Targeting the Mevalonate Pathway Suppresses VHL-Deficient CC-RCC through an HIF-Dependent Mechanism


Abstract

Clear cell renal cell carcinoma (CC-RCC) is a devastating disease with limited therapeutic options available for advanced stages. The objective of this study was to investigate HMG-CoA reductase inhibitors, also known as statins, as potential therapeutics for CC-RCC. Importantly, treatment with statins was found to be synthetically lethal with the loss of the von Hippel–Lindau (VHL) tumor suppressor gene, which occurs in 90% of CC-RCC, driving the disease. This effect has been confirmed in three different CC-RCC cell lines with three different lipophilic statins. Inhibition of mevalonate synthesis by statins causes a profound cytostatic effect at nanomolar concentrations and becomes cytotoxic at low micromolar concentrations in VHL-deficient CC-RCC. The synthetic lethal effect can be fully rescued by both mevalonate and geranylgeranylpyrophosphate, but not by squalene, indicating that the effect is due to disruption of small GTPase isoprenylation and not the inhibition of cholesterol synthesis. Inhibition of Rho and Rho kinase (ROCK) signaling contributes to the synthetic lethal effect, and overactivation of hypoxia-inducible factor signaling resulting from VHL loss is required. Finally, statin treatment is able to inhibit both tumor initiation and progression of subcutaneous 786-OT1-based CC-RCC tumors in mice. Thus, statins represent potential therapeutics for the treatment of VHL-deficient CC-RCC.

Mol Cancer Ther; 17(8); 1781–92. © 2018 AACR.

Introduction

Clear cell renal cell carcinoma (CC-RCC) is a life-threatening condition, especially in its metastatic manifestation. It is resistant to both radiation and chemotherapy (1), and although effective CC-RCC patients responding, the median overall survival remains at 2 years (2). In addition, toxicity to normal tissues is a limiting factor for current treatments. Thus, it is of primary importance to identify new therapeutics and their target pathways to successfully treat CC-RCC. Identified in 1993 as the tumor-suppressor gene affected in von Hippel–Lindau disease (3), the VHL gene is lost in 80% to 90% of CC-RCC (4). The goal of this study is to therapeutically target this large group of VHL-deficient CC-RCCs using a synthetic lethality approach.

Previously, we conducted a chemical library screen and identified Y-27632 and inhibition of its target (ROCK1) being synthetically lethal with VHL loss (5). While multiple ROCK inhibitors have shown success in topical treatments for glaucoma (6), systemic treatments were conducted only with two ROCK inhibitors. Fasudil was approved in Japan for treatment of cerebral vasospasm complicating intracranial hemorrhage (7); and AT13148 is currently in a phase 1 clinical trial (NCT01585701) for solid tumors other than CC-RCC (8). Thus, systemic use of ROCK inhibitors requires further investigation to determine a therapeutic window for cancer patients. On the other hand, HMG-CoA reductase inhibitors, also known as statins, can inhibit Rho/ROCK signaling in human patients (9), and their pharmacokinetics and doses are well established, including maximum tolerated doses (10).

Rho GTPases are upstream activators of ROCK (11). Rho/ROCK inhibition by statins occurs due to reduced synthesis of mevalonate and geranylgeranyl pyrophosphate (GGPP), in turn leading to inhibition of protein isoprenylation (9). This disrupts the intracellular trafficking of small GTPases like Rho, Ras, Rap1a, and Rac and their recruitment to the cell membrane required for their activity (12). Although statins are not specific toward Rho, they are safe and taken by hypercholesterolemia patients at up to 1 mpk (80 mg daily; ref. 13). In addition, lovastatin was evaluated as an anticancer agent for gastric carcinoma (14), anaplastic astrocytoma (15), and glioblastoma multiforme (15) in phase I and II clinical trials, and the maximum tolerated doses were established as high as 20 to 35 mpk daily for 7 days, with monthly repeats, resulting in responses in 3 of 18 patients (15). Accordingly, it has become clear that biomarkers are needed to stratify the patients into responders and nonresponders. In addition, studies addressing the mechanism of statins’ anticancer action are largely absent. Because our studies show that statins trigger synthetic lethality with VHL deficiency, VHL can be used as a biomarker for tumor sensitivity. Furthermore, the effect is dependent on the overactivation of HIF's
upon VHL loss, making HIF expression a second potential bio-
marker. Statin treatment selectively inhibits cell proliferation and
induces cell death. Our studies also reveal that this effect occurs
due to the disruption of GTPase isoprenylation and partially
through the inhibition of Rho/ROCK1 signaling. Statin treatment
is effective at inhibiting tumor initiation and tumor growth of
established tumors in vivo, confirming their potential as thera-
peutics for treating VHL-deficient CC-RCC.

