Inhibiting the Rho/ROCK pathway – the potential anti-cancer properties may not have been fully realised

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Abstract

The Rho/ROCK pathway is involved in many important cellular processes that have made it an object of intense study in cancer medicine, however, ROCK inhibitors are yet to make an appearance in the clinical setting. Their performance as an anti-cancer therapy has been heterogeneous in pre-clinical studies, but they have been shown to be effective vasodilators in the treatment of hypertension and post-ischaemic stroke vasospasm. The review provides a deeper understanding of the various roles Rho/ROCK plays in angiogenesis, tumour vascular tone and the tumour microenvironment and explores how ROCK inhibitors may be effective vascular normalising agents. ROCK inhibitors have the potential to enhance the delivery and efficacy of chemotherapy agents and improve the effectiveness of radiotherapy. Inhibition of the Rho/ROCK pathway may have under-appreciated effects which a comprehensive understanding of, may allow us to use these agents to our best advantage.

Highlights

- We explore the heterogeneity of the anti-proliferative effects of ROCK inhibitors
- The Rho/ROCK pathway is critical in many aspects of angiogenesis
- ROCK inhibitors may be effective vascular normalising and provascular agents
- The Rho/ROCK signalling is critical in the tumour microenvironment
- ROCK inhibitors may increase the delivery and efficacy of chemotherapeutic agents

Keywords: Chemoresistance, ECM, collagen, Rho GTPase, ROCK inhibitors, cancer stroma, vascular normalisation, provascular strategy, tumour oxygenation.

Cancer is now one of the leading causes of death worldwide, accounting for 8.2 million deaths in 2012 [1]. Although therapies for advanced disease are improving, overall, therapeutic options for patients are limited. In general, efficacy of chemotherapeutic agents is limited by adverse effects caused by their effects on normal tissues. Therefore, adjunctive treatments which specifically improve the delivery of cytotoxic therapies to the tumour may be of high value. Further, the efficacy of adjunctive therapies needs to be examined with regard to the effects on both tumour cells and the surrounding microenvironment.

The Rho/ROCK signalling pathway has been studied in both the fields of cancer and cardiovascular research. Its involvement in cellular proliferation and invasion make it an attractive target in cancer medicine however the full potential of ROCK inhibitors as anti-cancer therapies may not have been fully appreciated. The effects of the Rho/ROCK pathway on the vascular system have been extensively studied in the treatment of vascular disorders. Inhibition of Rho signalling within the hypoxic and abnormal tumour vasculature may lead to an improved anti-tumour efficacy of cytotoxic agents through the normalisation of the vascular supply to tumours. Moreover, the effects of ROCK inhibition on other key components of the tumour microenvironment, including activated (myo)fibroblasts, immune cells and extracellular matrix (ECM), may have an additional therapeutic value. This review summarises our current understanding of the diverse and complex roles of aberrant Rho/ROCK signalling in tumour development and progression, highlighting new avenues for the utilisation of ROCK inhibitors as anti-cancer therapy, increasingly in the context of modulating the tumour microenvironment.

The Rho family of small GTPases regulate a diverse array of cellular processes, including cytoskeletal dynamics, cell polarity, membrane transport and gene expression, which are integral in the growth and metastatic potential of cancer cells. The three best characterised members of this family are Rho (A, B & C), Rac (1, 2 & 3) and Cdc42[2]. They cycle between a GTP-bound active state and GDP-bound inactive state which is mediated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)[3, 4]. In their active state, they act on one of over 60 downstream targets which include Rho-associated

coiled-coil containing protein kinase (ROCK), mDia, PAK4-6, Par6 and WASP[5]. Although these members of the Rho-GTPase family all have distinct functions, they broadly regulate the polarization, migration, proliferation and survival of cells [2, 6]. ROCK, a downstream effector of Rho, has specific inhibitors including Y-27632 and Fasudil, which prevent activation of ROCK by competing with ATP for binding to the kinase [7-9]. Interestingly, fasudil has been shown to be safe for use in humans for the treatment of cerebral vasospasm with an acceptable side effect profile, making it an attractive drug for study [10].

Exploring the effects of inhibiting Rho/ROCK in cancer: the pre-clinical evidence

Numerous studies have thus far investigated the therapeutic efficacy of Rho/ROCK inhibition in various *in vitro* and *in vivo* models of cancer (summarised in Table 1). What can be ascertained from Table 1, is that blocking Rho-ROCK signalling in cancers cells can effectively reduce cellular proliferation, invasion, angiogenesis *in vitro* and reduce tumour growth and metastasis formation *in vivo*. What is interesting to note, however, is that the effects on proliferation are heterogeneous, with several studies reporting no effects at all [11-16], one study reporting an anti-proliferative effect of fasudil only when used at a high concentration [17], and one study only reporting anti-proliferative effects when combined with cisplatin [18]. Even more intriguing, is that of the studies reporting no effects on cellular proliferation *in vitro*, some report an effect on cellular invasion [11-14] and several go on to demonstrate an effect on the metastasis formation [11, 13, 14, 16] or average tumour size *in vivo* [15]. This disparity may be partially explained by the critical role RhoA plays in cellular invasion [19], but perhaps this is more reflective of the complex involvement Rho-ROCK has in cellular processes in cancer that cannot be accurately recapitulated in simple 2D assays. A deeper understanding of Rho/ROCK signalling activation *in vivo* is necessary to fully characterise the importance of inhibiting this pathway in cancer medicine.

