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MINI-REVIEW

Targeting the RhoGTPase/ROCK pathway for the treatment of VHL/HIF pathway-driven cancers

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ABSTRACT

The loss of the von Hippel-Lindau (*VHL*) tumor-suppressor is a major driver of Clear Cell Renal Cell Carcinoma (CC-RCC) resulting in the stabilization and overactivation of hypoxia inducible factors (HIFs). ROCK1 is a well-known protein serine/threonine kinase which is recognized as having a role in cancer including alterations in cell motility, metastasis and angiogenesis. We recently investigated and identified a synthetic lethal interaction between VHL loss and ROCK1 inhibition in CC-RCC that is dependent on HIF overactivation. Increased expression and activity of both HIFs and ROCK1 occurs in many types of cancer supporting the potential therapeutic role of ROCK inhibitors beyond CC-RCC. We also discuss future research required to establish prognostic markers to predict tumor response to ROCK inhibitors.

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Metastatic Clear Cell Renal Cell Carcinoma (CC-RCC) is a devastating disease with a 5-year survival 3 of 20%.[1] While surgical resection of the tumor is often curative for early stage disease, approximately one third of patients present with regional or metastatic CC-RCC.[2] Despite significant increases in T1a (< 4 cm) CC-RCC detection over the past few decades, with significant increases in surgical treatment, there has been no reduction in the number of patients presenting with more aggressive disease (regional or metastatic disease at time of diagnosis). As such, CC-RCC remains a major clinical challenge with great need for novel treatment strategies.

There are 2 types of FDA-approved therapies that are currently available for treating advanced-stage CC-RCC: immunotherapies (cytokines: interleukin-2 [IL2], interferon α [IFNα]; and programmed death-1 inhibitor [PD-1i]) and targeted therapies (receptor tyrosine kinase inhibitors [RTKis] and mammalian target of rapamycin inhibitors [mTORis]).

The first FDA-approved therapy for CC-RCC was IL2-based immunotherapy that has been shown to prolong overall patient survival to 17.5 months.[3] IL2 immunotherapy is both of limited efficacy and is associated with significant morbidity and some mortality. Since then, the first line RTKis – Sorafenib, Sunitinib, and Pazopanib – have been approved and prolong overall survival to 19.3 months,[4] 29.3, and 28.4 months respectively.[5] Second line RTKi Axitinib has been shown to prolong overall survival to 13.6 months in Sorafenib-refractory patients and to 29.9 months in cytokine-refractory patients.[6] While immunotherapy and RTKis are used to treat advanced stage CC-RCC, mTORis are used to specifically treat metastatic CC-RCC. First line mTORi Temsirolimus is approved for poor-prognosis metastatic CC-RCC patients and prolongs overall survival to 10.9 months as compared with cytokine-based immunotherapy, which prolongs overall survival to 7.3 months.[7] Second line mTORi Everolimus increases overall survival of RTKi-refractory metastatic CC-RCC patients by 14.8 months.[8] While patients initially respond to FDA-approved therapies, the majority of patients develop drug resistance.[9]

Recently, multiple next generation therapies, including 2 RTKis, Lenvatinib and Cabozantinib, and one immunotherapeutic, PD-1i Nivolumab, have been approved for CC-RCC. Along with inhibiting VEGF, both Lenvatinib and Cabozantinib also target additional RTKs that have been linked to RTKi drug resistance. Accordingly, upregulation of fibroblast growth factor receptor (FGFR) signaling has been shown to contribute to resistance to VEGFR inhibitors,[10] and Lenvatinib inhibits PDGFR, VEGFR, and FGFR. In RTKi refractory patients, Lenvatinib prolonged overall patient survival to 18.4 months in comparison to 17.5 months for

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Everolimus-treated patients. Combination treatment with both Lenvatinib and Everolimus was able to prolong overall patient survival to 25.5 months.[11] Overactivation of the hepatocyte growth factor receptor (HGFR, or MET) pathway has been also implicated in VEGFR inhibitor resistance,[12,13] and Cabozantinib inhibits both VEGFR and MET. Treatment with Cabozantinib prolonged overall survival to 21.4 months in RTKi refractory patients over Everolimus treatment, which only prolonged survival to 16.5 months. The approval of both Lenvatinib and Cabozantinib offers new options to overcome resistance to current RTKi, and further clinical trials are underway. Importantly, the therapeutic benefits of RTKis have been attributed to targeting of endothelial cells, and not direct targeting of cancer cells,[9,14] thus making the therapies, which directly target cancer cells, excellent candidates for combination treatment.

