Targeting the Loss of the von Hippel-Lindau Tumor Suppressor Gene in Renal Cell Carcinoma Cells

Patrick D. Sutphin, Denise A. Chan, James M. Li, Sandra Turcotte, Adam J. Krieg, and Amato J. Giaccia

Program in Cancer Biology, Department of Radiation Oncology, Stanford University, Stanford, California

Abstract
Late-stage clear cell renal carcinoma poses a formidable clinical challenge due to the high mortality rate associated with this disease. Molecular and genetic studies have identified functional loss of the von Hippel-Lindau (VHL) gene as a frequent and crucial event in the development of the malignant phenotype of clear cell renal carcinomas. Loss of VHL function thus represents a pathognomonic molecular defect for therapeutic exploitation. The objective of this study was to evaluate the possibility of targeting VHL loss through pharmacologic means. Chromomycin A3 (ChA3) was identified through in silico analysis of existing publicly available drug profiles from the National Cancer Institute as an agent that seemed to selectively target VHL-deficient clear cell renal carcinoma cells. Genotype-selective toxicity was first determined through short-term viability assays and then confirmed with clonogenic studies. Coculture of fluorescently labeled carcinoma cells. Genotype-selective toxicity was first determined through short-term viability assays and then confirmed with clonogenic studies. Coculture of fluorescently labeled VHL-deficient and VHL-positive cells showed discriminate killing of the VHL-deficient cells with ChA3. Mechanistically, overexpression of hypoxia-inducible factor (HIF)-2α in VHL-negative clear cell renal carcinoma cells phenocopied loss of VHL with respect to ChA3 toxicity, establishing ChA3 as a HIF-dependent cytotoxin. This study shows the feasibility of selectively targeting the loss of the VHL tumor suppressor gene in clear cell renal carcinoma for potential clinical benefit and may have greater ramifications in the development of new targeted therapies for the treatment of cancer and other genetic diseases. [Cancer Res 2007;67(12):5896–905]

Introduction
An estimated 51,190 people in the United States will be diagnosed with cancer of the kidney or renal pelvis in the year 2007. An estimated 12,890 people in the United States will die due to cancer of the kidney or renal pelvis in 2007 (1). For those presenting with locally confined stage I and II disease, surgical resection is sufficient for cure in >90% of patients. Unfortunately, ~25% of patients present with distant metastases or significant local-regional disease as symptoms arise only late in the course of disease. As with other tumor types, extension of tumor cells beyond the organ of origin into the surrounding tissue, lymph nodes, and distant metastasis poses a considerable clinical challenge. Individuals with stage IV disease have a median survival of 10 months and a 5-year survival rate of 10%. Systemic therapies are severely limited, as renal cell carcinoma (RCC) is notoriously refractory to standard treatment regimens, including chemotherapy and radiation therapy. Immunotherapy currently fills the gap with only moderate success. Complete and durable responses have been observed in select patients with this modality.

Many key insights into the understanding of RCC have developed over the last few years, much of which can be credited to the study of von Hippel-Lindau (VHL) disease. An autosomal-dominant, hereditary tumor syndrome, VHL disease is characterized by a narrow spectrum of tumors, which include clear cell RCC (CC-RCC), pheochromocytomas, and hemangioblastomas of the central nervous system and retina. Genetic studies of VHL disease led to the identification and cloning of the VHL gene. Tumor formation in VHL disease follows the Knudson two-hit model, whereby a defective copy of the VHL gene is inherited, whereas somatic inactivation occurs in the remaining allele, leading to malignancy. CC-RCC is a prominent feature in hereditary VHL disease, suggesting an etiologic role of the VHL gene in sporadic CC-RCC. Gnarra et al. (2) found that VHL is mutated in 57% of CC-RCC. An additional 10% to 20% of cases are due to inactivation of the VHL gene through hypermethylation (3). Thus, loss of VHL function occurs in a significant fraction of sporadic CC-RCC, approaching 70% to 80% of all cases.