The Rho/ROCK pathway is critical in angiogenesis and inhibition may result normalisation of tumour vasculature

Sustained angiogenesis is one of the hallmarks of tumour progression [20]. It is well documented that in response to tissue hypoxia, angiogenesis is constantly stimulated resulting in a highly abnormal vasculature [21, 22]. These vessels are immature, tortuous, have increased permeability and lead to intra-tumoural hypoxia, which can mediate resistance to anticancer therapies [23]. Moreover, hypoxia can lead to a more aggressive phenotype of cancer cell, which are more likely to metastasize. Angiogenesis is a complex process, which is largely controlled by VEFG and its membrane receptors. To begin the angiogenic process, endothelial cells (ECs) lose junctional integrity and increase permeability [24, 25]. There is degradation of the basement membrane and remodeling of the extracellular matrix (ECM) [26, 27], to allow ECs to migrate, proliferate and ultimately undergo morphogenesis in order for new vessels to develop.

The Rho/ROCK pathway has been shown to be an integral part of VEGF mediated angiogenesis and can not only be implicated in VEGF signalling, but also in many of the processes necessary for angiogenesis to occur. The Rho-ROCK pathway has been shown to be critical in VEGF induced angiogenesis [28]. It has been shown that adherin junctions between ECs need to be loosened in order for EC migration and proliferation to occur [29]. Rho/ROCK signalling acts via p-MLC to breakdown intracellular junctions and thereby increases vascular permeability [30]. In order for ECs to invade surrounding tissue and form new vessels, the basement membrane [31] and ECM must be disrupted via MMP secretion. Rho/ROCK activation has been shown to stimulate MMP-9 secretion [32] and is also linked to the MMP expression of tumours [33]. Once the BM and ECM are disrupted, EC migration and tube formation can occur. van Nieuw Amerongen *et al* [34] used HUVECs to show that not only does VEGF-induced changes in the EC cytoskeleton depend on RhoA, but also that growth of hMVECs into a fibrin matrix in response to VEGF is inhibited by Y-27632, suggesting that the Rho/ROCK pathway is necessary for ingrowth of ECs. Bryan *et al* [35] showed that disruption of the Rho/ROCK pathway inhibits VEGF-mediated changes to the cytoskeleton in ECs and also that ECs treated with Y-27632 failed to

assemble into recognisable vessel structures, highlighting the importance of the Rho/ROCK pathway in vasculogenesis. Hoang and Uchida [36, 37] both showed that inhibiting Rho/ROCK prevented ECs from forming organized vascular structures in a matrix. As the Rho/ROCK pathway has been established as being critical to multiple steps in angiogenesis, many studies have attempted to elucidate the importance of its involvement in the cancer setting. Croft *et al* [38] used a conditionally active form of ROCK II in colon carcinoma cells to show that increased ROCK signalling promoted increased tumour angiogenesis and tumour cell invasion *in vivo*. Using HUVEC and glioma cell co-culture techniques, Nakabayashi *et al* [17] showed that the ROCK inhibitor HA1077 suppressed tumour-induced angiogenesis and the migration of HUVEC cells through a transwell plate. Moreover, the same group also showed that the growth of T98G glioma xenografts was significantly inhibited when tumour-bearing mice were treated daily with HA1077 [17]. ROCK inhibitors also showed significant promise as anti-angiogenic agents in additional *in* vivo models, specifically HA1077 in Nakajima *et al* [12] used Lewis lung carcinoma cells to show that administration of the ROCK inhibitor Wf-536, reduced the number of spontaneous metastases and impaired angiogenesis *in vivo*. Somlyo *et al* [39] showed that mice bearing xenotransplants of PC3 cells had a reduction in tumour volume and increased survival when treated with Wf-536 combined with marimastat (MMP inhibitor). ROCK inhibitors have not been used in human trials *per se* however, much clinical data exists regarding the effects of VEGF inhibitors on various cancer subtypes. Using the modest effects that have been demonstrated thus far to guide us, perhaps employing a strategic use of ROCK inhibitors will allow us to fully realise their potential.

Rho/ROCK Inhibitors as vascular normalising agents

Clinical use of anti-angiogenic agents has generated disappointing results when used as monotherapy [40], but more success has been had when these agents are combined with cytotoxic chemotherapy. Some explanation for this may be that continual VEGF inhibition results in the accumulation of resistance mechanisms [41-44], and that excessive reduction in tumour vasculature renders it inefficient

