection of siRNA in Cytofectin™ in mice bearing breast tumour xenografts resulted in inhibition of tumour growth and angiogenesis indices based on IHC staining with platelet-endothelial cell-adhesion molecule (PECAM)-1 that was lower in tumours derived from mice treated with RhoA siRNA [26]. In a follow-up study, RhoA siRNA was encapsulated in chitosan-coated polyisohexylcyanoacrylate particles and injected intravenously in the same mouse model. Tumour growth was inhibited with this treatment and IHC staining revealed fewer ECs present in treated (versus untreated) tumours [61].

Post-translational modification inhibitors

Rho GTPases contain a CAAX motif at their C-terminus (C = cysteine, A = any aliphatic amino acid, X = any amino acid). The CAAX motif undergoes post-translational prenylation (farnesylation or geranylgeranylation) targeting the proteins to the endoplasmatic reticulum (ER). At the ER, the AAX section of the peptide is removed by RAS-converting enzyme (RCE)-1 and the carboxyl group of the cysteine is methylated by isoprenylcys-

teine carboxymethyltransferase (ICMT). Rho guanine nucleotide dissociation factors (GDIs) bind to the prenylation moiety and guide Rho GTPases to membranes. Prenylation of Rho GTPases can be targeted by mevalonate pathway inhibitors such as statins or bisphosphonates; prenylation inhibitors such as farnesyltransferase inhibitors; or post-prenylation inhibitors including REC1 and ICMT inhibitors [62].

Statins are widely used in the clinic as cholesterol-reducing agents and considered safe. Many studies demonstrate antiangiogenic effects of statins, for example simvastatin. In mouse corneal pocket assays fibroblast growth factor (FGF)-2- induced vascularization could be suppressed by simvastatin. In chick chorioallantoic membrane (CAM) assays VEGF-mediated angiogenesis was inhibited by simvasatin in a dose-dependent manner, which was also seen in an FGF-2/VEGF-stimulated tube formation of human dermal microvascular ECs (HDMECs) on a 3D collagen matrix. This effect could be reversed by transfecting the cells with a dominant activating mutant of RhoA, suggesting that the antiangiogenic effects of simvastatin are mediated by an effect on RhoA. Finally, simvastatin treatment of HDMECs decreased RhoA membrane localization. Geranylgeranyl pyrophosphate reversed the effects of simvastatin on tube formation and RhoA membrane localization, indicating that the effect is mediated via geranylgeranylation of RhoA [63]. However, several studies have shown conflicting proangiogenic effects of statin treatment [64]. The most likely hypothesis describes a biphasic effect of statins on angiogenesis where proangiogenic effects are observed at low concentrations, and antiangiogenic effects at high concentrations. As well as dosage, other factors such as cancer type or statin type could be of influence. The biphasic mechanism has been shown by Katsumoto *et al.* in FGF-2-induced neovascularization in CAM assays [65].

Migration and invasive properties of several melanoma cell lines could be dose-dependently inhibited by treatment with the statins lovastatin, mevastatin and simvastatin [66]. Bisphosphonates interfere with prenylation of Rho GTPases by inhibiting enzymes in the mevalonate pathway that are required for the production of farnesyl pyrophosphate and geranylgeranyl pyrophosphate [62]. Treatment of HUVECs with alendronate inhibited VEGF-mediated EC migration, tube formation and suppressed Rho activation. These effects could be restored by simultaneous treatment with geranylgeraniol, which is metabolized to geranylgeranyl pyrophosphate, indicating that the effects are caused by inhibition of Rho geranylgeranylation. Finally, alendronate treatment of mice bearing ovarian carcinoma xenografts inhibited intratumour angiogenesis by 75% when compared with non-treated mice [67]. Treatment of ECs derived from multiple myeloma patients (MMECs) with the bisphosphonate zoledronic acid inhibited cell proliferation, VEGF-mediated cell migration and tube formation. Furthermore, zoledronic acid inhibited *in vivo* angiogenesis in CAM assays when compared with sponges loaded with MMEC conditioned medium [68].

Despite strong antiangiogenic effects of post-translational modification inhibitors *in vitro*, results from clinical trials of these drugs as monotherapy only displayed modest effects, possibly because of the lack of specificity and requirement of high doses leading to toxicities. However, combinations with conventional anticancer therapies might improve the therapeutic outcome [62]. Moreover, the vast clinical experience with statins is beneficial for designing clinical trials and treatments that target Rho GTPases to interfere with tumour growth and angiogenesis [69].

Discussion

Rho proteins are rational targets to interfere with EC angiogenesis and invasion/metastasis of tumour cells. Indeed, it was suggested that tumour growth inhibition in studies performed with RhoA siRNA or Vav2/3-deficient mice might be a result of combined effects on angiogenesis and tumour cells [61,70]. Besides the direct effect in ECs, targeting Rho proteins might indirectly contribute to the overall antiangiogenic effect by inhibiting the production and subsequent secretion of proangiogenic factors, such as VEGF, that are produced by tumour cells [71]. At present, strategies exploring combinations of conventional anticancer therapies and targeting of Rho proteins are of particular interest. For example, potential beneficial effects of NSC23766 treatment of trastuzumab-resistant cancer cells [72]. Although conflicting outcomes have been reported previously, recent clinical trial data have suggested beneficial effects of statin or bisphosphonate treatment combined with conventional anticancer therapy [73,74]. There is still much to unravel about the mechanisms that are involved in the activation, regulation and downstream signalling of Rho GTPases. For this purpose, it is important to develop specific inhibitors of proteins involved in this pathway. It is now evident that Rho proteins are essential for tumour angiogenesis and invasion; and targeting this signalling network is a promising strategy to improve current antitumour therapy. An interesting challenge remains in determining which (combinations of) Rho proteins are the most promising druggable targets and how significant the beneficial effects of targeting this signalling network, in combination with conventional anticancer therapies, will be.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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