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ORIGINAL ARTICLE

Rho-associated kinase 1 inhibition is synthetically lethal with von Hippel-Lindau deficiency in clear cell renal cell carcinoma

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Clear cell renal cell carcinoma (CC-RCC) is the most lethal of all genitourinary cancers. The functional loss of the von Hippel-Lindau (*VHL*) gene occurs in 90% of CC-RCC, driving cancer progression. The objective of this study was to identify chemical compounds that are synthetically lethal with *VHL* deficiency in CC-RCC. An annotated chemical library, the library of pharmacologically active compounds (LOPAC), was screened in parallel on *VHL*-deficient RCC4 cells and RCC4VHL cells with re-introduced *VHL*. The ROCK inhibitor, Y-27632, was identified and validated for selective targeting of *VHL*-deficient CC-RCC in multiple genetic backgrounds by clonogenic assays. Downregulation of ROCK1 by small interfering RNA (siRNA) selectively reduced the colony-forming ability of *VHL*-deficient CC-RCC, thus mimicking the effect of Y-27632 treatment, whereas downregulation of ROCK2 had no effect. In addition, two other ROCK inhibitors, RKI 1447 and GSK 429286, selectively targeted *VHL*-deficient CC-RCC. CC-RCC treatment with ROCK inhibitors is cytotoxic and cytostatic based on bromodeoxyuridine (BrdU) assay, propidium iodide (PI) staining and growth curves, and blocks cell migration based on transwell assay. On the one hand, knockdown of hypoxia-inducible factor (HIF) β in the *VHL*-deficient CC-RCC had a protective effect against Y-27632 treatment, mimicking *VHL* reintroduction. On the other hand, CC-RCCVHL cells were sensitized to Y-27632 treatment in hypoxia (2% O_2). These results suggest that synthetic lethality between ROCK inhibition and *VHL* deficiency is dependent on HIF activation. Moreover, HIF1α or HIF2α overexpression in CC-RCCVHL cells is sufficient to sensitize them to ROCK inhibition. Finally, Y-27632 treatment inhibited growth of subcutaneous 786-OT1 CC-RCC tumors in mice. Thus, ROCK inhibitors represent potential therapeutics for *VHL*-deficient CC-RCC.

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INTRODUCTION

Renal cancer is the most deadly of all genitourinary cancers with 62 700 new cases and 14 240 deaths projected to occur in 2016.[1] Whereas surgical resection is often curative at early stages, metastatic renal cancer remains a devastating disease with a 5-year survival rate of less than 20%.[1,2] The poor survival rate is because of renal cancer's resistance to radiotherapy,[3] chemotherapy[2] and immunotherapy,[2] which has been linked to multidrug resistance mechanisms[4] and the lack of common solid tumor mutations.[5]

Clear cell renal cell carcinomas (CC-RCCs) account for 90% of all renal cancer cases, and the tumor-suppressor von Hippel-Lindau (*VHL*) is functionally lost in up to 90% of CC-RCC tumors.[6] *VHL* loss occurs early in the disease and drives its pathogenesis.[6] *VHL* is an E3 ubiquitin ligase that targets multiple proteins for proteasomal degradation, including the hypoxia-inducible factor (HIF) α subunits and the epidermal growth factor receptor.[7] Thus, upon *VHL* loss, CC-RCCs upregulate expression of epidermal growth factor receptor and other receptor tyrosine kinases, as well as HIFs, in turn upregulating proangiogenic genes, such as vascular endothelial growth factor. As a consequence, CC-RCCs are highly vascularized and aggressive. Accordingly, the majority of approved CC-RCC therapies inhibit angiogenesis. The receptor tyrosine kinase inhibitors sunitinib,[8] sorafenib[9] and axitinib,[10] which block vascular endothelial growth factor receptor and platelet-derived growth factor receptor, prolong progression-free survival for a median of 5 months when compared with placebo[9,11] or standard of care treatments such as interferon α.[12] Another class of CC-RCC therapeutics is represented by mammalian target of rapamycin inhibitors everolimus[13] and temsirolimus,[14] which prolong progression-free survival for a median of 3 months when used as single agents compared with standard of care. Whereas these treatments offer significant clinical benefit, resistance to both receptor tyrosine kinase inhibitor and mammalian target of rapamycin inhibitor therapeutics develops quickly creating the need for new and improved therapeutics.[15–17]

In this study we relied on a 'synthetic lethality' approach to identify new therapeutics for *VHL*-deficient CC-RCC. A large body of evidence supports the use of synthetic lethality screens for identifying specific chemical compounds or small hairpin RNAs (shRNAs) that cause cell death and/or inhibit cell proliferation in combination with a particular cancer mutation.[18,19] The principle underlying such screens is that cancer cells with a specific mutation will be more sensitive to targeted inhibition of a certain pathway than normal cells that do not have the same mutation. Thus, the resulting synthetic lethality compounds represent excellent candidates for therapies that target mutation-bearing cancer cells, but spare normal tissues. Several synthetic lethality screens have been conducted in CC-RCC to date.[20–25] Each of these screens utilized the loss of the *VHL* tumor suppressor to identify compounds that selectively target *VHL*-deficient CC-RCCs. The synthetic lethality screens relied on 'matched' cell lines, which were created by introducing either a vector control or the wild-type *VHL* expressing construct to *VHL*-deficient CC-RCC.[19] These matched cell lines were then used in chemical or shRNA library screens to identify chemical compounds or shRNAs that selectively target *VHL*-deficient CC-RCCs, while sparing their *VHL*-reconstituted 'matched' counterparts.

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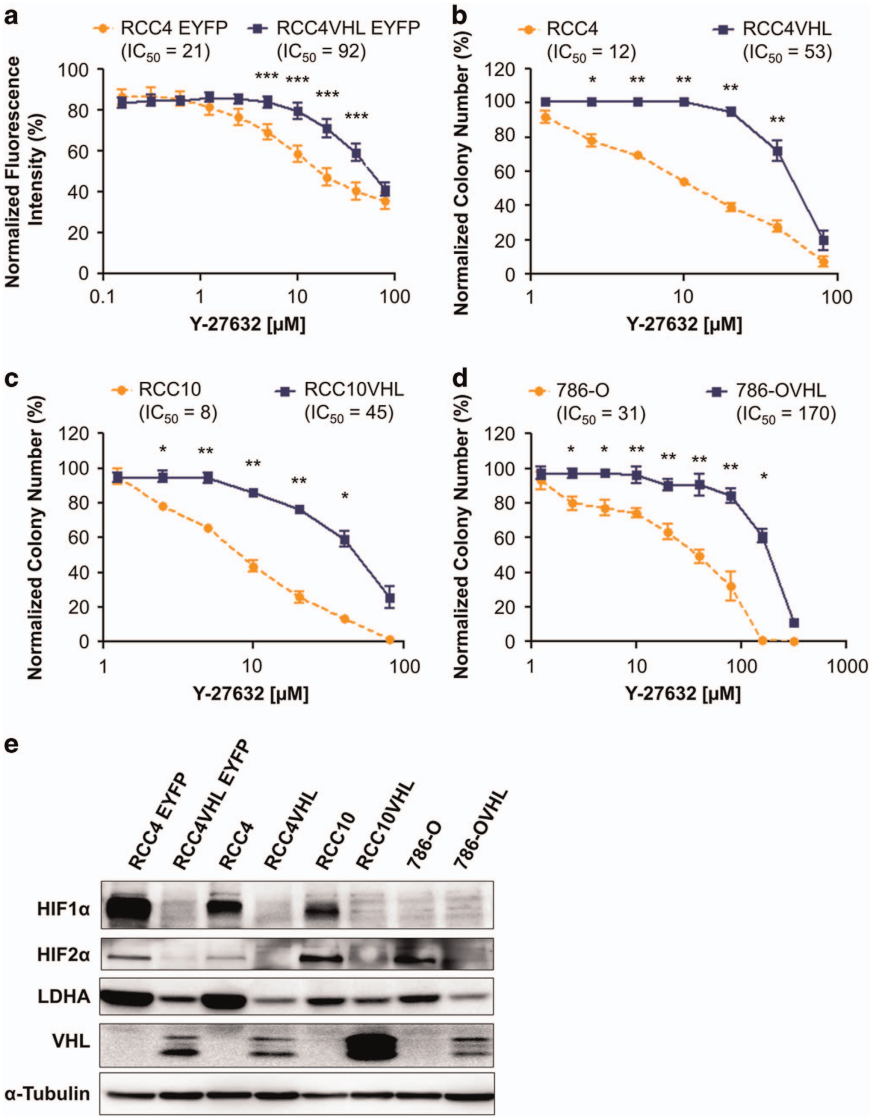