The strong association of VHL inactivation in sporadic and hereditary CC-RCC indicates a causative role for the loss of VHL in the molecular pathogenesis of CC-RCC. Tumor suppression by VHL was first established through tumor xenograft studies. Reintroduction of wild-type VHL into VHL-deficient CC-RCC cells inhibited the ability of these cells to form tumors in nude mice (4). Elegant molecular studies determined that VHL functions as an E3 ubiquitin, targeting hypoxia-inducible factor (HIF)-α for oxygen-dependent proteolysis (5). These studies suggest a role for HIF in the development of CC-RCC that is supported by molecular analyses of VHL disease-derived mutations. VHL patients with specific mutations resulting in HIF dysregulation are at the greatest risk for developing CC-RCC (6). Xenograft studies further support the role of unregulated HIF activity in the CC-RCC as shown that overexpression of a constitutively stable HIF-2α mutant phenocopies the loss of VHL, resulting in tumor growth (7).

Our improved understanding of the underlying molecular defects and deficits in CC-RCC provides a tremendous opportunity for the rational design of therapeutics to target this tumor type. A 70% probability of VHL functional loss in nonhereditary CC-RCC and the subsequent normoxic hyperactivity of HIF transcription factors lends itself to two possible therapeutic approaches: restoration of VHL tumor suppressor function through HIF inhibition or VHL inactivation-dependent cytoxicity. The literature has overwhelmingly focused on the identification and characterization of agents that inhibit the HIF family of transcription factors due to the applicability of HIF inhibitors to all solid tumors. We confined our study to a single tumor type with a specific
genetic lesion. Our strategy is tumor-selective cytotoxicity in CC-RCC through VHL functional loss, a strategy applicable to an estimated 70% of all new diagnoses of clear cell disease. In this study, we identify a group of agents that selectively kill cells with VHL defects, providing further validation to the concept of targeting the loss of tumor suppressor genes.

Materials and Methods

COMPARE analysis. The NCI60 cell lines were categorized into either VHL low expressers or VHL high expressers as described previously (8). Briefly, VHL expression was determined from previously reported publicly available microarray data (9). Based on these data, the NCI60 cell lines were divided into three categories: VHL low expressers, mid expressers, and high expressers. These categories were then used to design a seed pattern to reflect the toxicity profile of a drug that targets VHL-deficient cells. This seed pattern was then used in the COMPARE algorithm web located on the Developmental Therapeutics Program (DTP) Web site1 to identify candidate agents.

Cell culture and treatments. RCC4 and 786-O cells and their derivatives, including the VHL-matched counterparts RCC4/VHL and 786/VHL, were cultured in DMEM supplemented with 10% FCS. For 2,3-bis[2-methoxy-4-nitro-5-sulphonylphenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assays, 5,000 cells were plated in each well of a 96-well plate. The cells were allowed to attach overnight. The following day, vehicle or drug was added to each well. Four days later, regular DMEM culture medium was aspirated and replaced with phenol red–free medium with 0.3 mg/mL XTT and 2.65 μg/mL N-methyl dibenzoylamine methyl sulfate. The 96-well plates were returned to the 37°C incubator for 1 to 2 h. Metabolism of XTT was quantified by measuring the absorbance at 450 nm. IC50, which for each of the drugs were calculated using linear interpolation and listed in figure legends.

Drugs. Pyrazoloacridine (NSC 366140), chromomycin A3 (ChA3; NSC 58514), echinomycin (NSC 526617), and batracylin (NSC 320846) were kindly listed in figure legends.

Immunoblotting. Cells were lysed in urea lysis buffer [9 mol/L urea, 75 mmol/L Tris (pH 7.5), 150 mmol/L β-mercaptoethanol]. Cells were sonicated briefly (10 s). Fifty to 100 micrograms of protein (as determined by Bradford assay; Bio-Rad) were resolved on 8% or 12% SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. Total HIF-1α protein was detected with anti-HIF-1α antibody from Transduction Laboratories (clone 54) and HIF-2α with a mouse monoclonal antibody (clone ep190b) from Novus Biologicals. Anti-α-tubulin was from Research Diagnostics, Inc. (clone DM1A). Anti-VHL was from BD PharMingen (clone Ig32).