Materials and Methods

Cell culture and chemical treatments

All cells lines used in this study were grown in Dulbecco’s
Modified Eagle's Medium (DMEM; Caisson Labs #25-500,
North Logan, UT) + 10% fetal bovine serum (FBS; Omega
Scientific #EB-12) + 1% penicillin/streptomycin (Caisson Labs
#25-512) in 5% CO2, 21% O2, at 37°C. The cell lines used in
this study were a gift from Dr. Giaccia (Stanford). The identities
of RCC4, RCC10, and 786-O were confirmed via STR analysis
through the University of Arizona Genetics Core. 786-OT1
cells are a subline of 786-O described in ref. 5. Simvastatin,
pravastatin, mevalonate, GGPP, squalene (Sigma-Aldrich), flu-
vastatin, lovastatin (Selleck Chemicals), and arachidonic acid
(AA; MP Biomedicals). Fluvasatin and pravastatin were diluted
in dimethyl sulfoxide (DMSO) and serially diluted for each
experiment. Simvastatin and lovastatin were dissolved in
ethanol and activated in 0.1N NaOH by incubation at 50°C
for 20 minutes. The vehicle control was sub-
dilution to 20 mmol/L in DMSO. The vehicle control was sub-
jected to the same process and is approximately 20% ethanol
and 80% DMSO.

Clonogenic assay

Clonogenic assays were performed using 300 cells/plate as
previously described (5). For rescue experiments, fluvasatin
(0.6125 or 1.25 μmol/L) and metabolite [GGPP (10 and 20
μmol/L), mevalonate (500, 1,000, and 2,000 μmol/L), or squal-
en (10 and 100 μmol/L)] were dosed together, and the clono-
ogenic assays were analyzed 7 days after treatment.

Live/dead cell viability assay

The live/dead cell viability assays were performed by plating
300 cells per well into a 96-well plate, allowing them to
attach overnight, and treated the following day with statins.
On the 6th day, calcein AM fluorescent dye from Thermo Fisher
(1:1,000) and propidium iodide (PI) from Sigma Aldrich
(1:250) were added to each well, incubated for 10 minutes at
37°C, and then images were obtained on a Nikon TI-E at 4×.
The live cells (calcein-positive) and dead cells (PI-positive)
counted per field. Treatments were normalized to vehicle
controls for proliferation calculations. Normalized % viability
was calculated by dividing the number of dead PI-positive cells
by the total number of cells (calcein-positive + PI-positive),
subtracting the result from 1, and then multiplying by 100%.
Treatments were conducted in quadruplicate and each exper-
iment was repeated three times.

Western blot analysis

After treatments, cells were lysed, and Western blot was con-
ducted as previously described (5). Proteins were visualized using
primary antibodies recognizing HIF1α, VHL (BD Biosciences,
#610959, #564183), HIF2α (Novus Biological, #NB100-122
and abcam, #ab199), α-tubulin (Fitzgerald, #10R-842), β-actin
(Sigma, #A5441), Phospho-LIMK1 (Thr508)/LIMK2 (Thr505),
LIMK1, Rap1a (Cell Signaling Technology, #3841S, #3842S,
and #4938S), unprenylated Rap1a (Santa Cruz Biotechnology,
#SC-1482), LDHA, CAIX (GeneTex, #GTX101416, #GTX70020);
and Horseradish peroxidase conjugated Goat anti-Rabbit
IgG and Goat anti-Mouse IgG secondary antibodies (Thermo
Scientific, #31460 and #31430). Blots were imaged using a
Chemidoc XR+ (BioRad).

Cholesterol detection assay

The Cholesterol Cell-Based Detection Assay Kit (Cayman
Chemical, #10009779) was performed following the manu-
facturer's protocol. Cells were treated 24 hours before fixation.
U-18666A, at 1.25 μmol/L, served as a positive control and came
with the kit.

Lentiviral constructs and virus production

HEK 293T cells were transfected with lentiviral plasmids
(pLKO.1shARNT: 5'AAATAAACCATCCTGACCTTCC3; target
sequence, OpenBiosystems) or pLKO.1shScr: 5'CCTAAGGT1AA
GTCGCCCTGGCC3 (target sequence, Addgene, #1864), along
with packaging plasmids, pVSVG and ΔR8.2. Virus collection
and infection were conducted as previously described (5).

Cell-cycle analysis

A total of 50,000 cells were seeded per well of a 6-well plate
and treated the following day with vehicle (DMSO) or fluvast-
atin for 6 days. BrdU analysis was performed using the
FITC BrdU Flow Kit (BD Biosciences, #559619) following the
manufacturer’s protocol.

In vivo experiments

All in vivo experiments were conducted in accordance
with and approval of the UCI IACUC. Twenty-five RAG1
mice (B6.129S7-Rag1tm1J.1gfl/J, The Jackson Laboratory) mice
(11–20 weeks old) were injected subcutaneously into the right
flank with 5 × 106 786-OT1 cells resuspended in 50 μL of
50%PBS/50% matrigel (BD Bioscience, #354248) mixture.
See details in the Results section. Kaplan–Meier curves were
derived using GraphPad software and statistical significance
was calculated based on a Mantel–Cox test. Tumor volume was
calculated using the formula: $V = \frac{a(b)^2}{2}$, where $a$ is the
shorter measurement of length/width. Statistical analysis was
performed using a one-way ANOVA between the two groups
day.