Figure 1. The ROCK inhibitor Y-27632 causes synthetic lethality with *VHL* loss in multiple CC-RCC cell lines. (**a**) The LOPAC hit Y-27632 was validated in the RCC4 EYFP and RCC4VHL EYFP matched cell lines, showing selective toxicity toward *VHL*-deficient cells. Each dose of Y-27632 within each experiment was tested in quadruplicate, and the experiment was repeated three times. Fluorescence intensity of EYFP-labeled cells was used as a surrogate for cell number. (**b–d**) Clonogenic assays in (**b**) RCC4 ± VHL, (**c**) RCC10 ± VHL and (**d**) 786-O ± VHL matched cell lines, confirming that Y-27632 causes synthetic lethality with *VHL* loss in multiple CC-RCC genetic backgrounds. Each dose of Y-27632 within each experiment was tested in duplicate, and the experiment was repeated three times. IC_{50s} are indicated. Statistical analysis in **a–d** was performed using a paired *t*-test between the matched cell lines at each dose (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$); s.e.m.'s are shown. (**e**) Western blot showing the effect of VHL re-expression in CC-RCC cell lines on HIF1α and HIF2α expression, and the expression of their downstream target lactate dehydrogenase A (LDHA). α-tubulin serves as a loading control.

Although both unannotated chemical library[20–24] and shRNA[25] screens have been conducted, to date no screens have been conducted using an annotated chemical library.

In this study we screened the annotated library of pharmacologically active compounds (LOPAC). This approach simultaneously revealed the exact molecular pathways responsible for selective targeting of *VHL*-deficient cells, and chemical compounds that inhibit them. Herein, we report a chemical hit identified in a LOPAC screen, Y-27632 an inhibitor of the Rho-associated coiled-coil-containing protein kinase (ROCK) that selectively targets *VHL*-deficient CC-RCC. The ROCK proteins are regulated by the small GTP-binding protein Rho and are best known for their role in regulating cell morphology and motility by controlling actin–myosin contractile force.[26] This role is mediated through phosphorylation of their downstream substrates, including Myosin Light Chain, Myosin Light Chain 2, Myosin Phosphatase Target 1 (MYPT1) and LIM Kinases.[26] ROCK expression is commonly upregulated in bladder,[27] testicular,[28] breast,[29] prostate[30] and renal cancer,[31] and has been shown to contribute to tumor metastasis in bladder,[27] breast[32] and prostate cancer.[30] In addition, certain ROCK substrates induce cell proliferation[30] and apoptosis,[33] and inhibit autophagy.[34] The two ROCK isoforms, ROCK1 and ROCK2, are differentially expressed throughout the body, with ROCK1 being expressed ubiquitously and ROCK2 being expressed predominantly in the brain, muscle, heart and lungs.[35] Although the two isoforms are highly homologous and have redundant functions, they also have unique functions and substrates.[26]

In the present study we show that ROCK inhibitors selectively target *VHL*-deficient CC-RCC to reduce cell proliferation, induce cell death and block migration, which is mediated through inhibition of ROCK1 and not ROCK2. Our studies also reveal that HIF

overactivation caused by *VHL* loss is both necessary and sufficient to cause synthetic lethality with ROCK inhibitors. Importantly, treatment with ROCK inhibitors blocks tumor growth *in vivo*, validating ROCK inhibitors as potential therapeutics for *VHL*-deficient CC-RCC.

RESULTS

Identification of chemical hit Y-27632 targeting *VHL*-deficient CC-RCC

To identify novel chemical compounds that selectively target *VHL*-deficient CC-RCC, we screened the LOPAC composed of 1280 compounds annotated with their protein targets (unpublished data), which allowed us to identify not only the chemicals but also the molecular pathways necessary for survival/proliferation of *VHL*-deficient CC-RCC. The screen utilized the RCC4 ± VHL matched cell lines. RCC4 cells lack both alleles of *VHL* and, as a consequence, HIF1α and HIF2α expression and activity are dramatically elevated compared with cell lines expressing *VHL* tumor suppressor.[6,36,37] RCC4VHL cells were generated by stably transfecting a full-length wild-type *VHL* expression construct to RCC4.[38] Both RCC4 and RCC4VHL cells were labeled with enhanced yellow fluorescent protein (EYFP) and the matched cell lines were treated in parallel with the LOPAC compounds at concentrations ranging from 0.3 to 20 μM in 384-well plates. Fluorescence intensity, a surrogate measure of cell numbers per well, was measured 96 h following the treatment. The ROCK inhibitor Y-27632 (structure shown in Supplementary Figure 1a) was identified in this screen and selectively targeted *VHL*-deficient RCC4 while sparing RCC4VHL. The structures of the other two ROCK inhibitors, used later in this study, are shown in Supplementary Figures 1b and c. We validated Y-27632 as a 'hit' by fluorescence-based viability assay (Figure 1a).

To further validate Y-27632 as a chemical hit, we conducted clonogenic assays on RCC4 and RCC4VHL cell lines (Figure 1b and Supplementary Figure 2a). Importantly, *VHL*-deficient RCC4 cells were four to five times more sensitive to Y-27632 treatment than RCC4VHL in both assays (Figures 1a and b).

Treatment with ROCK inhibitor Y-27632 selectively targets *VHL*-deficient CC-RCCs of multiple genetic backgrounds

Next, we tested whether the synthetic lethality effect could be reproduced in multiple genetic backgrounds. We repeated the clonogenic assays in two more *VHL*-matched CC-RCC cell lines based on RCC10 expressing both HIF1α and HIF2α and 786-O expressing only HIF2α (Figures 1c and d; Supplementary Figures 2b and c). Similar to the results obtained in RCC4, Y-27632 treatment specifically targeted the *VHL*-deficient RCC10 and 786-O cell lines while sparing the CC-RCCVHL. Y-27632 treatment not only reduced colony numbers selectively in *VHL*-deficient CC-RCC (Supplementary Figures 2a–c), but also caused reduced colony staining intensity because of a reduction in cell numbers per colony (Supplementary Figures 2d–f). For each CC-RCC/CC-RCCVHL cell line pair the IC_{50} for *VHL*-deficient CC-RCC was approximately five times lower than that for CC-RCCVHL (Figures 1b–d). VHL expression in each CC-RCCVHL cell line was confirmed with western blot analysis, and it caused a reduction in HIF1α and HIF2α expression compared with the respective CC-RCC cell line (Figure 1e).