for drug delivery [45]. VEGF inhibition can increase tumour oxygenation when used in a transient manner, a process called vascular normalisation [46-48]. Exploiting this process to improve the efficacy of standard cytotoxic therapies is attractive. Several pre-clinical and clinical studies have explored this concept thus far: In several *in vivo* solid tumour models, pre-treatment of tumour-breaing mice with a VEGF neutralizing antibody prior to radiation, lead to significant inhibition of tumour growth compared to radiation alone [49]. Lee *et al* [50] concluded that blocking VEGF in glioblastoma or colon adenocarcinoma compensates for hypoxia induced radiation resistance. They showed that using an anti-VEGF antibody resulted in greater tumour growth delay when combined with radiation, than radiation alone. Falcon *et al* [51] demonstrated that PDGF-B blockade on Lewis lung carcinoma tumours increased tumour vessel efficiency *in vivo*. They also showed that the combination of imatanib with cyclophosphamide improves the delivery of cyclophosphamide to the tumour and the tumour burden was reduced *in vivo*. The Rho/ROCK pathway has been specifically tested: Arder *et al* [52] *in vivo* induction of dominant negative Rho (RhoBN19) to show that inhibiting Rho decreased tumour cell survival after irradiation and moreover, tumours had improved oxygenation and decreased vessel density. The critical aspect of optimal timing of administration of combinations involving anti-VEGF therapies and cytotoxic agents was further explored by Winkler *et al* [53]. Treatment of glioblastoma xenografts with a VEGFR2 monoclonal resulted in a significant reduction in tumour hypoxia on day 2 that was almost abolished on day 5 and increased again on day 8. Further, radiation therapy produced a synergistic effect when given on days 4-6. This suggests that after VEGFR blockade, there is an initial increase in tumour oxygenation during which, the effects of radiation therapy are increased, but with continual VEGFR blockade, the tumour becomes hypoxic and this synergism with radiation is lost. Several randomised trials have shown that the addition of bevacizumab to chemotherapy and radiotherapy improves progression free survival in patients with central nervous system malignancies [54, 55], and a phase I trial specifically testing the vascular normalisation strategy have shown this holds considerable promise in patient care. Here, patients with rectal cancer receiving neoadjuvant chemotherapy plus radiation, were exposed to the VEGF inhibitor, bevacizumab.

Interestingly, bevacizumab treatment led to normalisation of the tumour vasculature, increased tumour cell apoptosis and resulted in a complete pathological response in two patients [56]. Therefore, Rho/ROCK pathway inhibitors may be particularly useful as vascular normalising agents, increasing the effectiveness of conventional cytotoxic therapies by affecting the VEGF signalling pathway.

Rho/ROCK inhibitors have the potential to act as provascular agents

In addition to normalising the tumour vasculature, a provascular strategy has been explored where transient vasodilation improves blood supply and exposure of tumour cells to circulating chemotherapeutics and/or sensitises cells to radiation. Because most vasodilators dilate both the tumour and systemic vasculature, there can be unpredictable effects on the tumour vasculature. If the tumour vessels are in series with the systemic circulation, systemic vasodilation can increase tumour blood flow, however if the tumour vasculature is in parallel, then systemic vasodilation will cause a reduction in tumour blood flow (vascular steal phenomenon) [57]. An ideal provascular agent, therefore, would be one that preferentially targets the tumour vascular bed. There have been a number of studies that have shown some success with this strategy, suggesting the idea has merit. Gallez and Sonveaux [58, 59] both demonstrated that it is possible to increase tumour blood flow using vasodilators. Jordan and Sonveaux [60, 61] used pre-clinical *in vivo* models to show that administration of nitric oxide not only increased tumour blood flow, but sensitised tumours to the effects of radiation. Wood *et al* [62] showed that calcium channel blockers used at small doses increased radiosensitivity of tumous, however when used in high doses, actually reduced tumour perfusion. A systematic review of clinical trials assessing the effects of improving tumour oxygenation to radiosensitise tumours, suggests there may be clinical benefit with an odds ratio of 0.77 [63]. In terms of improving the delivery of chemotherapy, Masunaga [64] described an increased cellular uptake of cisplatin when tumour bearing mice were injected with nicotinamide. Martinive [65] also showed that using an endothelin-1 receptor antagonist improved access of cyclophosphamide to the tumour

compartment. With this strategy in mind, it would be interesting to explore the vasodilatory effects of ROCK inhibitors. ROCK inhibitors reduce vasospasm via reduction in smooth muscle contraction and down regulation of endothelial nitric oxide synthase, leading to their use in the treatment of ischaemic stroke [10, 66, 67], with significant efficacy in reducing post stroke cerebral vasospasm and showing an acceptable side effect profile. ROCK inhibitors have been shown to normalise smooth muscle contraction and suppress vascular lesion formation, making them a therapy of interest in hypertension, pulmonary hypertension, hypertensive vascular disease and ischaemic heart disease [68], [69]. It is possible therefore, that Rho-ROCK inhibitors can act as provascular agents, improving tumour blood flow and increasing exposure of cells to chemotherapy and/or sensitising cells to the effects of radiation.

The importance of the Rho/ROCK pathway in the tumour microenvironment

A deeper understanding of the effects of the tumour microenvironment on the behaviour of cancer cells is interestingly being gained. The interplay between tumour cells, stromal cells and ECM affect cancer initiation, progression, metastasis and also, chemoresistance [70]. Rho GTPases have been shown to be implicit in a number of stromal processes that contribute to the invasiveness and metastatic potential of cancer cells. It has been long understood that the presence of high density stroma in breast tissue confer an increased risk of developing breast cancer. Women with high mammographic densities have increased proliferation of stromal or epithelial tissue on histological examination and this has been correlated with an increased risk of breast cancer. It was further hypothesised that interactions between the stroma and epithelium ultimately lead to cancer formation [71]. In an effort to better understand this phenomenon, Lisanti *et al* [72] conducted genome-wide transcriptional profiling of low density (LD) breast fibroblasts, compared with high density (HD) breast fibroblasts. This revealed several key processes including stress response, inflammation, stemness and signal transduction. The authors postulated that the presence of HD fibroblasts could be considered a pre-cancerous phenotype and