Tumor sample processing

Tumor samples were fixed in formalin overnight and stored in
70% ethanol until processing on a Leica Tissue Processor
(TP1020) following the manufacturer's protocol. Samples were
then embedded in paraffin on a Leica EG 1150 embedding/
cooling station and sectioned on a Leica RM2255.

Ki67 staining

Samples were baked at 65°C overnight and then deparaaffi-
nized and rehydrated. Antibody retrieval was performed in
10 mmol/L sodium citrate, and samples were blocked in goat
serum. Samples were then incubated overnight in Ki67 pri-
mary antibody (Genetex, #GTX16667) at 4°C, followed by
incubation for 1 hour in secondary antibody ( Goat anti-Rabbit IgG H&L; Alexa Fluor 488; Abcam, #ab150077) at room temperature. Cell nuclei were visualized with DAPI using Vectashield with DAPI (VWR, #101098-044). Images were obtained on a Nikon Ti-E.

**TUNEL assay**

TUNEL analysis was conducted using the DeadEnd Fluorometric TUNEL System (Promega, #G3250) following the manufacturer’s protocol for paraffin-embedded tissue sections. The positive control was prepared by treating 786-O cells with 2 μmol/L staurosporine for 4 hours. The cells were then trypsinized, washed with PBS, and spun down onto slides using a cytopsin centrifuge.

**Growth curves and statistical analysis**

Dose response and cell growth curves were generated using GraphPad Prism. IC_{50} values were calculated by transforming the X axis using X = Log(X), normalizing the transformed data to the vehicle control with 0 as 0%, and then fitting the normalized transformed data with a nonlinear trend line using either a normalized response (‘log(inhibitor) vs. normalized response’) or a variable slope (‘log(inhibitor) vs. normalized response – variable slope’). The correct nonlinear trend line was selected using GraphPad’s comparison of fits, which directly compares both fit lines statistically using an extra sum-of-squares F test. 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 ANOVA between cell lines with a P value of less than 0.05 considered statistically significant. All error bars represent the SEMs. The number of biological replicates is indicated in each figure legend.

**Results**

**Treatment with statins selectively targets VHL-deficient CC-RCC of multiple genetic backgrounds**

Because statins inhibit Rho/ROCK signaling (9) and we recently showed that ROCK1 inhibition is synthetically lethal with VHL loss in CC-RCC (5), we decided to test whether statin treatment would be synthetically lethal with VHL loss. Isogenic cell line pairs were generated from the parental VHL-deficient CC-RCC cell lines by reexpressing the full-length wild-type VHL cDNA (16). VHL is a substrate-recognition subunit of an E3 ubiquitin ligase complex that targets the α subunits of hypoxia inducible factors 1 and 2 (HIF1 and HIF2) for proapoptotic degradation in the presence of oxygen (in normoxia) (17). Accordingly, VHL loss causes overexpression of HIF1α and HIF2α in RCC4 and RCC10 cells and HIF2α in 786-O cells, and VHL reintroduction causes a decrease in HIF expression (Supplementary Fig. S1). We conducted clonogenic assays and showed that both simvastatin (Fig. 1A–C; Supplementary Fig. S2A–S2C) and lovastatin (Fig. 1D–F; Supplementary Fig. S2D–S2I) treatments are synthetically lethal with VHL loss. Both RCC4 (Fig. 1A and D) and RCC10 (Fig. 1B and E) showed sensitivity to statin treatment and a nearly 15-fold difference in IC_{50} values over respective RCCVHL cell lines. 786-O (Fig. 1C and F) showed a 5-fold difference in IC_{50} values over 786-OVHL.

We also tested the effect of lovastatin and pravastatin on the colony-forming ability of the RCC4 + VHL isogenic cell lines. RCC4 (Supplementary Fig. S3A) showed sensitivity to lovastatin treatment and a 9-fold difference in IC_{50} values over RCC4VHL. Because treatment with pravastatin up to 80 μmol/L did not reduce the colony-forming ability of both RCC4 and RCC4VHL (Supplementary Fig. S3B), we assessed the inhibitory effect of each statin on isoprenylation of Rap1α, which depends on the mevalonate pathway. Unprenylated Rap1α was detected by Western blot (18) after treatment with all statins except pravastatin (Supplementary Fig. S3C), which is consistent with the lack of the effect of pravastatin on colony-forming ability of RCC4 cells. Unlike lipophilic statins (simvastatin, lovastatin, and pravastatin), pravastatin is hydrophilic and requires a liver-specific transporter OATP1B1 (19) to be delivered inside the cells; thus, it is likely not delivered to CC-RCC cells. Together, these data indicate that treatment with multiple lipophilic statins is synthetically lethal with VHL loss in several CC-RCC genetic backgrounds.