Y-27632's ability to inhibit ROCK activity was assayed via western blot analysis of MYPT1 Thr^{696} phosphorylation (ROCK substrate[39]). Y-27632 treatment for 2 h was effective at inhibiting MYPT1 phosphorylation (Supplementary Figure 3). Interestingly, *VHL*-deficient CC-RCCs have decreased basal MYPT1 phosphorylation in comparison with CC-RCCVHL. Together, these results indicate that Y-27632 inhibits ROCK in CC-RCC and selectively targets *VHL*-deficient CC-RCC while sparing *VHL*-reconstituted CC-RCC in multiple genetic backgrounds.

Synthetic lethality occurs through inhibition of ROCK1

We aimed to confirm that the synthetic lethal effect of Y-27632 is 'on-target' through blocking ROCKs as annotated in LOPAC. As Y-27632 inhibits both ROCK family members, ROCK1 and ROCK2, we sought to determine which ROCK was responsible for the synthetic lethal effect. To do this, we knocked down ROCK1 or ROCK2 with selective small interfering RNAs (siRNAs). To control for the off-target effects of the siRNAs, we used two siRNAs to knockdown ROCK1 (siROCK1#1 and siROCK1#2) and two to knockdown ROCK2 (siROCK2#1 and siROCK2#2). Our data showed that knockdown of ROCK1, but not ROCK2, reduced the colony-forming ability and colony size of *VHL*-deficient RCC4 cells, sparing RCC4VHL cells, thus mimicking the effect of Y-27632 treatment (Figure 2a and Supplementary Figure 4). The ROCK1 and ROCK2 knockdowns were confirmed by western blot analysis. Importantly,

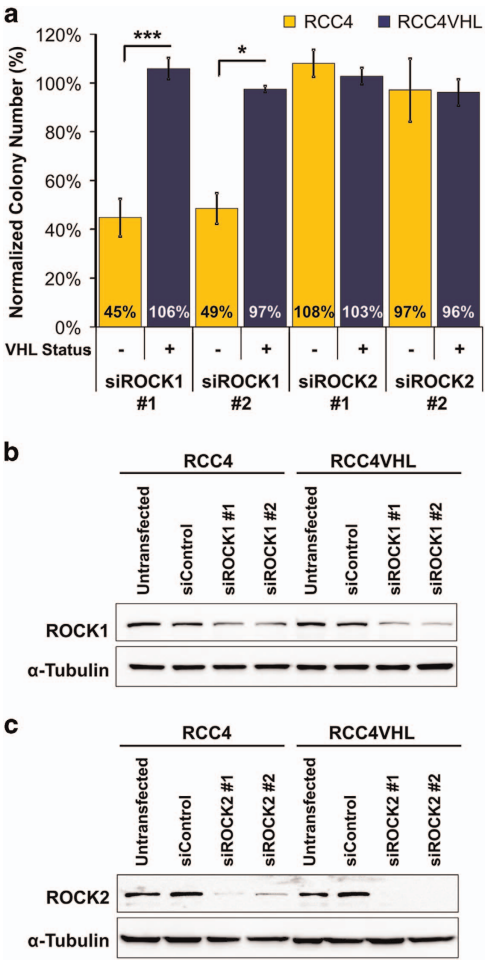


Figure 2. Synthetic lethality of Y-27632 with *VHL* loss is mimicked by siRNA downregulation of ROCK1, not ROCK2. RCC4 ± VHL matched cell lines were transfected with siRNAs targeting ROCK1, ROCK2 or non-targeting siRNA control (siControl). Twenty-four hours after transfection, cells were plated for a clonogenic assay. Each transfection was carried out in triplicate, followed by clonogenic assays conducted in triplicate, and the experiments were repeated at least two times. (**a**) Transfection with siROCK1, but not siROCK2, resulted in significant reduction in RCC4 colony numbers in comparison with RCC4VHL. Thus, ROCK1 downregulation mimics the effect of Y-27632 treatment on viability of RCC4 cells, making it a likely target for Y-27632 causing synthetic lethality effect. Statistical analysis was performed using a paired *t*-test comparing numbers of colonies in each siROCK group to siControl (*$P < 0.05$ and ***$P < 0.001$); s.e.m.'s are shown. (**b** and **c**) The degree of each target knockdown by its specific siRNA (as indicated) was assessed by western blot.

the knockdowns were equal or greater in the RCC4VHL cells for each siRNA used (Figures 2b and c). These data were reproduced in 786-O and 786-OVHL matched cell lines (Supplementary Figure 5). In summary, siRNA knockdown of ROCK1, but not ROCK2, selectively inhibits colony formation of *VHL*-deficient CC-RCC.

RKI 1447 and GSK 429286 ROCK inhibitors target *VHL*-deficient CC-RCC

As there are several commercially available ROCK inhibitors, and all of them differ in their potency and selectivity toward ROCK1 versus ROCK2, we tested two additional ROCK inhibitors in clonogenic assays: RKI 1447 (structure shown in Supplementary Figure 1b) and GSK 429286 (structure shown in Supplementary Figure 1c). As RKI 1447 showed the strongest potency, it was tested in all three matched cell lines. RKI 1447, similar to Y-27632 treatment and ROCK1 knockdown, selectively reduced the number of colonies and cells per colony in the *VHL*-deficient CC-RCC (Figures 3a–c and Supplementary Figure 6). The potencies of Y-27632, RKI 1447 and GSK 429286 were compared in RCC10 ± VHL in Figures 1c, 3b and Supplementary Figure 7, respectively. The overall inhibitor potencies based on IC_{50s} are as follows: RKI 1447 (0.8 μM) > GSK 429286 (6.4 μM) > Y-27632 (8.2 μM). As GSK 429286 was less potent than RKI 1447, we did not test it further. We also observed that repeat, daily, treatment with 2 μM RKI 1447 led to an enhanced synthetic lethal effect in each of the *VHL*-deficient CC-RCC, while minimally affecting matched *VHL*-expressing CC-RCCVHL (Figures 3d–f). For visual comparison representative images were obtained on day 14 of treatment for RCC4 and 786-O, and day 9 of treatment for RCC10 (Supplementary Figure 8). Thus, multiple ROCK inhibitors specifically target *VHL*-deficient CC-RCC.

Treatment with ROCK inhibitors reduces CC-RCC proliferation and induces cell death

The results from the clonogenic assays pointed to both cell death (reduced colony numbers) and proliferation defect (reduced colony size) as biological outcomes of Y-27632 treatment (Figures 1b–d; Supplementary Figure 2). To confirm these biological outcomes, we assessed cell cycle progression using a fluorescein isothiocyanate-bromodeoxyuridine (BrdU) assay. Treatment of RCC4 and RCC4VHL cells with Y-27632 at 10, 20 and 40 μM resulted in an increase in the apoptotic/debris population and a decrease in the S phase and G_0/G_1 phase populations; however, the effects were more pronounced in RCC4 than in RCC4VHL (Figure 4a and Supplementary Figure 9). To determine whether apoptosis was responsible for the increase in the apoptotic/debris population, we assessed whether Y-27632 stimulated caspase 3 cleavage in CC-RCC cells by western blot analysis. Our results show that Y-27632 induced caspase 3 cleavage in both RCC4 and RCC4VHL, but did not induce caspase 3 cleavage in RCC10 ± VHL or 786-O ± VHL over the basal level, thus ruling out apoptosis as a cause of selective cell death in *VHL*-deficient CC-RCC (Supplementary Figure 10).