that Rho GTPases (along with JNK1, iNOS, FGF-R, EGF-R and PDGF-R) were identified as a key biological processes here [72]. Further work in breast cancer has shown that breast cancer cells grown in a 3D floating matrix differentiate into tubular structures, however if the same matrix is attached to the dish, the cells do not differentiate, but proliferate and spread [73]. In the same study, differentiation could be disrupted by increasing the density of the matrix. Interestingly, it was also shown that tubulogenesis required contraction of the 3D matrix which was dependent on the Rho-ROCK pathway and that RhoA activity was found to be down-regulated in differentiated cells [73]. Following on from this work, it was shown that p190RhoGAP-B mediates down regulation of RhoA activity and ductal morphogenesis. RhoA activity was reduced at cell-cell adhesions versus activity at cell-ECM adhesions [74]. The stromal compartment of tumours has long been thought to contribute to the aggressive phenotype of cancers, and cancer associated fibroblasts (CAFs) have been shown to provide tumour cells with proliferative and anti-appototic signals affecting angiogenesis and ECM remodelling. Cadamuro *et al* [75] showed that PDGF-D plays a major role in CAF recruitment and acts on the Rho/ROCK pathway to promote fibroblast migration. Also, it has been shown that an increase in palladin expression of CAFs is associated with increased growth and metastasis of PC cells by increasing their ability to remodel the ECM and thereby promotes tumour invasion [76]. Gaggioli *et al* [77] demonstrated that SCC cells required fibroblasts to invade a 3D organotypic matrix. They went on to show that inhibition of Rho/ROCK in fibroblasts (not in the SCC cells) reduced invasion of the SCC cells. This suggests that the presence of fibroblasts is necessary for cancer cell invasion and that the Rho/ROCK pathway is critical here. Similarly, Sanz-Moreno *et al* [78] demonstrated a role for cytokine signaling through GP130-IL6ST/JAK1 to regulate ROCK dependent actomyosin contraction, which drives matrix remodeling by CAFs and migration of melanoma cells. *In vivo*, cells must breach the endothelial barrier to metastasise [79]. The process of intercalation is where cancer cells first adhere to ECs, open the EC junctions, stimulate EC retraction and then insert into the endothelial monolayer. It has been shown that Cdc42 depletion impairs intercalation in PC3 cells and also that Cdc42, RAC1 and RhoA impair EC junction opening. Mice injected with Cdc42

depleted PC3 cells developed significantly less metastasis, highlighting the importance of the RhoGTPases in intercalation [80]. It is clear that the Rho-ROCK pathway is critically important in the functioning of the tumour microenvironment, particularly in the processes which must be undertaken for a cell to move through the ECM and metastasise.

Conclusion

The Rho/ROCK pathway has been a popular field of study for cancer researchers, but unfortunately, despite ROCK inhibitors being demonstrated to be safe for human use, these drugs have not yet made it to the clinic. These agents have well documented effects on cellular proliferation, however their effects on cell invasion, tumour growth and metastasis appear to be more robust. Taking this into account, a more broad view of how the Rho-ROCK pathway affects cellular function in the cancer cell is required, to elucidate how these drugs may be most effectively used to treat cancer. In addition to their effects on cellular proliferation and movement, these agents modulate angiogenesis and vascular tone and thus could potentially improve the delivery and efficacy of standard cytotoxic therapies. The Rho/ROCK pathway is also important in regulating the dynamic cross-talk between tumour cells and their microenvironment which may also be therapeutically exploited to inhibit metastasis formation. Finally, the therapeutic potential of ROCK inhibitors as an adjunct to cytotoxic chemotherapy is yet to be realised and systematically examined. Further understanding of Rho pathway modulating in the various tumour compartments will determine whether the inhibitors to Rho GTPase signalling are best used for newly diagnosed or recurrent tumours and will establish the optimum combinations with radiation, cytotoxic chemotherapy, and other targeted molecular compounds. Importantly, these agents may improve the delivery of chemotherapy to the tumour, perhaps enhancing efficacy, reducing the effective dose required or overcoming some of the chemoresistance mechanisms.