**Treatment with statins is cytostatic and cytotoxic in VHL-deficient CC-RCC**

We next sought to determine if the effect of statins on colony-forming ability of VHL-deficient CC-RCC was caused by cell death, inhibition of proliferation, or both. We treated RCC4 + VHL cells with simvastatin at doses ranging from ~600 μmol/L to 10 μmol/L. We found that simvastatin decreases RCC4 cell proliferation starting at nanomolar doses and increases RCC4 cell death starting at low micromolar doses based on calcein-based Live/Dead assay (Fig. 2A–C). We confirmed these effects in the 786-O + VHL cells (Supplementary Fig. S4). Thus, statin treatment is predominantly cytostatic in VHL-deficient CC-RCC, but becomes cytotoxic as the concentration increases.

Because each of the RCCVHL cell lines used above was genetically modified to overexpress VHL, we then asked whether endogenous VHL expression could protect against the cytostatic effects of simvastatin treatment. We compared the sensitivity of four VHL-deficient and four VHL-expressing kidney cancer cell lines with 5 μmol/L simvastatin by calcein-based assay. As expected, the VHL-deficient RCC4, RCC10, 786-O, and A498 were all more sensitive to simvastatin treatment in comparison with the VHL-expressing ACHN, SN12C, SN12Li, and TK10 (Fig. 2D). VHL expression and HIF expression in these cell lines was confirmed by Western blot (Fig. 2E). Thus, endogenous VHL expression is sufficient to protect cells against simvastatin treatment.

**Synthetic lethality depends on statins’ blocking effect on small GTPase isoprenylation**

Next, we sought to confirm that the synthetic lethal interaction between statins and VHL loss was due to the inhibition of HMG-CoA reductase (see Supplementary Fig. S5 for schematic). Because HMG-CoA reductase catalyzes generation of mevalonate, we performed experiments with exogenous mevalonate to see if we could rescue cell proliferation of simvastatin-treated RCC4. As expected, 500 and 1,000 μmol/L mevalonate treatments were able to partially and fully rescue the colony-forming ability of simvastatin-treated RCC4 (Fig. 3A). The effectiveness of treatment by statins and rescue by mevalonate was assessed by their effect on Rap1α isoprenylation at 24 hours, which was blocked by lovastatin and rescued by mevalonate (Fig. 3C). Because 500 μmol/L mevalonate was able to fully restore Rap1α
isoprenylation at 24 hours, but provided just the partial rescue of colony-forming ability at 10 days, we assume that mevalonate stability and/or metabolic rate over the prolonged period of time contributes to the partial rescue. Furthermore, the addition of mevalonate to the RCC4/VHL cells treated with high doses of simvastatin (10 and 20 μmol/L) resulted in a partial rescue of proliferation and a complete rescue of cell death in the Live/Dead Assay (Supplementary Fig. S6). BrdU cell-cycle analysis revealed that statin treatment selectively decreases S phase progression and increases apoptotic/debris cells in RCC4 cells, but not in RCC4VHL cells (Supplementary Fig. S7).

Because the mevalonate pathway has multiple downstream metabolic products, we next sought to elucidate which arm of the pathway is involved in the synthetic lethal effect. It is important to note that in our experiments we used medium containing cholesterol from the serum. Because we were seeing the difference in RCC and RCCVHL colony-forming ability in that medium, it suggested that this arm of the mevalonate pathway was not important for the synthetic lethal effect. In addition, to rescue cholesterol synthesis in fluvastatin-treated RCC4/VHL cells, we added up to 100 μmol/L squalene (cholesterol precursor) and conducted clonogenic assays. The inability of squalene to rescue colony numbers (Supplementary Fig. S8) confirmed that cholesterol synthesis does not contribute to the synthetic lethal effect.

Previous studies have identified the inhibitory effect of statins on small GTPases, including the Rho GTPase and Rap1a GTPase (refs. 9, 18, 20, 21; used as a readout of statin inhibitory action on the mevalonate pathway in Fig. 3C and Supplementary Fig. S2B). One of the arms of the mevalonate pathway generates GGPP, which is used as a substrate for isoprenylation of small GTPases by GGTPase, directing GTPases' membrane localization (see Supplementary Fig. S5 for schematic). In order
Figure 2.
Statin treatment is cytostatic and cytotoxic in VHL-deficient CC-RCC. Live/dead assay measuring live cell numbers via calcein staining (A) and dead cell numbers via PI staining (B) reveals that simvastatin treatment inhibits RCC4 cell proliferation at nanomolar and micromolar doses and triggers cell death at higher micromolar doses. RCC4/VHL cells were treated with simvastatin or vehicle control (80% DMSO/20% ethanol) for 6 days. Calcein-positive cells in A were normalized to the vehicle control. Statistical analysis in A and B was performed using a paired t test between the matched cell lines at each dose (*, P < 0.05; **, P < 0.01); SEMs are shown. C, Representative images of the live/dead assay. D, VHL-deficient CC-RCC are more sensitive to simvastatin treatment than renal cancer cell lines endogenously expressing VHL. Cell lines were treated with 5 μmol/L simvastatin for 6 days, and the live cell number was assessed by calcein staining. The results were normalized to vehicle-treated cells. Statistical significance was determined using a one-way ANOVA followed by Tukey post hoc analysis (***, P < 0.001); SEMs are shown. The experiment was conducted in duplicate and repeated two times. E, Western blot confirming VHL expression in cell lines used in D; HIF1α and HIF2α expression is also shown. α-Tubulin serves as a loading control.
The inhibitory effect of statins on the Rho/ROCK pathway is important for synthetic lethality with VHL loss