In vivo experiments. Male nude mice, 4 to 6 weeks old, were obtained from Charles River Laboratories. 786-O and 786/VHL cells were counted and resuspended in sterile PBS. Five million cells were injected into each flank of the animal (786-O cells in one flank and 786/VHL cells in the opposite flank). The extensive collection of data about in vivo treatment with ChA3 (NSC 58514) was reviewed on the DTP Web site. The in vivo data were used as a guide for the dosage and scheduling of our treatments. Based on these studies, we completed a small-scale testing of tolerated dose (data not shown). We found that i.p. injection of 2 and 2.5 mg/kg led to mortality within 3 days of injection. We chose a treatment schedule of 0.4 and 0.6 mg/kg i.p. injection every 3 days based on our findings, in addition to the data from the DTP Web site. Tumor-bearing mice (tumor size, ~200 mm3) were randomized into either vehicle control group or the ChA3 treatment group. Mice were injected i.p. every 3 days for a total of five times with PBS, ChA3 in PBS at 0.4 mg/kg, or ChA3 in PBS at 0.6 mg/kg.

Results

COMPARE algorithm identifies drugs based on VHL expression patterns in NCI60 panel. A tissue-directed anticancer drug screen was created in 1990 by the NCI to evaluate compounds for antineoplastic activity. A cell line panel consisting of 60 tumor cell lines from nine different tissue types deemed the NCI60 was established. Importantly, these 60 cell lines span a spectrum of molecular defects, allowing for the analysis of drug activity with respect to specific molecular alterations. To examine the relationship between VHL expression and drug sensitivity, we divided the 60 cell lines into three categories based on VHL expression. VHL low expressers (n = 16), VHL mid expressers (n = 35), and VHL high expressers (n = 9), as measured by microarray analysis and described previously (8, 9). These VHL expression categories were then used to construct a theoretical drug activity pattern, or seed pattern, reflective of a drug that targets VHL-deficient cells or, more specifically, VHL low expressers (Fig. 1A). In the design of the seed pattern, cells designated low expressers were set to be the most sensitive to the theoretical drug, whereas the high expressers were set to be the least sensitive to the drug. All remaining cell lines were placed into the mid expressers category and given a neutral sensitivity to the theoretical drug. A schematic of the theoretical seed pattern is shown in Fig. 1A. The X axis represents VHL expression, whereas the Y axis represents drug sensitivity.

The COMPARE algorithm (10), a pattern recognition algorithm, was then used to evaluate the seed pattern individually against each of the drugs in the Standard Agent Database of the DTP at the NCI (11). The COMPARE algorithm returned a rank-ordered list of compounds most similar to our seed pattern based on the concentration of drug at which growth is inhibited by 50% (GI50) and the Pearson correlation coefficient. The top 10 rank-ordered compounds returned by the COMPARE algorithm are shown in Fig. 1B. Included in the table are the NSC identifier, the common name, the average GI50 of the VHL low expressers, and the average GI50 of the high expressers as well as a P value from a t test comparing GI50s between the two categories.

All 60 cell lines were used in the COMPARE analysis. Due to the large size of the mid-expresser group, 35 cell lines, which were all set to a neutral sensitivity, the analysis was biased toward identifying drugs with a relatively flat GI50 distribution. COMPARE analysis results were thus further scrutinized by examining the average GI50s in the VHL low expressers versus the VHL high expressers. Ideally, we hypothesized we would identify drugs with a high potency (large negative log GI50) and significant difference

1 http://dtp.nci.nih.gov
between the low and high expressers (low $P$ value). Despite having a statistically significant difference in the $\text{GI}_{50}$ between VHL high and low expressers, nitroestrone (NSC 321803) is a low-potency agent with log $\text{GI}_{50}$s of $-3.12 \text{ mol/L}$ and $-3.32 \text{ mol/L}$, respectively. Thus, it was not considered for further study. ChA3 (NSC 58514) is a highly potent agent with a log $\text{GI}_{50}$ of $-11.24 \text{ mol/L}$ and $-10.78 \text{ mol/L}$ in the low and high VHL expressers, respectively. Based on these results, we tested the top four compounds for activity against VHL-deficient cells. This analysis provided a rapid and inexpensive means of identifying compounds potentially capable of targeting VHL-deficient tumors.