Study	Cells used	Inhibitor used	Effect on proliferation	Effect on invasion	Effects on angiogenesis	In vivo	Additional
Abe [81]	Bladder cancer cells UM-UC3	HA-1077	↓	↓	-	-	↓ migration
Deng 2010 [82]	Human GBM T98G	Fasudil	↓	↓	-	Reduction in tumour growth, invasion and prolonged survival	
Genda 1999 [83]	Human HCC PLC-PRF-5 & HepG2	Dominant negative p160 ROCK	-	-	-	Dominant negative transfected cells had a reduced number of metastases	Dominant negative transfected cells had reduced cell motility
Horiuchi 2008 [84]	Ovarian SKOV3, OVCAR3	Y-27632, Lovastatin	-	↓	-	Reduction in number of metastatic sites when treated with Lovastatin	
Igishi 2003 [85]	Lung carcinoma A549	Y-27632	↓ when Y-27632 given prior to cisplatin	-	-	-	
Itoh 1999 [11]	Rat hepatoma MM1	Y-27632	No effect	↓	-	Reduced incidence of tumour dissemination, number of metastases and volume of ascites	
Lane 2008 [86]	Human breast MDA-MB-231	Y-27632; Knock down of ROCK I & II	-	↓	-	No difference in tumour volume when knockdown cells were injected into mice	
Liu 2009 [87]	Human breast MCF-7, MDA-MB-231 & SUM 1315	Y-27632	↓	↓	-	No effect on tumour weight but reduction in number of metastases	
Nakabayashi 2011 [17]	Human glioma T98G & U87MG	Fasudil	Reduced at 100uM	-	↓	Reduction in average tumour volume	
Nakajima 2003 [12]	Mouse Lewis lung cancer	Wf-536	No effect	↓	↓	Reduced angiogenesis	
Nakajima 2003 [13]	Mouse melanoma B16BL6 & B16F10	Wf-536	No effect	↓	-	Reduction in number of metastases. Synergistic effect seen with paclitaxel	
Nakajima 2003 [88]	Human fibrosarcoma Ht1080	Wf-536	-	↓	-	-	Reduction in HGF induced hyperpermeability of ECV304 cells
Ogata 2009 [14]	Human ovarian Caov-3 & SKOV3ip1	Fasudil	No effect	↓ LPA induced invasion	-	Reduction in tumour burden and ascites (SKOV3ip1)	
Ohta 2012 [18]	Human cancer A2780 (cis sensitive) & A2780CP (cis resistant)	Fasudil, Y-27632	↓ in combination with cisplatin	-	-	-	Knockdown of ROCK I & II increased antiproliferative effects of cisplatin
Pille 2005 [89]	Human breast MDA-MB-231	anti-RhoA/C siRNA	↓	↓	↓	Reduction in tumour volume and vascularisation	
Routhier 2010 [15]	Mouse B16F1, Human uveal UvMel 1.3, 1.5 & 270	Y-27632	No effect	↓	-	Reduction in average tumour volume (B16F1 cells)	
Somlyo 2000 [16]	Human prostate PC3	Y-27632	No effect	-	↓	Reduction in number and size of tumours, increased survival	
Somlyo 2003 [39]	Human prostate PC3	Wf-536	-	-	↓	No effect on in vivo growth alone. When combined with Marimastat, reduced tumour volume with or without paclitaxel	
Takamura 2001 [90]	Human HCC Li7	Y-27632	-	-	-	Reduction in number of metastases	
Ueno 2011 [91]	Clear renal cell A-498, 769-P	Knock down of ROCK1	-	↓	-	-	
Voorneveld [92]	HCT116, HT29 colorectal cancer cells	Y-27632	-	-	-	Reduction in number of metastases formed	↓ migration
Xue 2008 [33]	Murine HCC CB0140C12	Y-27632	-	↓		Reduction in number and size of tumours.	Treated tumours had reduced MMP-9 staining
Yang 2010 [93]	Human lung 95D	Fasudil	↓	↓	-	-	Reduced adhesion demonstrated
Ying 2006 [94]	Breast MDA-MB-231, fibrosarcoma HT1080, rat hepatoma MM1	Fasudil	↓	-	-	Reduction in peritoneal dissemtination (MM1), lung seeding (HT1080) and increase in number of tumour free mice (MDA-MB-231)	
Zhang [95]	Prostate cancer PC 3 cells	Y-27632	↓	-	-	Reduction in size of tumours and number of metastases formed	
Zhu 2011 [96]	Lung cancer A549	Fasudil	↓	↓	-	-	
Zohrabian 2009 [97]	Human GBM LN-18	Y-27632	↓	-	-	-	

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1. Globocan. GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. In. International Agency for Research on Cancer 2012.
2. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002; 420: 629-635.
3. Hart MJ, Eva A, Evans T et al. Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. Nature 1991; 354: 311-314.
4. Ueda T, Kikuchi A, Ohga N et al. Purification and characterization from bovine brain cytosol of a novel regulatory protein inhibiting the dissociation of GDP from and the subsequent binding of GTP to rhoB p20, a ras p21-like GTP-binding protein. The Journal of biological chemistry 1990; 265: 9373-9380.
5. Bishop AL, Hall A. Rho GTPases and their effector proteins. The Biochemical journal 2000; 348 Pt 2: 241-255.
6. Rath N, Olson MF. Rho-associated kinases in tumorigenesis: re-considering ROCK inhibition for cancer therapy. EMBO reports 2012.
7. Narumiya S, Ishizaki T, Uehata M. Use and properties of ROCK-specific inhibitor Y-27632. Methods in enzymology 2000; 325: 273-284.
8. Breitenlechner C, Gassel M, Hidaka H et al. Protein kinase A in complex with Rho-kinase inhibitors Y-27632, Fasudil, and H-1152P: structural basis of selectivity. Structure 2003; 11: 1595-1607.
9. Ishizaki T, Uehata M, Tamechika I et al. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. Molecular pharmacology 2000; 57: 976-983.
10. Shibuya M, Hirai S, Seto M et al. Effects of fasudil in acute ischemic stroke: results of a prospective placebo-controlled double-blind trial. Journal of the neurological sciences 2005; 238: 31-39.
11. Itoh K, Yoshioka K, Akedo H et al. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. Nature medicine 1999; 5: 221-225.
12. Nakajima M, Hayashi K, Katayama K et al. Wf-536 prevents tumor metastasis by inhibiting both tumor motility and angiogenic actions. European journal of pharmacology 2003; 459: 113-120.
13. Nakajima M, Hayashi K, Egi Y et al. Effect of Wf-536, a novel ROCK inhibitor, against metastasis of B16 melanoma. Cancer chemotherapy and pharmacology 2003; 52: 319-324.
14. Ogata S, Morishige K, Sawada K et al. Fasudil inhibits lysophosphatidic acid-induced invasiveness of human ovarian cancer cells. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 2009; 19: 1473-1480.
15. Routhier A, Astuccio M, Lahey D et al. Pharmacological inhibition of Rho-kinase signaling with Y-27632 blocks melanoma tumor growth. Oncology reports 2010; 23: 861-867.
16. Somlyo AV, Bradshaw D, Ramos S et al. Rho-kinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells. Biochemical and biophysical research communications 2000; 269: 652-659.
17. Nakabayashi H, Shimizu K. HA1077, a Rho kinase inhibitor, suppresses glioma-induced angiogenesis by targeting the Rho-ROCK and the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signal pathways. Cancer science 2011; 102: 393-399.