While only 12.5% of CC-RCC patients enter partial remission with cytokine-based immunotherapy,[15] second line Nivolumab-based immunotherapy increases overall survival of RTKi-refractory metastatic CC-RCC patients by 25 months, as compared with Everolimus, which increases overall survival of RTKi-refractory metastatic CC-RCC patients by 19.6 months.[16] This represents a significant advance for immunotherapy, but there remains the need for novel targeted therapies to treat CC-RCC patients belonging to the group of poor-responders to immunotherapy.

The genetics of sporadic CC-RCC has been extensively studied and loss of function of the von Hippel-Lindau (*VHL*) tumor-suppressor occurs in up to 90% of CC-RCC patients.[17-19] In addition, the *VHL* gene is affected by mutations/deletions in familial VHL disease, a syndrome, predisposing affected individuals to hemangioblastoma, pheochromocytoma, and CC-RCC.[20] This genetic alteration sets the stage for a synthetic lethality screen to find novel therapeutics specifically targeting *VHL*-deficient cancer cells and sparing *VHL*-expressing normal tissue. The principle underlying synthetic lethality screens is that cancer cells with a specific genetic alteration (e.g. *VHL* deficiency) will be more sensitive to targeted inhibition of a certain pathway than normal cells, where genetic alterations of tumor-suppressor genes are rare.[21] Thus, the resulting synthetic lethality compounds represent excellent candidates for therapies that target mutation-bearing cancer cells, but spare surrounding normal tissues.

We recently reported on a chemical library screen that revealed a synthetic lethal interaction of *VHL* deficiency with the Rho-associated protein kinase (ROCK) inhibitors – Y-27632, RKI 1447, and GSK 429286 – confirmed in several CC-RCC genetic backgrounds (see Fig. 1 for VHL and ROCK pathway overview).[22] siRNA-mediated knockdown of ROCK1, but not ROCK2, replicated the synthetic lethality effect, suggesting that inhibition of ROCK1 by ROCK inhibitors is critical for targeting *VHL*-deficient CC-RCC. Importantly, we have shown that the hypoxia-inducible factor (HIF) pathway, which gets over-activated as a result of *VHL* loss, is critical for sensitivity to ROCK inhibitors. Based on these findings

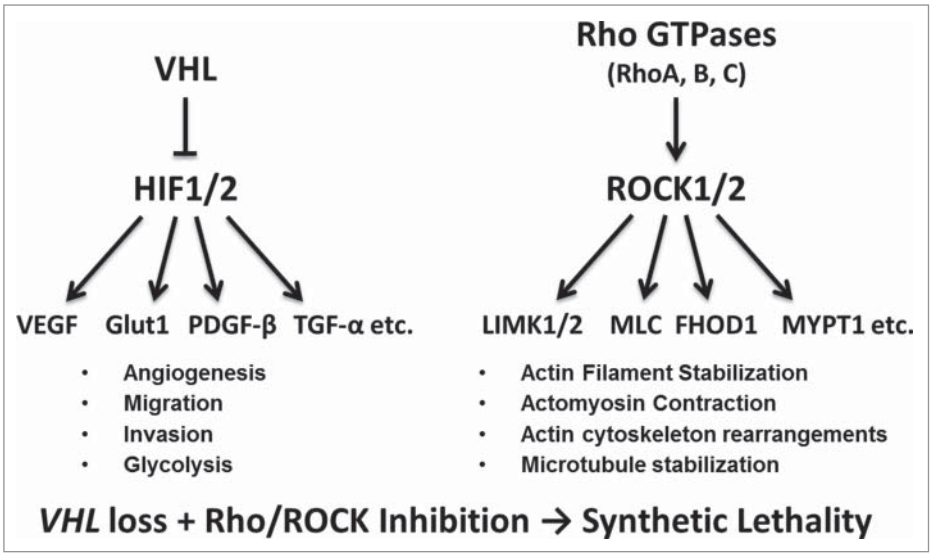

Figure 1. Overview of VHL/HIF and Rho/ROCK signaling pathways. VHL, left, is a part of an E3 ubiquitin ligase complex that targets HIF-1α and HIF-2α for degradation. The loss of VHL stabilizes HIFs, leading to elevated expression of a multitude of HIF-target genes, involved in angiogenesis, migration, invasion, glycolysis, etc. ROCK signaling, right, is dependent on activation by RhoGTPases that bind to ROCK1 and ROCK2. ROCK family kinases are major regulators of actin organization within the cell controlling actin filament stabilization, actomyosin contraction, actin cytoskeleton rearrangements, microtubule stabilization, etc. The combination of *VHL* loss leading to HIF overactivation and Rho/ROCK pathway inhibition triggers synthetic lethality.

we expect that ROCK inhibitors would represent potential therapeutics not only for *VHL*-deficient CC-RCC, but also for CC-RCC with certain *VHL* mutations. In addition, ROCK inhibitors should target hemangioblastoma and pheochromocytoma with *VHL* deficiencies and certain mutations, as well as cancer types with overactivation of HIF pathway arising independent of *VHL* loss of function.