Previously, we reported that ROCK1 inhibition results in a synthetic lethal interaction with VHL loss in CC-RCC (5). Thus, we hypothesized that inhibition of Rho GTPase isoprenylation and subsequent inhibition of ROCK by statins is responsible for the synthetic lethality with VHL loss. First, we assessed whether treatment with statins causes Rho/ROCK pathway inhibition. We treated the RCC4 ± VHL cells with fluvastatin for 24 hours and observed a decrease in phosphorylation of LIMK1 at Thr508 and LIMK2 at Thr505 (Fig. 4A). This effect was also replicated in the RCC10 ± VHL and the 786-O ± VHL (Supplementary Fig. S9).

Second, in order to rescue ROCK pathway activity in fluvastatin-treated RCC4 ± VHL cells, we added 100 μmol/L AA, which binds and activates ROCK by releasing it from its own autoinhibition (22, 23), and conducted clonogenic assays. AA was able to partially rescue the colony-forming ability of fluvastatin-treated RCC4 cells (Fig. 4B). AA treatment activated ROCK signaling as judged by increased phosphorylation of LIMK1/2 (Fig. 4C). Together, these results indicate that inhibition of the Rho/ROCK pathway by statins contributes to synthetic lethality with VHL loss in CC-RCC.

The synthetic lethal interaction between statin treatment and VHL loss is dependent on the activation of HIFs

Because mutation or deletion of VHL results in the stabilization and activation of HIF1α and HIF2α (24–26), we conducted three experiments to test if the synthetic lethal effect of statin treatment with VHL loss is dependent on overactivation of the HIF pathway. In the first experiment, we knocked down the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT), which heterodimerizes with both HIF1α and HIF2α and is required for their activity (27). We then treated shARNT- and shScramble-transduced CC-RCC cells with fluvastatin and conducted clonogenic assays. shARNT-transduced RCC4, RCC10, and 786-O cells were protected from fluvastatin treatment in comparison with respective shScramble-transduced control cells (Fig. 5A). ARNT knockdown led to reduced expression of HIF target gene LDHA as compared to the DMSO vehicle control. RCCVHL cell lines were sensitized to fluvastatin treatment in hypoxia mimics VHL reintroduction, indicating that the synthetic lethal effect is dependent on HIF signaling.

In the second experiment, we treated RCC4 ± VHL, RCC10 ± VHL, and 786-O ± VHL cells with fluvastatin and subjected them to clonogenic assays in normoxia (21% oxygen) or hypoxia (2% oxygen). Each treatment was then normalized to the DMSO vehicle control. RCCVHL cell lines were sensitized to fluvastatin treatment in hypoxia having decreased colony-forming ability in hypoxia in comparison with normoxia (Fig. 5C). Activation of HIF1α and HIF2α and induction of their downstream target in hypoxia were confirmed by Western blot (Fig. 5D). The sensitization of the RCCVHL cells to fluvastatin treatment in hypoxia mimics VHL loss, indicating that the synthetic lethal effect is dependent on HIF signaling.

In the third experiment, we used 786-OVHL cell line expressing a nondegradable constitutively active hemagglutinin-tagged HIF1α (CA-HA-HIF1α) or HIF2α (CA-HA-HIF2α; Fig. 5E), which sensitized 786-OVHL cells to fluvastatin treatment in comparison with vector control cells (Fig. 5F).
Expression of CA-HA-HIF1α in 786-O cells did not further sensitize them to fluvastatin treatment in comparison with the vector control cells (Fig. 5F). Together, these results confirm that the synthetic lethal effect between statins and VHL loss is dependent on the stabilization and overactivation of either HIF1α or HIF2α signaling.