18. Ohta T, Takahashi T, Shibuya T et al. Inhibition of the Rho/ROCK pathway enhances the efficacy of cisplatin through the blockage of hypoxia-inducible factor-1alpha in human ovarian cancer cells. Cancer Biology & Therapy 2012; 13: 25-33.
19. Timpson P, McGhee EJ, Morton JP et al. Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53. Cancer Research 2011; 71: 747-757.
20. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.
21. Gazit Y, Baish JW, Safabakhsh N et al. Fractal characteristics of tumor vascular architecture during tumor growth and regression. Microcirculation 1997; 4: 395-402.
22. Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. Microcirculation 2010; 17: 206-225.
23. Gerlowski LE, Jain RK. Microvascular permeability of normal and neoplastic tissues. Microvascular research 1986; 31: 288-305.
24. Gavard J, Gutkind JS. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. Nature cell biology 2006; 8: 1223-1234.
25. Esser S, Lampugnani MG, Corada M et al. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. Journal of cell science 1998; 111 (Pt 13): 1853-1865.
26. Liu Y, Senger DR. Matrix-specific activation of Src and Rho initiates capillary morphogenesis of endothelial cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2004; 18: 457-468.
27. Montesano R, Orci L. Tumor-promoting phorbol esters induce angiogenesis in vitro. Cell 1985; 42: 469-477.
28. Yin L, Morishige K, Takahashi T et al. Fasudil inhibits vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. Molecular Cancer Therapeutics 2007; 6: 1517-1525.
29. Dejana E. Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. The Journal of clinical investigation 1996; 98: 1949-1953.
30. Wojciak-Stothard B, Ridley AJ. Rho GTPases and the regulation of endothelial permeability. Vascular pharmacology 2002; 39: 187-199.
31. Mikulits W, Schranzhofer M, Bauer A et al. Impaired ferritin mRNA translation in primary erythroid progenitors: shift to iron-dependent regulation by the v-ErbA oncoprotein. Blood 1999; 94: 4321-4332.
32. Turner NA, O'Regan DJ, Ball SG, Porter KE. Simvastatin inhibits MMP-9 secretion from human saphenous vein smooth muscle cells by inhibiting the RhoA/ROCK pathway and reducing MMP-9 mRNA levels. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2005; 19: 804-806.
33. Xue F, Takahara T, Yata Y et al. Blockade of Rho/Rho-associated coiled coil-forming kinase signaling can prevent progression of hepatocellular carcinoma in matrix metalloproteinase-dependent manner. Hepatology research : the official journal of the Japan Society of Hepatology 2008; 38: 810-817.

34. van Nieuw Amerongen GP, Koolwijk P, Versteilen A, van Hinsbergh VW. Involvement of RhoA/Rho kinase signaling in VEGF-induced endothelial cell migration and angiogenesis in vitro. Arteriosclerosis, thrombosis, and vascular biology 2003; 23: 211-217.
35. Bryan BA, Dennstedt E, Mitchell DC et al. RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2010; 24: 3186-3195.
36. Hoang MV, Whelan MC, Senger DR. Rho activity critically and selectively regulates endothelial cell organization during angiogenesis. Proceedings of the National Academy of Sciences of the United States of America 2004; 101: 1874-1879.
37. Uchida S, Watanabe G, Shimada Y et al. The suppression of small GTPase rho signal transduction pathway inhibits angiogenesis in vitro and in vivo. Biochemical and biophysical research communications 2000; 269: 633-640.
38. Croft DR, Sahai E, Mavria G et al. Conditional ROCK activation in vivo induces tumor cell dissemination and angiogenesis. Cancer Research 2004; 64: 8994-9001.
39. Somlyo AV, Phelps C, Dipierro C et al. Rho kinase and matrix metalloproteinase inhibitors cooperate to inhibit angiogenesis and growth of human prostate cancer xenotransplants. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2003; 17: 223-234.
40. Giantonio BJ, Catalano PJ, Meropol NJ et al. Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2007; 25: 1539-1544.
41. Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer cell 2005; 8: 299-309.
42. Fernando NT, Koch M, Rothrock C et al. Tumor escape from endogenous, extracellular matrix-associated angiogenesis inhibitors by up-regulation of multiple proangiogenic factors. Clinical cancer research : an official journal of the American Association for Cancer Research 2008; 14: 1529-1539.
43. Bergers G, Song S, Meyer-Morse N et al. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. The Journal of clinical investigation 2003; 111: 1287-1295.
44. Rubenstein JL, Kim J, Ozawa T et al. Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption. Neoplasia 2000; 2: 306-314.
45. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nature clinical practice. Oncology 2006; 3: 24-40.
46. Hansen-Algenstaedt N, Stoll BR, Padera TP et al. Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockade, hormone ablation, and chemotherapy. Cancer Research 2000; 60: 4556-4560.