VHL loss of function due to deletions, mutations, and promoter hypermethylation occurs in over 90% of sporadic CC-RCC cases.[23] The frequency of *VHL* mutations in CC-RCC tumors ranges from about 46% to 82% depending on the study,[17,24] and loss of heterozygosity occurs in up to 98% of cases.[25] In addition, *VHL* promoter hypermethylation occurs in about 10 to 20% of CC-RCC tumors.[19,26,27] Accordingly, *VHL* loss of function occurs by multiple mechanisms and is a hallmark of sporadic CC-RCC tumors, which has been shown to be a major driver of the disease.

Besides sporadic cases, CC-RCC frequently occurs in people affected by familial VHL disease. VHL disease is a heritable autosomal-dominant neoplastic syndrome with an incidence of 1 in 36,000[28] that is associated with the development of renal cysts (60 to 70% of patients), with some cysts progressing to CC-RCC (40% of patients), spinal cord (60 to 80% of patients) and retinal (60% of patients) hemangioblastomas (tumors originating from the vasculature), and pheochromocytoma (adrenal gland tumors) (5% of patients).[29,30] Patients with VHL disease are born lacking one functional copy of the *VHL* gene, and during their lifetime lose a second functional copy due to mutation, deletion, or promoter hypermethylation in certain tissues, triggering cancer development. The disease is divided into distinct subtypes based on *VHL* status.[31] Type 1 VHL disease is associated with deletions and mutations in *VHL* that completely disrupt its function and are associated with high risk of CC-RCC and hemangioblastoma formation. Type 2 VHL disease is further split into 3 additional subsets, 2A-C, and is associated with *VHL* missense mutations.[32] Type 2A is associated with hemangioblastoma, pheochromocytoma, and a low risk for CC-RCC; type 2B is associated with hemangioblastoma, pheochromocytoma, and CC-RCC; whereas type 2C is associated with pheochromocytoma only.[23,31] Since our data indicate that *VHL* loss causing HIF stabilization sensitizes CC-RCC to ROCK inhibitors, we expect that tumors harboring *VHL* mutations which either completely disrupt VHL function (like those occurring in type 1 VHL disease, e.g., C162F[31]), or specifically disrupt VHL's ability to regulate HIF activity (like those occurring in type 2A disease, e.g., Y98H, Y112H, A149T, T157I, and 2B disease, e.g., Y98N, Y112N[33,34]) will be sensitive to ROCK inhibitors. The resulting overactivation of HIFs in these tumors will make them candidates for ROCK inhibitor-based therapies. Since VHL regulates many targets besides HIFα, including activated epidermal growth factor receptor, RNA Pol II subunits, protein kinase C, and others[35] further investigation is needed to establish if, in addition to disruption of HIF regulation, disruption of any of these VHL functions by certain missense mutations is important for ROCK inhibitor sensitivity.

Overactivation of HIFs is a frequent event in cancer. Both HIF1α and HIF2α have been shown to be overexpressed in tumor samples compared with matched normal tissues in multiple cancer types besides CC-RCC including bladder, brain, breast, colon, ovarian, gastric, lung, melanoma, pancreatic, and prostate cancers.[36,37] While HIF activation often occurs in perinecrotic regions of solid tumors that lack adequate vasculature and oxygen supply,[38] there are multiple mechanisms by which HIFs are activated under normoxic conditions apart from *VHL* loss. For instance, the loss of *p53* tumor suppressor leads to disruption of human homolog of mouse double minute 2 (HDM2)-mediated degradation of HIFα subunits resulting in HIF overactivation.[39] Similarly, the loss of phosphatase and tensin homolog (PTEN) leads to deregulation of phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt) activity resulting in HIF overactivation.[40] Furthermore, disruption of prolyl hydroxylases (PHDs) by mutations blocks hydroxylation of the HIFα subunits and inhibits VHL-mediated HIFα degradation.[41] Multiple oncogenes that are commonly activated by mutations or overexpressed in cancer have also been shown to result in HIF overactivation, including Ha-ras[42] (via PI3K signaling), v-Src,[43] and c-Myc.[44] Although these findings suggest that HIF overactivation occurs frequently in multiple cancers, it is important to keep in mind that the magnitude of HIF activity is often less than in the case of *VHL* loss or hypoxic exposure, which are the main players of pathway controlling HIF activity.[37] Thus, additional experiments establishing the sensitivity of cancer cell lines with the genetic alterations listed above to ROCK inhibitors in normoxia and hypoxia are required to drive the conclusions on their sensitivity and utility of ROCK inhibitors for their targeting.