Fluvastatin delays tumor initiation and inhibits tumor growth in vivo

There are reports suggesting that statins reduce the risk of developing cancer (28–30), while other studies suggest that statins could serve as cancer therapeutics (21, 31). Accordingly, we decided to test if statins delay CC-RCC tumor initiation and also inhibit tumor growth. For in vivo studies, we used 786-OT1 cells, which were established from a 786-O–based tumor and are characterized by fast tumor growth kinetics in vivo as previously described (5). 786-OT1 showed similar sensitivity to fluvastatin in vitro as the parental 786-O cell line (Supplementary Fig. S10). 786-OT1 cells were injected subcutaneously into the right flank of 25 Rag1−/− mice. The mice were then randomized into three groups, and two of three groups were treated daily with 1% DMSO in PBS vehicle control (n = 8) or 10 mpk fluvastatin (n = 8) via intraperitoneal (i.p.) injection. The third group was left untreated (n = 9) until the tumors reached approximately 300 mm³. Tumors were examined daily, and palpable tumors were recorded. Treatment with 10 mpk fluvastatin inhibited tumor initiation as shown by Kaplan–Meier curves (Fig. 6A). Once tumors had formed, tumor size was measured biweekly by calipers; tumor volume increased rapidly in the vehicle-treated control group, while tumor volume in the 10 mpk fluvastatin-treated group increased at a significantly slower rate (Fig. 6B). When tumors reached approximately 300 mm³ in the third group (at day 54), we began treatments with 15 mpk fluvastatin daily for 21 days. Tumor size was measured triweekly and while tumor size constantly increased in the control group, treatment with 15 mpk fluvastatin resulted in a significant regression in tumor size (Fig. 6C). Both treatments resulted in a reduction of tumor weight and size (Fig. 6D and E). We also confirmed that 10 and 15 mpk fluvastatin treatments resulted in increased unprenylated Rap1a in tumor samples by Western blot, indicative of effective fluvastatin delivery to tumor tissue (Fig. 6F). The group treated with 10 mpk did not show a statistically significant difference in both proliferation (Ki67 staining) and apoptosis (TUNEL assay), which is consistent with the primary tumor growth curves in Fig. 6, where at the time of sacrifice the slope of the curves for PBS-treated and 10 mpk statin-treated groups becomes similar. As expected, Ki67 staining of the tumor tissue revealed that 15 mpk fluvastatin treatment resulted in decreased tumor cell proliferation in vivo at the time of sacrifice (Fig. 6G; Supplementary Fig. S11A). At the same time, TUNEL staining revealed that statin treatment slightly increased apoptotic cell death in vivo (Fig. 6H; Supplementary Fig. S11B). In summary, in vivo treatment with fluvastatin successfully inhibited tumor initiation and decreased tumor growth in established tumors.

Figure 4.
The inhibitory effect of statins on the Rho/ROCK pathway contributes to synthetic lethality with VHL loss. A, Western blot showing that 24-hour treatment with fluvastatin is sufficient to inhibit phosphorylation of LIMK1 (Thr508) and LIMK2 (Thr505) (ROCK substrates) in RCC4 ± VHL. Unprenylated Rap1a is used as a readout for the disruption of GTPase isoprenylation by treatment with fluvastatin. B, The effect of fluvastatin on RCC4 colony-forming ability can be partially rescued by administration of 100 μmol/L AA (ROCK activator). Each dose of fluvastatin or vehicle control (DMSO) within each experiment was tested in duplicate, and the experiment was repeated three times. Statistical analysis was performed using a paired t test between the matched cell lines at each dose (*, P < 0.05; **, P < 0.01); SEMs are shown. C, Western blot showing that 24-hour treatment with fluvastatin inhibits phosphorylation of LIMK1/2, and cotreatment with AA rescues phosphorylation of LIMK1/2 in the RCC4 ± VHL. A–C, α-Tubulin serves as a loading control.
Figure 5. HIF activation sensitizes CC-RCC to fluvastatin. A, Clonogenic assay showing that CC-RCC cells transduced with shARNT are protected against fluvastatin treatment and their colony-forming ability is comparable with RCCVHL cells. B, Western blot confirming the downregulation of ARNT in shRNA-transduced cells, accompanied by downregulation of HIF’s downstream target LDHA. Cells transduced with scramble shRNA (shScr) serve as controls. C, RCCVHL cells were treated with 1.25 μmol/L fluvastatin, plated for clonogenic assays, and replicate plates were subjected to either normoxia (21% O2) or hypoxia (2% O2) for the duration of the experiment. Colony numbers were normalized to the DMSO vehicle control. RCC4VHL, RCC10VHL, and 786-OVHL cells were sensitized to fluvastatin treatment in hypoxia. D, Western blot showing the induction of HIF1α and HIF2α and their downstream target LDHA in hypoxia (2% O2, 24 hours). E, Western blot confirming the overexpression of constitutively active (CA) nondegradable hemagglutinin-tagged HIF1α or HIF2α (CA-HA-HIF1α and CA-HA-HIF2α). Cells transduced with vector control (VC) serve as controls. Western blots shown are from the same gel. F, Clonogenic assay showing that overexpression of CAHIF1α or CAHIF2α sensitizes the indicated cell lines to fluvastatin treatment. Each assay in A, C, and F was performed in duplicate and repeated three times, and statistical analysis was performed using a paired t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001); SEMs are shown. α-Tubulin serves as a loading control in B, D, and E.
Fluvastatin prevents tumor initiation and inhibits tumor growth in vivo. A, $5 \times 10^6$ 786-OT1 cells were injected subcutaneously into 25 RAG1 mice. The mice were then randomized into three groups: vehicle control ($n = 8$), 10 mpk fluvastatin ($n = 8$), and no treatment ($n = 9$). Treatment was administrated immediately for the vehicle control and 10 mpk fluvastatin groups. Fluvastatin treatment (10 mpk) inhibited tumor initiation (A) as shown by Kaplan-Meier analysis and inhibited tumor growth (B). When the “vehicle control” and “no treatment” tumors reached approximately 300 mm$^3$, 15 mpk fluvastatin treatment was initiated for the “no treatment” group, which inhibited tumor growth (C). D, Administration of fluvastatin to both groups (10 and 15 mpk) resulted in reduced tumor weight in comparison with the vehicle-control group at sacrifice. E, Representative images of tumors. F, Representative Western blot of two showing that 10 and 15 mpk fluvastatin treatments resulted in appearance of unprenylated Rap1a in the tumor samples in comparison with the vehicle control, indicating that the drug was effectively delivered to the tumors. Administration of 15 mpk fluvastatin resulted in (G) decreased proliferation measured by Ki67 staining and (H) increased apoptotic cell death measured by TUNEL assay in comparison with the vehicle-control group at sacrifice. Statistical analysis in A was conducted using log-rank (Mantel-Cox) test ($P = 0.0007$) and Gehan-Breslow-Wilcoxon test ($P = 0.0015$). Statistical analysis in B and C was conducted using a paired t test between doses and a two-way ANOVA comparing the response of each treatment group over time. Statistical analysis in D, G, and H was conducted using a one-way ANOVA comparing treatments (*, $P < 0.05$; **, $P < 0.01$;***, $P < 0.001$) and Dunnett post hoc with vehicle set as the control group; SEMs are shown.
Discussion