47. Hoang T, Huang S, Armstrong E et al. Enhancement of radiation response with bevacizumab. Journal of experimental & clinical cancer research : CR 2012; 31: 37.
48. Rao SS, Thompson C, Cheng J et al. Axitinib sensitization of high Single Dose Radiotherapy. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2014; 111: 88-93.
49. Gorski DH, Beckett MA, Jaskowiak NT et al. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. Cancer Research 1999; 59: 3374-3378.
50. Lee CG, Heijn M, di Tomaso E et al. Anti-Vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. Cancer Research 2000; 60: 5565-5570.
51. Falcon BL, Pietras K, Chou J et al. Increased vascular delivery and efficacy of chemotherapy after inhibition of platelet-derived growth factor-B. The American journal of pathology 2011; 178: 2920-2930.
52. Ader I, Delmas C, Bonnet J et al. Inhibition of Rho pathways induces radiosensitization and oxygenation in human glioblastoma xenografts. Oncogene 2003; 22: 8861-8869.
53. Winkler F, Kozin SV, Tong RT et al. Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer cell 2004; 6: 553-563.
54. Lai A, Tran A, Nghiemphu PL et al. Phase II study of bevacizumab plus temozolomide during and after radiation therapy for patients with newly diagnosed glioblastoma multiforme. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2011; 29: 142-148.
55. Narayana A, Kunnakkat SD, Medabalmi P et al. Change in pattern of relapse after antiangiogenic therapy in high-grade glioma. International journal of radiation oncology, biology, physics 2012; 82: 77-82.
56. Willett CG, Boucher Y, Duda DG et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2005; 23: 8136-8139.
57. Sonveaux P. Provascular strategy: targeting functional adaptations of mature blood vessels in tumors to selectively influence the tumor vascular reactivity and improve cancer treatment. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 2008; 86: 300-313.
58. Gallez B, Jordan BF, Baudelet C, Misson PD. Pharmacological modifications of the partial pressure of oxygen in murine tumors: evaluation using in vivo EPR oximetry. Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine 1999; 42: 627-630.
59. Sonveaux P, Dessy C, Martinive P et al. Endothelin-1 is a critical mediator of myogenic tone in tumor arterioles: implications for cancer treatment. Cancer Research 2004; 64: 3209-3214.

60. Jordan BF, Beghein N, Aubry M et al. Potentiation of radiation-induced regrowth delay by isosorbide dinitrate in FSaII murine tumors. International journal of cancer. Journal international du cancer 2003; 103: 138-141.
61. Sonveaux P, Dessy C, Brouet A et al. Modulation of the tumor vasculature functionality by ionizing radiation accounts for tumor radiosensitization and promotes gene delivery. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2002; 16: 1979-1981.
62. Wood PJ HD. Modification of tumour response by calcium antagonists in the SCVII/St tumour implanted at two different sites. International journal of radiation biology 1989; 56: 355.
63. Overgaard J. Hypoxic radiosensitization: adored and ignored. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2007; 25: 4066-4074.
64. Masunaga S, Ono K, Akuta K et al. Enhancement of chemosensitivity of quiescent cell populations in murine solid tumors using nicotinamide. Chemotherapy 1994; 40: 418-426.
65. Martinive P, De Wever J, Bouzin C et al. Reversal of temporal and spatial heterogeneities in tumor perfusion identifies the tumor vascular tone as a tunable variable to improve drug delivery. Molecular Cancer Therapeutics 2006; 5: 1620-1627.
66. Shibuya T, Watanabe Y. [Central effect of Ca2+ channel blockers: multiple sites of action]. Nihon yakurigaku zasshi. Folia pharmacologica Japonica 1992; 100: 239-247.
67. Rikitake Y, Kim HH, Huang Z et al. Inhibition of Rho kinase (ROCK) leads to increased cerebral blood flow and stroke protection. Stroke; a journal of cerebral circulation 2005; 36: 2251-2257.
68. Oka M, Fagan KA, Jones PL, McMurtry IF. Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension. British journal of pharmacology 2008; 155: 444-454.
69. Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. Trends in pharmacological sciences 2007; 28: 296-302.
70. Tredan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. Journal of the National Cancer Institute 2007; 99: 1441-1454.
71. Boyd NF, Lockwood GA, Byng JW et al. Mammographic densities and breast cancer risk. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 1998; 7: 1133-1144.
72. Lisanti MP, Tsirigos A, Pavlides S et al. JNK1 stress signaling is hyper-activated in high breast density and the tumor stroma: connecting fibrosis, inflammation, and stemness for cancer prevention. Cell cycle 2014; 13: 580-599.
73. Wozniak MA, Desai R, Solski PA et al. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. The Journal of cell biology 2003; 163: 583-595.
74. Ponik SM, Trier SM, Wozniak MA et al. RhoA is down-regulated at cell-cell contacts via p190RhoGAP-B in response to tensional homeostasis. Molecular biology of the cell 2013; 24: 1688-1699, S1681-1683.