To confirm that Y-27632 treatment induces cell death, we treated RCC4 and RCC4VHL cells with 20 μM Y-27632 for 24 h and then stained the cells with propidium iodide (PI). Imaging of the RCC4 cells showed a 5.4-fold increase in the number of PI-positive dead cells, whereas RCC4VHL showed a 1.5-fold increase (Figure 4b and Supplementary Figure 11a). In addition, siRNA knockdown of ROCK1, but not ROCK2, resulted in a more than fivefold increase in PI-positive dead cells (Figure 4c and Supplementary Figure 11b). Together, these results indicate that ROCK1 inhibition induces cell death and blocks proliferation in *VHL*-deficient CC-RCC.

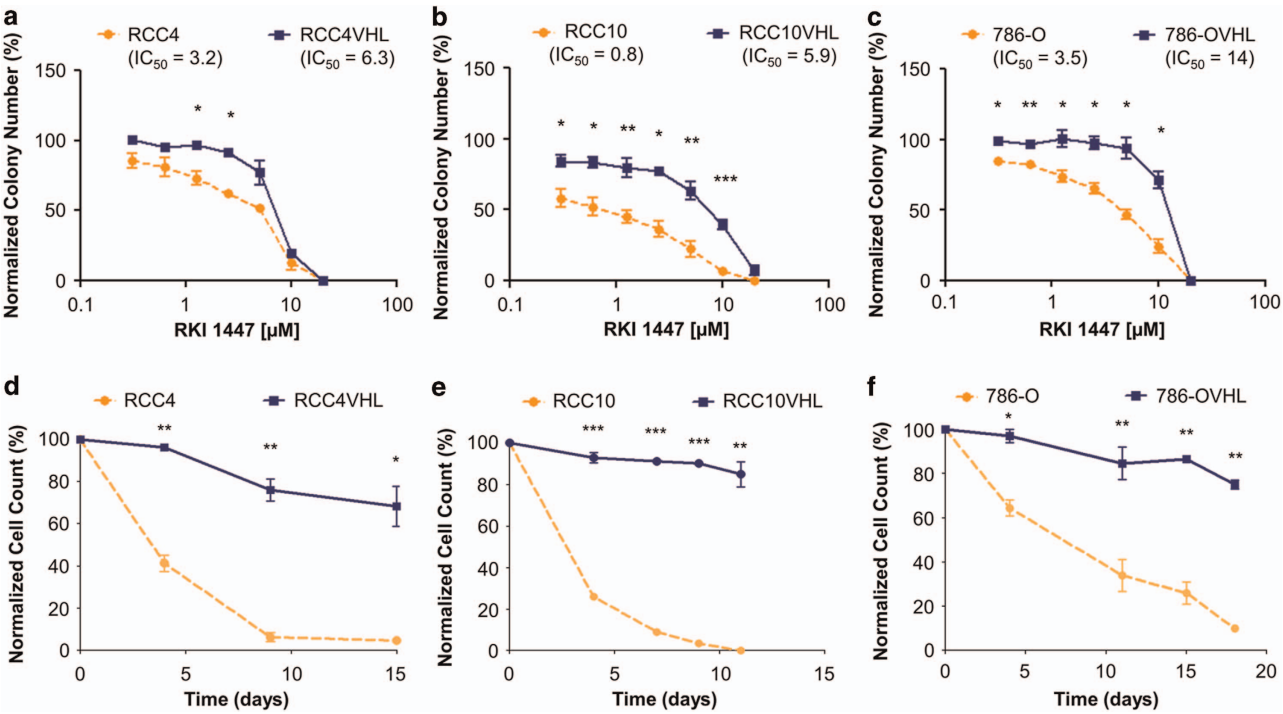


Figure 3. ROCK inhibitor RKI 1447 causes synthetic lethality with *VHL* deficiency similar to Y-27632. Clonogenic assays in RCC4 ± VHL- (**a**), RCC10 ± VHL- (**b**) and 786-O ± VHL (**c**) matched cell lines confirmed the synthetic lethality of ROCK inhibitors with *VHL* loss. Each dose of RKI 1447 within each experiment was tested in duplicate, and each experiment was repeated three times. (**d–f**) Long-term repeat administration of RKI 1447 enhances the synthetic lethality effect. Repeated daily treatment of RCC4 ± VHL (**d**), RCC10 ± VHL (**e**) and 786-O ± VHL (**f**) with 2μM RKI 1447 causes *VHL*-deficient CC-RCC cell numbers to decline, while CC-RCCVHL cells continue to proliferate. Daily treatment with DMSO was used as a control. Cells were counted and passaged at 1:10 when the DMSO-treated *VHL*-expressing cells became >80% confluent. Statistical analysis was performed using a paired *t*-test between the matched cell lines at each dose (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$); s.e.m.'s are shown.

ROCK inhibition blocks CC-RCC migration

Owing to the known role of ROCKs in the regulation of cell adhesion, migration and invasion,[32,40,41] we decided to assess the contribution of ROCKs to CC-RCC migration. First, we noticed that treatment with Y-27632 results in a change in cell morphology (cells become elongated and spindly), likely because of ROCK's role in regulating actin cytoskeleton re-organization and acto-myosin contraction[26] (Supplementary Figure 12). When we stopped the compound treatment at 48 h, cells reverted to their non-elongated phenotype (Supplementary Figure 12). Second, both Y-27632 and RKI 1447 caused a dramatic reduction of RCC10 and 786-O cell migration in a transwell migration assay (Figure 4d). To rule out the cytotoxic/proliferation-inhibitory effect of Y-27632 and RKI 1447 on migrating cells, we conducted all of the migration experiments at short 6-h time points. At 6 h the live cell numbers were assessed using PI vital dye exclusion flow cytometry and no changes were detected (Supplementary Figure 13). Thus, ROCK inhibitors have the potential to reduce CC-RCC primary tumor growth through their cytotoxic and cytostatic effects and may inhibit metastasis through blocking cell migration.