In this study, we have shown that fluvastatin may serve as a potential therapeutic to target VHL-deficient CC-RCC. We have further determined that the therapeutic effect depends on blocking GTPase isoprenylation, including blocking of the Rho/ROCK pathway. Importantly, overactivation of the HIF pathway triggered by VHL loss contributes to CC-RCC sensitivity to statins. Treatment of VHL-deficient CC-RCC with statins in vitro inhibits proliferation and induces cell death. Accordingly, in vivo fluvastatin is effective at both preventing tumor initiation and at inhibiting tumor growth of established xenografts, confirming its therapeutic potential.

Multiple synthetic lethal interactions have been identified in VHL-deficient CC-RCC, including stimulation of autophagy (32), inhibition of Glut1 (26), CDK6 (33), MET (33), MEK1 (33), protein translation (34, 35), and inhibition of ROCK1 (5). Interestingly, with the exception of MET inhibition, statin target each of these synthetic lethal partners of VHL. Statin treatment has been shown to increase autophagy through inhibition of Rho and Ras (36), inhibit glucose uptake and glucose metabolism in cancer cells (37), inhibit CDKs (refs. 31, 38; including CDK6, ref. 31), inhibit the MEK pathway (39) through inhibition of Ras, and finally inhibit the ROCK pathway through inhibition of Rho (20, 21). Because our studies show only partial reliance on Rho/ROCK pathway inhibition for statins' selective targeting of VHL-deficient CC-RCC, statins' inhibitory effect on other synthetic lethality targets likely contributes to the observed therapeutic effect. Furthermore, statins' inhibitory effect on all of these synthetic lethality targets, except for the inhibition of glucose uptake, can be rescued by GPP (21, 36, 38, 39), indicating that inhibition of small GTPase isoprenylation is important.

The majority of patients with CC-RCC lose the function of VHL (17), which results in constitutive activation of HIFs, which is vital to the pathogenesis of the disease. Our finding that the synthetic lethal interaction is dependent on HIF overactivation suggests that other cancer types with overactivation of the HIF pathway might be sensitive to statin treatment. Thus, the stratification based on HIF activity and the status of small GTPase signaling needs to be tested to predict tumor's sensitivity to statins.

In this study, we chose fluvastatin for our in vivo studies because it has been shown to be the only statin that is able to achieve micromolar peak plasma concentrations ($C_{\text{max}}$) with oral doses that are currently approved for treating hypercholesterolemia. This is due to fluvastatin's saturable first-pass metabolism (40), which results in greater than proportional increases in $C_{\text{max}}$ as the dose is increased in patients. Thus, fluvastatin at hypercholesterolemia doses reaches $C_{\text{max}}$ concentrations that are greater than the IC50, determined in our study (Fig. 1D–F). Corsine and colleagues (10) showed that one 40 mg oral dose has a $C_{\text{max}}$ of 448 ng/mL (1.09 µmol/L) in patients. Another study by Siekmeier and colleagues (40) found that an 80 mg dose has an average $C_{\text{max}}$ of 1024.7 ng/mL (2.49 µmol/L). These studies make fluvastatin a statin of choice for evaluation as CC-RCC therapeutic.

In addition, the doses of statins used in anticancer clinical trials far exceed the doses used for hypercholesterolemia, and although they have side effects, they are tolerated by patients. Lovastatin was evaluated as an anticancer agent for gastric carcinoma (14), anaplastic astrocytoma (15), and glioblastoma multiforme (15) in phase I and II clinical trials, and the maximum tolerated doses were established as high as 20 to 35 mpk/day daily for 7 days, with monthly repeats (15). Hartman and colleagues reported that rhesus monkeys tolerated well 48/84/108 mpk/day doses of fluvastatin for 26 weeks (41). López-Aguilar and colleagues established a maximum tolerated dose for fluvastatin in pediatric patients at 8 mpk/day (42). The dose limiting side effect of statin treatment is apoptosis of skeletal muscle cells, which is preventable by the coadministration of ubiquinone (43). Accordingly, a maximum tolerated dose for lovastatin can be increased from 25 mpk/day to at least 45 mpk/day by coadministration of ubiquinone (maximum tolerated dose was not reached in the study; ref. 43). Thus, statin dosing and regimen for treatment of CC-RCC need to be carefully evaluated in patients.