75. Cadamuro M, Nardo G, Indraccolo S et al. Platelet-derived growth factor-D and Rho GTPases regulate recruitment of cancer-associated fibroblasts in cholangiocarcinoma. Hepatology 2013; 58: 1042-1053.
76. Goicoechea SM, Garcia-Mata R, Staub J et al. Palladin promotes invasion of pancreatic cancer cells by enhancing invadopodia formation in cancer-associated fibroblasts. Oncogene 2014; 33: 1265-1273.
77. Gaggioli C, Hooper S, Hidalgo-Carcedo C et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nature cell biology 2007; 9: 1392-1400.
78. Sanz-Moreno V, Gaggioli C, Yeo M et al. ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. Cancer cell 2011; 20: 229-245.
79. Friedl P, Maaser K, Klein CE et al. Migration of highly aggressive MV3 melanoma cells in 3-dimensional collagen lattices results in local matrix reorganization and shedding of alpha2 and beta1 integrins and CD44. Cancer Research 1997; 57: 2061-2070.
80. Reymond N, Im JH, Garg R et al. Cdc42 promotes transendothelial migration of cancer cells through beta1 integrin. The Journal of cell biology 2012; 199: 653-668.
81. Abe H, Kamai T, Hayashi K et al. The Rho-kinase inhibitor HA-1077 suppresses proliferation/migration and induces apoptosis of urothelial cancer cells. BMC cancer 2014; 14: 412.
82. Deng L, Li G, Li R et al. Rho-kinase inhibitor, fasudil, suppresses glioblastoma cell line progression in vitro and in vivo. Cancer Biology & Therapy 2010; 9: 875-884.
83. Genda T, Sakamoto M, Ichida T et al. Cell motility mediated by rho and Rho-associated protein kinase plays a critical role in intrahepatic metastasis of human hepatocellular carcinoma. Hepatology 1999; 30: 1027-1036.
84. Horiuchi A, Kikuchi N, Osada R et al. Overexpression of RhoA enhances peritoneal dissemination: RhoA suppression with Lovastatin may be useful for ovarian cancer. Cancer science 2008; 99: 2532-2539.
85. Igishi T, Mikami M, Murakami K et al. Enhancement of cisplatin-induced cytotoxicity by ROCK inhibitor through suppression of focal adhesion kinase-independent mechanism in lung carcinoma cells. International journal of oncology 2003; 23: 1079-1085.
86. Lane J, Martin TA, Watkins G et al. The expression and prognostic value of ROCK I and ROCK II and their role in human breast cancer. International journal of oncology 2008; 33: 585-593.
87. Liu S, Goldstein RH, Scepansky EM, Rosenblatt M. Inhibition of rho-associated kinase signaling prevents breast cancer metastasis to human bone. Cancer Research 2009; 69: 8742-8751.
88. Nakajima M, Katayama K, Tamechika I et al. WF-536 inhibits metastatic invasion by enhancing the host cell barrier and inhibiting tumour cell motility. Clinical and experimental pharmacology & physiology 2003; 30: 457-463.
89. Pille JY, Denoyelle C, Varet J et al. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. Molecular therapy : the journal of the American Society of Gene Therapy 2005; 11: 267-274.

90. Takamura M, Sakamoto M, Genda T et al. Inhibition of intrahepatic metastasis of human hepatocellular carcinoma by Rho-associated protein kinase inhibitor Y-27632. Hepatology 2001; 33: 577-581.
91. Ueno K, Hirata H, Shahryari V et al. Tumour suppressor microRNA-584 directly targets oncogene Rock-1 and decreases invasion ability in human clear cell renal cell carcinoma. British journal of cancer 2011; 104: 308-315.
92. Voorneveld PW, Kodach LL, Jacobs RJ et al. Loss of SMAD4 alters BMP signaling to promote colorectal cancer cell metastasis via activation of Rho and ROCK. Gastroenterology 2014; 147: 196-208 e113.
93. Yang X, Zhang Y, Wang S, Shi W. Effect of fasudil on growth, adhesion, invasion, and migration of 95D lung carcinoma cells in vitro. Canadian journal of physiology and pharmacology 2010; 88: 874-879.
94. Ying H, Biroc SL, Li WW et al. The Rho kinase inhibitor fasudil inhibits tumor progression in human and rat tumor models. Molecular Cancer Therapeutics 2006; 5: 2158-2164.
95. Zhang C, Zhang S, Zhang Z et al. ROCK has a crucial role in regulating prostate tumor growth through interaction with c-Myc. Oncogene 2013.
96. Zhu F, Zhang Z, Wu G et al. Rho kinase inhibitor fasudil suppresses migration and invasion though down-regulating the expression of VEGF in lung cancer cell line A549. Medical oncology 2011; 28: 565-571.
97. Zohrabian VM, Forzani B, Chau Z et al. Rho/ROCK and MAPK signaling pathways are involved in glioblastoma cell migration and proliferation. Anticancer research 2009; 29: 119-123.