Synthetic lethality between ROCK inhibition and *VHL* deficiency is dependent on HIFs

One of the best-studied consequences of *VHL* loss/mutation in CC-RCC is the massive stabilization and activation of HIF1α and HIF2α[18–20] (Figure 1e). Thus, we hypothesized that the synthetic lethal effect between ROCK inhibition and *VHL* deficiency would

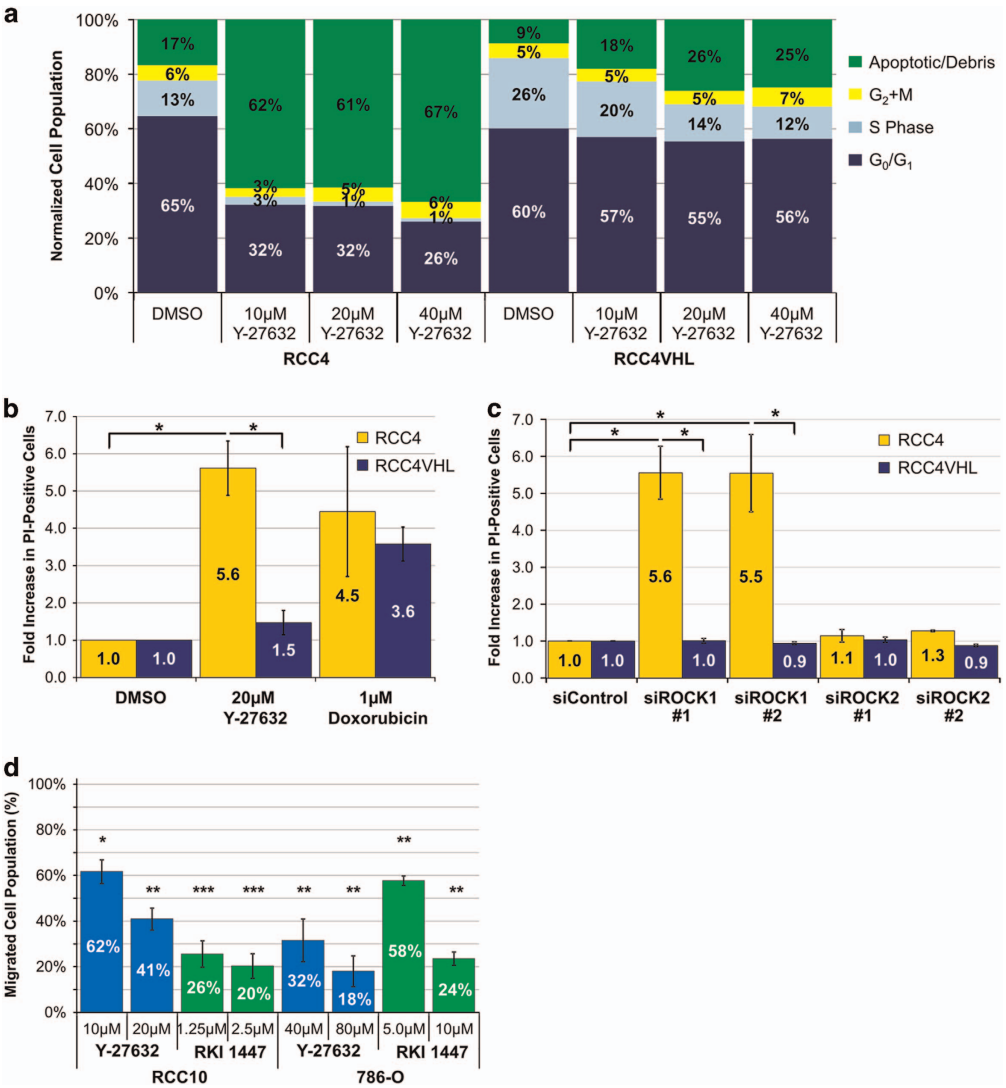


Figure 4. ROCK inhibition in *VHL*-deficient CC-RCC cells decreases proliferation, induces cell death, and blocks cell migration. (**a**) BrdU assay reveals that Y-27632 treatment is both cytotoxic and cytostatic in RCC4. RCC4 cells acquire a large fraction of apoptotic/debris cells and greatly reduce the S phase upon treatment with Y-27632 for 72 h as opposed to RCC4VHL. The graph shows the representative experiment of two experiments performed. (**b**) RCC4 cells treated with 20 μM Y-27632 for 24 h show a more than fivefold increase in cell death, whereas RCC4VHL cells are minimally affected. Cells were stained with the vital dye PI and imaged at 4×. The number of PI-positive cells was then counted for each field. The data were normalized to DMSO-treated cells. (**c**) Knockdown of ROCK1, but not ROCK2, induces cell death in the *VHL*-deficient RCC4. Forty-eight hours post siRNA transfection, RCC4-matched cells were stained with PI and imaged. Knockdown of ROCK1 resulted in over a fivefold increase in PI-positive cells. The data were normalized to siControl-treated cells. (**b** and **c**) Each experiment was performed in triplicate and repeated three times. (**d**) ROCK inhibition blocks CC-RCC migration in a transwell assay. RCC10 or 786-O cells were treated with Y-27632, RKI 1447 or DMSO vehicle (as indicated) for 6 h. The assay was performed in duplicate and the experiment was repeated three times. Statistical analysis in (**b**–**d**) was performed using a paired *t*-test comparing each normalized dose to the negative control (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$); s.e.m.'s are shown.

be dependent on HIF activation. To test this hypothesis, we acquired RCC4 and RCC10 cell lines where we stably knocked down HIFβ, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) with a specific shRNA. As ARNT forms a heterodimer with either HIF1α or HIF2α, and is essential for HIF transcriptional activity, its knockdown inhibited HIF activity. This resulted in a reduction of the lactate dehydrogenase A (LDHA) HIF target gene expression (Figure 5a). As predicted, knockdown of ARNT in the *VHL*-deficient RCC4 and RCC10 cell lines had a protective effect against Y-27632 treatment (Figure 5b), mimicking *VHL* reintroduction. These results indicate that synthetic lethality between ROCK inhibition and *VHL* deficiency is dependent on HIF activation.

To further confirm our findings, cells from the matched cell lines RCC4 ± VHL, RCC10 ± VHL and 786-O ± VHL were treated with Y-27632, plated for clonogenic assays and replicate plates were subjected to either normoxia (21% O_2, low HIF level and activity) or hypoxia (2% O_2, high HIF level and activity). Each Y-27632 treatment was normalized to the dimethyl sulfoxide (DMSO) vehicle control in both normoxia and hypoxia groups. The normalized colony numbers for Y-27632-treated *VHL*-deficient CC-RCC cell lines were not affected by oxygen concentration (Figure 5c). In contrast, CC-RCCVHL cell lines were sensitized to Y-27632 treatment in hypoxia having reduced colony-forming ability in hypoxia compared with normoxia (Figure 5c). Hypoxic induction of HIF1α and HIF2α was confirmed by western blot analysis (Supplementary Figure 14). These results confirm that the synthetic lethal interaction between ROCK inhibition and *VHL* deficiency is HIF-dependent.

As 786-O cells were the most resistant to Y-27632 out of the three matched cell lines tested (Figures 1b–d) and they do not express HIF1α, although RCC4 and RCC10 do, we hypothesized that HIF1α re-expression in 786-O would sensitize them to Y-27632. To test this hypothesis, we generated a 786-O cell line expressing a non-degradable constitutively active HA (hemagglutinin)-tagged HIF1α (CA-HA-HIF1α). The 786-O CA-HA-HIF1α cells showed increased sensitivity to both Y-27632 (Figure 5d) and RKI 1447 (Figure 5e) when compared with the 786-O vector control expressing cell line. In addition, we generated 786-OVHL cells expressing either the CA-HA-HIF1α or CA-HA-HIF2α. Expression of either HIF1α or HIF2α in 786-OVHL was sufficient to cause the synthetic lethal effect with ROCK inhibition, with 786-OVHL CA-HA-HIF1α showing a more pronounced effect than 786-OVHL CA-HA-HIF2α (Figures 5f and g). Altogether, these results indicate that the synthetic lethal interaction of *VHL* loss with ROCK inhibition is because of the resulting constitutive activation of HIF in *VHL*-deficient CC-RCC.