The top four compounds identified by COMPARE analysis were evaluated in a short-term viability assay in two well-characterized CC-RCC cell lines (Fig. 1C). We measured metabolic activity, a surrogate marker of viability, by the capacity of the cells to convert the tetrazolium salt XTT to a colored formazan (Fig. 1C). Pyrazoloacridine and batracylin led to an equivalent reduction in XTT metabolism between the VHL-deficient cells (RCC4 and 786-O) and the VHL-positive cells (RCC4/VHL and 786/VHL) at each of the concentrations examined, indicating that these two drugs are equitoxic regardless of VHL status. In contrast, ChA3 and echinomycin showed a selective reduction in XTT metabolism in the VHL-deficient cell lines relative to the VHL-positive cell lines, suggesting that these two drugs induce a decrease in cellular proliferation and/or an increase in cell death through a mechanism dependent on the loss of VHL.

**ChA3 targets VHL-deficient cells.** A short-term analysis of candidate drug toxicity versus VHL expression status in CC-RCC cells with the XTT viability screen revealed that pyrazoloacridine and batracylin are equitoxic regardless of VHL status, whereas ChA3 and echinomycin are significantly more toxic to VHL-deficient cells. However, clonogenic survival assays are the classic means of determining the efficacy of a candidate agent on cell growth. ChA3 toxicity was examined in RCC4 and 786-O CC-RCC

---

**Table 1.** Comparison of drug potency between VHL low and high expressers.

<table>
<thead>
<tr>
<th>NSC#</th>
<th>Name</th>
<th>Low Log $\text{GI}_{50}$</th>
<th>High Log $\text{GI}_{50}$</th>
<th>$t$-test $P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>366140</td>
<td>Pyrazoloacridine</td>
<td>-6.693</td>
<td>-6.637</td>
<td>0.337</td>
</tr>
<tr>
<td>58514</td>
<td>ChA3</td>
<td>-11.239</td>
<td>-10.783</td>
<td>0.076</td>
</tr>
<tr>
<td>526417</td>
<td>Echinomycin</td>
<td>-11.26</td>
<td>-11.286</td>
<td>0.455</td>
</tr>
<tr>
<td>320846</td>
<td>Batracylin</td>
<td>-4.561</td>
<td>-4.491</td>
<td>0.316</td>
</tr>
<tr>
<td>339555</td>
<td>Bryostatin 1</td>
<td>-5.253</td>
<td>-5.242</td>
<td>0.466</td>
</tr>
<tr>
<td>321803</td>
<td>Nitroestrone</td>
<td>-3.319</td>
<td>-3.123</td>
<td>0.016</td>
</tr>
<tr>
<td>293015</td>
<td>ChA3</td>
<td>-4.155</td>
<td>-4.316</td>
<td>0.278</td>
</tr>
<tr>
<td>328464</td>
<td>Methyl GAG</td>
<td>-4.667</td>
<td>-4.723</td>
<td>0.411</td>
</tr>
<tr>
<td>22413</td>
<td>PALA</td>
<td>-3.549</td>
<td>-3.267</td>
<td>0.154</td>
</tr>
</tbody>
</table>