There is another study reporting on the effect of statins on kidney cancer proliferation, migration, and tumor growth (44). The study did not concentrate on VHL-deficient CC-RCC and included several types of VHL-positive and -negative kidney cancers. Simvastatin was found to block tumor cell proliferation via the inhibition of the AKT/mTOR, ERK, and JAK2/STAT3 pathways at µmol/L doses (44), and block tumor growth of A498-based xenografts. Our data show that A498 cells are the least sensitive to statin treatment among the VHL-deficient cell lines tested (Fig. 2D), and the reported simvastatin $C_{\text{max}}$ concentrations in the blood (10) are far lower than those reported for fluvastatin. At the same time, we provide additional mechanisms of action for statins in CC-RCC via VHL loss and the resulting overactivation of the HIF pathway and inhibition of small GTPases, including Rho GTPase. Importantly, Rho GTPase was reported to regulate AKT signaling in melanoma (45), making our findings consistent with the ones discussed above. We also provide data on tumor initiation in addition to tumor progression.

Although statin administration led to reduction of tumor growth in both 786-OT1-based and A498-based (44) xenografts, additional evaluation of statins in vivo would be valuable. Although CC-RCCs tend to lose HIF1α during tumor progression (46) and multiple CC-RCC tumors and cell lines are expressing HIF2α only, including 786-O and A498, the efficacy of statins on HIF1α/HIF2α tumors in vivo would strengthen our conclusions. Furthermore, two autochthonous CC-RCC mouse models with the intact immune system have recently been developed (47, 48), which could be used as future testing platforms for statins.

While a number of epidemiologic studies have been conducted on the ability of statins to reduce the risk of CC-RCC, there is conflicting literature on the subject. Although there are studies showing that people on statins have a lower risk of CC-RCC development (28), and that CC-RCC patients on statins have a better overall survival and lower risk of progression after surgery (29, 30), there are two studies that found no correlation of statin intake and CC-RCC recurrence-free and progression-free survival (49, 50). This discrepancy may be explained by the absence of stratification of CC-RCC patients by VHL status, difference in pharmacokinetics ($C_{\text{max}}$ achieved) for different statins, and intake of lipophilic versus hydrophilic statins, targeting both hepatic and nonhepatic tissues versus mainly hepatic tissue, respectively. Taking into consideration the above factors, a more careful epidemiologic analysis should be conducted to draw the conclusions.
In addition, we see a conceptual difference between tumors arising in patients already on statins (those tumors should be statin-resistant), and VHL-deficient statin-naïve tumors (those tumors should be statin-sensitive and respond to statin therapy). Accordingly, we propose that VHL-deficient CC-RCC patients, who were never on statins before, may benefit from lipophilic statin intake; and that VHL-deficient CC-RCC tumor patients, who are on hydrophilic statins, may benefit from switching to lipophilic statins. Furthermore, patients with VHL disease, lacking one copy of VHL at birth, may benefit from taking lipophilic statins to prevent initiation of CC-RCC, hemangioblastoma, and pheochromocytoma (17).

In conclusion, statin treatment is synthetically lethal with VHL loss in CC-RCC, and fluvastatin could serve as a viable therapy for the disease. Treatment with fluvastatin has a profound effect on VHL-deficient CC-RCC cells inhibiting proliferation, inducing cell death, and inhibiting both tumor initiation and growth. It is expected that patient stratification by the HIF and small GTPase signaling status will predict the response to lipophilic statin therapy; it is also expected that the reanalysis of the existing epidemiologic data on the CC-RCC initiation taking into account the administrated statin’s response to lipophilic statin therapy; it is also expected that the tumors should be statin-sensitive and respond to statin therapy).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** J.M. Thompson, D.A. Fruman, O.V. Razorenova

**Development of methodology:** J.M. Thompson, Q.H. Nguyen

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.M. Thompson, A. Alvarez, M.K. Singha, M.W. Pavesic, Q.H. Nguyen, L.J. Nelson, O.V. Razorenova

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.M. Thompson, M.W. Pavesic, Q.H. Nguyen, O.V. Razorenova

**Writing, review, and/or revision of the manuscript:** J.M. Thompson, L.J. Nelson, D.A. Fruman, O.V. Razorenova

**Study supervision:** O.V. Razorenova

**Acknowledgments**

This work was supported by an NCI/NIH R03 to O.V. Razorenova (R03 CA22563-01), an R01 RSG-170150-01-CDD to O.V. Razorenova, an NIH T32 (T32CA090954-36A1) to J.M. Thompson, and an NIH ICTS (UL1 TR001414) to L.J. Nelson.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 1, 2017; revised November 19, 2017; accepted April 24, 2018; published first May 2, 2018.

**References**