Y-27632 inhibits tumor growth *in vivo*

786-OT1 cells were isolated from a 786-O tumor grown in a RAG1 mouse and re-established *in vitro* to acquire a cell subline capable of fast growth *in vivo*. 786-OT1 were injected subcutaneously (sc) into the right flank of 18 RAG1 mice. After the tumors reached ~ 500 mm^3, the mice were randomized and either daily treated with a vehicle control (phosphate-buffered saline (PBS)) or 10 mg kg^{-1} Y-27632 via intraperitoneal (ip) injection for 18 days. Y-27632 was selected for *in vivo* experiments based on abundant literature, reporting its maximum tolerated dose and treatment regimens in mouse experiments[41–44] in comparison with RKI 1447 used in a single study.[40] Tumor size was measured every day during treatment, and tumor volume constantly increased in the control group ($n = 9$), whereas tumor volume remained static in the Y-27632 group ($n = 9$; Figure 6). The treatment was well tolerated with no weight loss in the mice (Supplementary Figure 15). The antitumor effects of Y-27632 support the concept that ROCK inhibitors can be used to selectively target *VHL*-deficient CC-RCC *in vivo*.

DISCUSSION

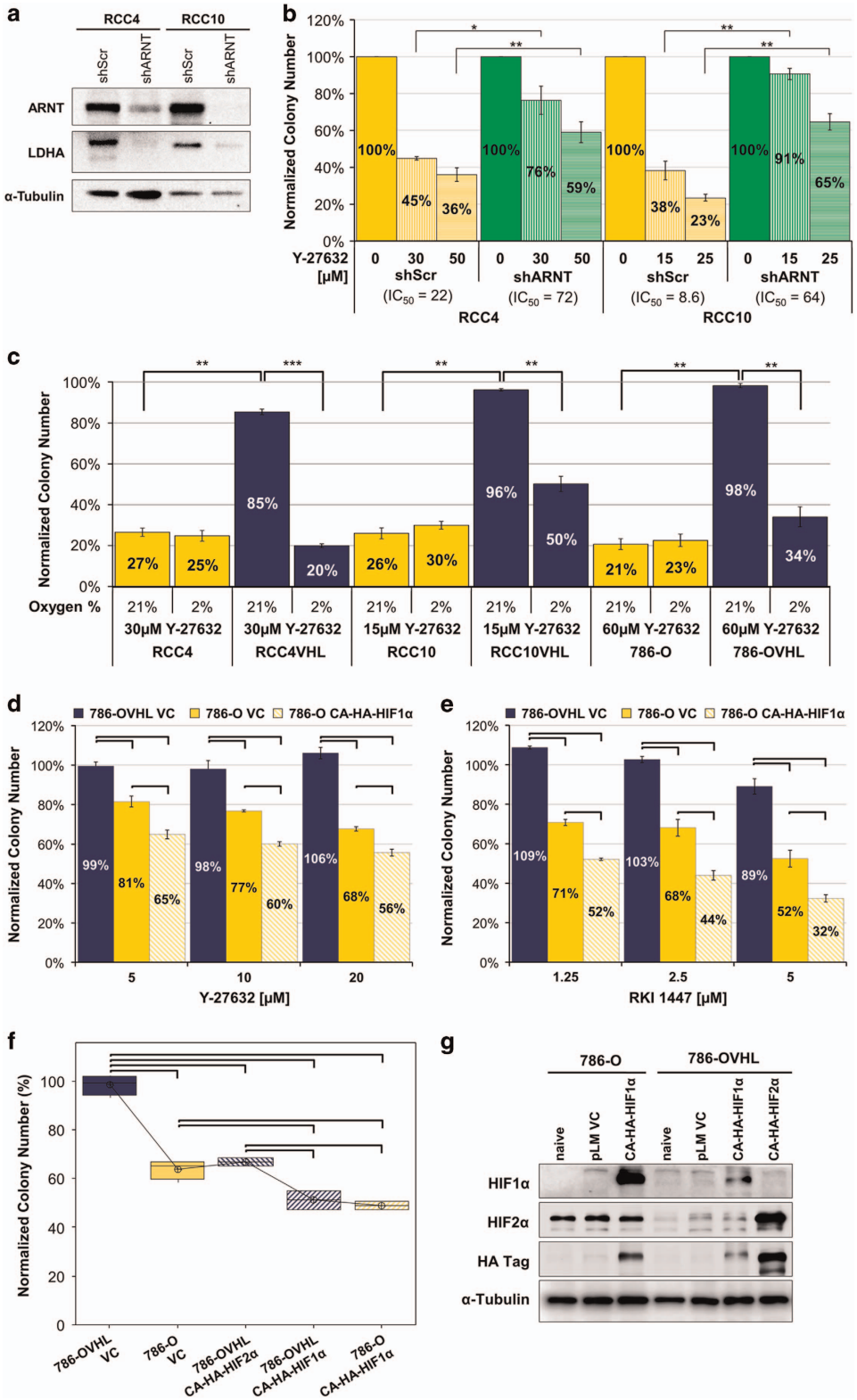
In this study we identified a synthetic lethal interaction between the ROCK inhibitor Y-27632 and the loss of *VHL* in CC-RCC. We have focused on validating ROCK inhibitors (Y-27632 and RKI 1447), which exhibited cytotoxic and cytostatic effects on *VHL*-deficient CC-RCC, making them candidate novel therapeutics for CC-RCC. First, the vast majority of CC-RCCs have lost *VHL* expression/function,[6] making over 90% of CC-RCC potentially sensitive to ROCK inhibition. Second, we have shown that the ROCK inhibitor Y-27632 suppresses CC-RCC tumor growth *in vivo*. Third, ROCK inhibitors reduce CC-RCC cell migration, indicating that they may have the potential to inhibit CC-RCC metastasis. Finally, ROCK1 and ROCK2 knockout mice are viable, indicating that both are dispensable under physiological conditions,[26] predicting no normal tissue toxicity. As we have shown that synthetic lethality of ROCK inhibition with *VHL* deficiency occurs primarily through ROCK1, one future direction would be to acquire ROCK inhibitors specifically targeting ROCK1 and not ROCK2.

Previous synthetic lethality studies of *VHL* deficiency have identified 'hits' being HIF-dependent[20,22] and HIF-independent.[21,25] Our data in Figure 5 indicate that the synthetic lethal interaction of ROCK inhibitors with *VHL* loss is HIF-dependent. Exposure of *VHL*-reconstituted CC-RCC to hypoxia conferred sensitivity to ROCK inhibitors, whereas the knockdown of *ARNT* in *VHL*-deficient CC-RCC conferred resistance to ROCK inhibitors. Re-expression of non-degradable HIFs also sensitized 786-OVHL cells to ROCK inhibition. Importantly, *VHL*-deficient CC-RCC patient's tumors differ in their repertoire of HIF subunits: 69% of patients express both HIF1α and HIF2α, whereas 31% express only HIF2α.[45] ROCK inhibition is synthetically lethal in both tumor types, although the cell lines RCC4 and RCC10 expressing both HIF1α and HIF2α are more sensitive to ROCK inhibition than 786-O expressing HIF2α only. In support of the role of HIF1α in the sensitization to ROCK inhibition, the same increase in sensitivity to ROCK inhibitors was observed in the cell lines expressing HIF1α (786-OVHL CA-HA-HIF1α and 786-O CA-HA-HIF1α) over those only expressing HIF2α (786-O and 786-OVHL HIF2α).