Another important factor that needs to be taken into consideration for the prediction of sensitivity to ROCK inhibitors is expression of the drug target, ROCK1, in cancers other than CC-RCC. In this respect, elevated ROCK1 expression was reported in breast[45] and prostate[46] cancers at the protein level and lung cancer at the mRNA level.[47] Activating ROCK1 somatic mutations have been also reported in breast and lung cancers,[48] affecting the autoinhibitory region of ROCK1, resulting

in increased activity even though protein levels remain unchanged. Interestingly, ROCK1 has been reported to be a HIF-target gene in breast cancer,[49] although the regulation likely involves cell-type-specific components since we found ROCK1 expression to be similar in *VHL*-deficient CC-RCC and CC-RCC with re-introduced *VHL*.[22] In addition, overexpression at the mRNA and protein levels of upstream regulators of ROCK – Rho GTPases RhoA and RhoC[50] – occurs in breast, prostate, lung, bladder, colon, ovarian, gastric, melanoma, and pancreatic cancers.[51] While somatic mutations resulting in increased Rho activity are rare,[52] regulators of Rho, guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine-nucleotide dissociation inhibitors (GDIs), have been shown to be deregulated in cancer[51] contributing to the activity of the Rho/ROCK pathway. Currently, a specific Rho GTPase inhibitor, Cethrin, was developed for the management of spinal cord injuries compatible with intrathecal delivery,[53] but feasibility of its systemic delivery needs evaluation. RhoGTPases can also be targeted indirectly, e.g., by statins (HMG-CoA Reductase inhibitors), which inhibit Rho GTPase isoprenylation and translocation to the plasma membrane,[54] although statins are far from being specific toward Rho, and also inhibit Ras and Rac GTPases dependent on isoprenylation.[54] Together, these data suggest that the Rho/ROCK pathway is active and can be targeted in multiple cancer types.

Overall, ROCK and HIF co-activation frequently occurs in several cancer types, suggesting that ROCK inhibitors should be effective for targeting those cancers. Accordingly, ROCK inhibitors have shown an anti-cancer effect in breast,[55] prostate,[46] ovarian,[56] and melanoma[57] cancers both *in vitro* and *in vivo* in mouse models. Recently, AT13148, a multi-kinase inhibitor targeting ROCK, has shown an anti-cancer effect in mouse models of breast,[58] prostate,[58] lung,[58] uterine,[58] gastric,[59] and melanoma[57] types of cancer. Currently, there is an ongoing phase I clinical trial (NCT01585701) of AT13148 administered to breast, prostate, and ovarian cancer patients, which will be evaluated for normal tissue toxicity and possible anti-tumor response. It is likely that the synthetic lethal interaction between ROCK inhibition and HIF overactivation contributes to sensitivity of these forms of cancer to ROCK inhibitors. Additional studies are required to develop reliable markers for prediction of ROCK inhibitor anti-tumor response.

In conclusion, the synthetic lethal interaction between ROCK inhibition and HIF overactivation is important since it justifies ROCK inhibitors as candidate therapeutics for multiple forms of cancer. While ROCK inhibitors represent good candidates for targeting hypoxic regions of solid tumors, where HIFs are stabilized due to the lack of adequate vasculature and oxygen supply, ROCK inhibitors are also expected to target cancers where HIFs are overactivated by mutations in their upstream regulators. It is also worth investigating which *VHL* mutations can confer sensitivity to ROCK inhibitors to a degree similar to *VHL* loss in CC-RCC, hemangioblastoma, and pheochromocytoma. Since ROCK1 is expressed in multiple cancers, it represents a druggable molecule. Further research is needed to evaluate the impact of the discovered synthetic lethal interaction on sensitivity of other forms of cancer besides CC-RCC to ROCK inhibitors; and develop the plan for patient stratification into ROCK inhibitor-sensitive and -insensitive groups.

Abbreviations

Akt	protein kinase B
CC-RCC	Clear Cell Renal Cell Carcinoma
GAPs	GTPase-activating proteins
GDIs	guanine-nucleotide dissociation inhibitors
FGFR	fibroblast growth factor receptor
GEFs	guanine-nucleotide exchange factors
HIFs	hypoxia inducible factors
IFNα	interferon α
IL-2	interleukin-2
mTORis	mammalian target of rapamycin inhibitors
PD-1i	programmed death-1 inhibitor
PHD	prolyl hydroxylases
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
ROCK	Rho-associated protein kinase
RTKis	receptor tyrosine kinase inhibitors
VHL	von Hippel-Lindau

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Author contributions

JMT, JL, and OVR wrote the paper.

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