Figure 5. The synthetic lethal interaction between *VHL* loss and ROCK inhibition is HIF-dependent. (**a**) Western blot showing the efficiency of ARNT knockdown by shRNA (shARNT) in *VHL*-deficient RCC4 and RCC10. The scrambled shRNA was used as a control (shScr). ARNT inhibition causes a decrease in HIF1α and HIF2α activity as evidenced by the decrease in expression of a HIF target gene LDHA. (**b**) Clonogenic assay showing that CC-RCC cells transduced with shARNT exerted resistance to ROCK inhibition in comparison with shScr-transduced CC-RCC cells. In that respect, CC-RCC cells transduced with shARNT behaved similarly to the cell lines with re-introduced *VHL*. Each treatment was normalized to the DMSO control. (**c**) CC-RCC ± VHL cells were treated with Y-27632 at the indicated concentrations, plated for clonogenic assays and replicate plates were subjected to either normoxia (21% O_2) or hypoxia (2% O_2). Each assay was performed in duplicate and repeated three times. Colony numbers were normalized to the vehicle control. RCC4VHL, RCC10VHL and 786-OVHL cells were sensitized to ROCK inhibition in hypoxia. Statistical analysis was performed using a paired *t*-test (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$); s.e.m.'s are shown. (**d** and **e**) Clonogenic assay showing that HIF1α expression sensitizes 786-O cells to Y-27632 (**d**) and RKI 1447 (**e**). Each dose was compared statistically using a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc*. Tie bars indicate significant differences with a *P*-value < 0.01. (**f**) Clonogenic assay showing that non-degradable HIF1α and HIF2α expression are sufficient to induce the synthetic lethal effect with 20 μM Y-27632 treatment. Statistical analysis was conducted using a one-way ANOVA ($P < 0.001$) followed by Tukey's *post hoc*. There were three statistically significant groups: 786-OVHL > 786-O, 786-OVHL CA-HA-HIF2α > 786-OVHL CA-HA-HIF1α and 786-O CA-HA-HIF1α.

The dependence of the synthetic lethal effect of ROCK inhibition on HIF overexpression is important as ROCK inhibitors may serve as potential therapeutics for other types of solid tumors besides CC-RCC, where both HIF and ROCK are expressed. In addition to CC-RCC,[31] ROCK overexpression occurs commonly in multiple cancer types[46] including lung,[35] breast,[29,32] osteosarcoma[47] and prostate cancer.[30] On the other hand, a large fraction of solid tumors possesses hypoxic regions (where HIFs are stabilized[48]) or stabilize HIF1α and HIF2α by *VHL*-independent mechanisms, including Phosphatase and Tensin homolog (PTEN) loss or Harvey rat sarcoma viral oncogene homolog (H-Ras) activation.[48,49] Thus, we predict that ROCK inhibitors will be effective against several more tumor types besides CC-RCC.

The crosstalk between HIF and ROCK has been investigated previously.[50–53] On the one hand, two studies show that RhoA and



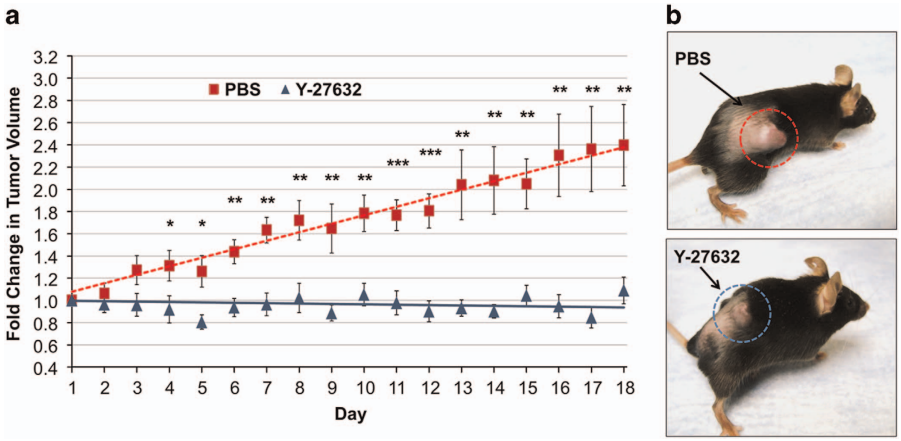


Figure 6. Y-27632 inhibits tumor growth *in vivo*. (**a**) RAG1 mice were injected with 5 × 106 786-OT1 cells into the right flank. After 1 month, mice were randomized into two groups and treated daily with PBS vehicle ($n = 9$) or 10 mg kg^{-1} Y-27632 ($n = 9$) by ip injection. The fold change in tumor volume was analyzed statistically using a one-way ANOVA with treatment as the factor (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$); s.e.m.'s are shown. The solid line represents the linear trend fit of the data for each treatment group. (**b**) Representative images of a control mouse (top) and a Y-27632-treated mouse (bottom) on day 14 of treatment are shown.

ROCK1 are HIF target genes in breast cancer[50] and trophoblast cells.[51] In addition, Turcotte *et al.*[52] showed that RhoA expression and activity are hypoxia-inducible in renal cancer, although it does not depend on HIF activity. If this regulation is maintained in renal cancer, the loss of *VHL* would be predicted to induce ROCK1 upregulation. We do not see increased ROCK1 expression (Figures 2b and c; Supplementary Figure 5) and actually observe decreased phosphorylation of the ROCK substrate MYPT1 in *VHL*-deficient cells (Supplementary Figure 3), thus not supporting this type of regulation. On the other hand, multiple studies show that the Rho/ROCK pathway stimulates HIF activity by multiple mechanisms, which are likely to be cell-type-specific.[52–54] These data for CC-RCC are missing, and the crosstalk needs to be investigated.

In summary, ROCK1 inhibition is synthetically lethal with *VHL* loss in CC-RCC, and ROCK inhibitors could serve as novel therapeutics for the disease. ROCK inhibitors would complement currently approved angiogenesis inhibitors as ROCK inhibitors selectively induce tumor cell death, reduce proliferation and migration, ultimately leading to inhibition of tumor growth and potentially metastasis.

MATERIALS AND METHODS

Cell culture and chemical treatments

The CMV-EYFP-labeled RCC4 ± VHL matched cell lines were previously described.[22] Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Caisson Labs #25-500, North Logan, UT, USA)+10% fetal bovine serum (Omega Scientific #FB-12, Tarzana, CA, USA)+1% penicillin/streptomycin (Caisson Labs #25-512) in 5% CO_2, 21% O_2 at +37 °C. Cell lines used in this study were validated using short tandem repeat profiling. Y-27632 and GSK 429286 were obtained from Tocris (Minneapolis, MN, USA). RKI 1447 was obtained from Selleck Chemicals (Houston, TX, USA). All compounds were diluted in DMSO and serially diluted for each experiment.

Cell viability assay based on measurements of fluorescence intensity

RCC4 EYFP and RCC4VHL EYFP were plated at 5000 cells per well in black 96-well tissue culture plates in fetal bovine serum-free DMEM media. The following day, DMSO vehicle or varying compound concentrations were prepared in 20% fetal bovine serum DMEM by serial dilution, and an equal volume was added to the cells. Cells were incubated for 72 h. Wells were washed with PBS. Then, 100 µl of PBS was added to each well and fluorescence intensity was measured on a BioTek Synergy HT Microplate Reader (Winooski, VT, USA) at 488 nm. Each experiment was performed three times in quadruplicate per treatment.

Clonogenic cell survival assay

Clonogenic assays were performed plating 300 cells per plate as previously described.[36]

Long-term repeat treatment experiments

CC-RCC ± VHL cell lines were plated at 5×10^4 cells per well into a six-well plate and treated with vehicle (DMSO) or 2 µM RKI 1447 daily. Each day, the media was aspirated and fresh media with DMSO vehicle or 2 µM RKI 1447 was added to each well. When the vehicle control plate was at 80% confluency, the cells were passaged 1:10 into new plates. Owing to the different growth kinetics each cell line was passaged and counted at different time points: RCC4 on day 4, 9 and 15; RCC10 on 4, 7, 9 and 11; and 786-O on 4, 11, 15 and 18.

Gene knockdowns by siRNAs

RCC4 ± VHL cell lines were plated at 200 000 cells per well of a six-well plate in fetal bovine serum-free DMEM. The following day, the cells were transfected with 6 µl DharmaFECT1 (Dharmacon, Lafayette, CO, USA) and up to 100 nM siRNA according to the manufacturer's protocol. The siRNAs for ROCK1 (#1: SASI_Hs01_00065573 and #2: SASI_Hs01_00065570), ROCK2 (#1: SASI_Hs01_00204253 and #2: SASI_Hs01_00204251) and MISSION(R) Universal Negative Control #1 siRNA were obtained from Sigma (St Louis, MO, USA). The following day, transfected cells were plated for the clonogenic cell survival assay. Replicate plates were lysed after 72 h, and ROCK1 and ROCK2 expression analyzed using western blot analysis.

Western blot analysis

After treatments, cells were lysed and western blot analysis was conducted as previously described.[55] Proteins were visualized using primary antibodies recognizing ARNT, HIF1α (BD Biosciences, #611078, #610959, San Jose, CA, USA), HIF2α (Novus Biological, #NB100-122, Littleton, CO, USA), α-tubulin (Fitzgerald, #10 R-842, North Acton, MA, USA), LDHA (Genetex, #GTX101416, Irvine, CA, USA), MYPT1-P Thr696 (EMD Millipore, #ABS45, Temecula, CA, USA), MYPT1, (Abcam, #ab32393, Cambridge, MA, USA), Cleaved Caspase 3, VHL, HA (Cell Signaling, #9661, #2738, #3724, Danvers, MA, USA), HSP70 (Sigma, #H5147, St Louis, MO, USA), ROCK1, ROCK2 (Thermo Scientific, #PA5-22262, #PA5-21131, Grand Island, NY, USA) and horseradish peroxidase-conjugated Goat anti-Rabbit IgG and Goat anti-Mouse IgG secondary antibodies (Thermo Scientific, #31460, #31430). Blots were imaged using a Bio-Rad ChemiDoc XRS$^+$ (Bio-Rad, Hercules, CA, USA).

PI-immunofluorescence staining

RCC4 ± VHL cells were cultured in the presence of DMSO, 20 µM Y-27632 or 1 µM doxorubicin. After 24 h, 1 µg ml^{-1} PI was added to each well and the cells were imaged on a Nikon TI-E at ×4 and the PI-positive cells were

counted per field. For the siRNA experiments, siRNAs were transfected at up to 100 nM following Dharmacon's protocol and imaged at ×10 after 48 h. Each transfection was conducted in triplicate.

Transwell migration assay

Polyethylene terephthalate transwells (8.0 μm; Corning, Corning, NY) were coated with fibronectin as previously described.[55] Overall, 70 000 RCC10 or 35 000 786-O cells were used per transwell.

Cell cycle analysis

For this experiment, 10^5 cells were seeded per well of a six-well plate and treated the following day with vehicle (DMSO) or Y-27632 for 72 h. BrdU analysis was performed using the fluorescein isothiocyanate BrdU Flow Kit (BD Biosciences, Catalog #559619) following the manufacturer's protocol.

shRNA expression constructs, lentivirus packaging and infection of target cells

HEK 293 T cells were transfected with lentiviral plasmids (pLKO.1shARNT: 5′AAATAAACCATCTGACTTCTC3′ (OpenBiosystems, Huntsville, AL, USA)) or pLKO.1shScrambled (Addgene, #1864, Cambridge, MA, USA) along with packaging plasmids, pVSVG and ΔR8.2, as previously described.[56]

Tumor growth analysis

Briefly, 18 RAG1 (B6.129S7-Rag1^{tm1Mom}/J, Jackson Labs, Bar Harbor, ME, USA) mice (11–20-week old) were injected sc into the right flank with 5×10^6 786-OT1 cells. Before each injection, cells were resuspended in 50 μl PBS/matrigel (BD Bioscience #354248) mixture at 50/50 ratio. One month post injections, when the tumors had reached the size of ~500 mm^3, littermates were randomized into two groups. Y-27632 (10 mg kg^{-1}) or PBS diluent was administered ip daily for 18 days. Tumor size was measured daily with a digital caliper. On day 18, the mice were sacrificed. Tumor volume was calculated using the formula: $V = (a)(b^2/2)$, where '*a*' is the shorter measurement of length/width. Every measurement for each mouse was normalized to the day 1 measurement to show the fold change over time. Statistical analysis was performed using a one-way analysis of variance between the two groups per day.

Growth curves and statistical analysis

Dose–response and cell growth curves were generated using GraphPad Prism, San Diego, CA, USA. IC_{50} values were calculated by transforming the *x* axis using $x = \text{Log}(x)$, normalizing the transformed data to the vehicle control with 0 as 0%, and then fitting the normalized transformed data with a nonlinear trendline either using a normalized response ('log(inhibitor) versus normalized response') or a variable slope ('log(inhibitor) versus normalized response – variable slope'). The correct nonlinear trendline was selected using GraphPad's comparison of fits, which directly compares both fit lines statistically using an extra sum-of-squares F test. The fit line is not shown in the figures. The IC_{50} values for each experiment were then calculated from the best-fit values. Statistical analysis was conducted in Minitab 16 using a paired *t*-test or analysis of variance between cell lines with a *P*-value of less than 0.05 considered statistically significant. All error bars represent the s.e.m.'s.

ABBREVIATIONS

ANOVA, analysis of variance; ARNT, Aryl hydrocarbon receptor nuclear translocator; BrdU, Bromodeoxyuridine; CC-RCC, Clear cell renal cell carcinoma; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl sulfoxide; EYFP, Enhanced Yellow Fluorescent Protein; HA, hemagglutinin; HIF, Hypoxia-Inducible Factor; ip, intraperitoneal; LDHA, Lactate Dehydrogenase A; LOPAC, Library of pharmacologically active compounds; mTORi, Mammalian Target of Rapamycin Inhibitors; MYPT1, Myosin Phosphatase Target 1; PBS, Phosphate-buffered saline; PI, Propidium iodide; ROCK, Rho-Associated, coiled-coil-containing protein kinase; sc, subcutaneous; shRNA, small hairpin RNA; siRNA, small interfering RNA; VHL, von Hippel-Lindau.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

OVR and JMT designed the study. JMT, QHN, MS, MWP, IN, LJN, ACL and OVR conducted experiments and analyzed the data. JMT and OVR wrote the paper.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)