**Figure 1.** COMPARE algorithm seed pattern and results. A, a seed pattern for the COMPARE algorithm was created based on VHL expression. Cells with low VHL expression levels were set to high sensitivity and cells with high VHL expression were set to low sensitivity. B, rank-ordered list returned by the COMPARE algorithm. Included in the table are the NSC identifier, name, VHL low-expresser $\text{GI}_{50}$, VHL high-expresser $\text{GI}_{50}$, and $P$ value from a $t$-test done to compare the $\text{GI}_{50}$ values between the low expressers and the high expressers. C, the top four drugs, pyrazoloacridine (PZA), ChA3, echinomycin (Ech), and batracylin (Bat), were evaluated by XTT assay against the VHL-deficient RCC cell lines RCC4 and 786-O with a matched VHL-positive cell line. Bars, SE. Pyrazoloacridine IC$\text{_{50}}$: RCC4, 362 ng/mL; RCC4/VHL, 278 ng/mL; 786-O, 140 ng/mL; and 786/VHL, 164 ng/mL. ChA3 IC$\text{_{50}}$: RCC4, 9.0 ng/mL; RCC4/VHL, >10.0 ng/mL; 786-O, 8.7 ng/mL; and 786/VHL, 17.0 ng/mL. Echinomycin IC$\text{_{50}}$: RCC4, 1.55 ng/mL; RCC4/VHL, >10.0 ng/mL; 786-O, 1.26 ng/mL; and 786/VHL, 2.30 ng/mL. Batracylin IC$\text{_{50}}$: RCC4, >10 ng/mL; RCC4/VHL, >10.0 ng/mL; 786-O, 5.6 ng/mL; and 786/VHL, 6.9 ng/mL.
The cells were fixed and photographed. The cells were rinsed with PBS and refed with normal medium. Four days later, equal concentrations. ChA3 was added for 4 h the following day, after which C, ECFP-labeled RCC4 cells and EYFP-labeled RCC4/VHL cells were mixed at done as described above except with incubation period of 12 to 14 d. The following day, ChA3 was added. At the conclusion of a 9- to 11-d incubation period, the colonies were stained with crystal violet and counted. The average of the three plates is graphed as percentage survival relative to the untreated plates. Bars, SE. Photograph of plates with 3,000 cells treated with vehicle or increasing concentrations of ChA3 for 4 h. At the conclusion of the treatment, the cells were rinsed with PBS and refed with fresh medium and allowed to grow for an additional 4 days. Figure 2C shows that ChA3 results in a dose-dependent selective loss of the ECFP-labeled VHL-deficient RCC4 cells, whereas the EYFP-labeled RCC4/VHL cells continued to proliferate, critically showing the concept of targeting tumors based on VHL status. More importantly, this serves as proof to the larger idea of genotype-selective toxicity based on loss of function of tumor suppressor gene function.

**VHL mutants are more sensitive to ChA3.** Complete disruption of VHL function sensitizes cells to ChA3-induced toxicity as shown by clonogenic survival studies and mixing experiments of VHL-deficient and VHL-positive cell lines. As HIF-α subunit stabilization is the best-characterized consequence of VHL disruption, we hypothesized that a compromise in the ability of VHL to mediate HIF-α subunit degradation enhances ChA3 toxicity. Several clinically derived mutations found in VHL disease have been studied for their ability to bind and ubiquitylate HIF-α subunits, including the Y98H and Y112H mutations (6, 12). We examined these two VHL mutations for their ability to modify sensitivity to ChA3. These mutations are clinically derived from patients with type 2A VHL disease and have been shown to retain the ability to suppress normoxic stabilization of the HIF-α subunit in vivo, although with diminished activity relative to wild-type VHL (6). To evaluate the role of the diminished ability of VHL mutants to degrade the HIF-α subunit as a mechanism of enhancing the toxic effects of ChA3, we studied the toxicity of ChA3 in RCC4 cells stably transfected with VHL Y98H and VHL Y112H, RCC4/VHL Y98H, and RCC4/VHL Y112H (Fig. 3A). Consistent with previous results, the RCC4 cells were more sensitive than the RCC4/VHL cells. Both RCC4 cell lines stably expressing the type 2A VHL mutants showed increased sensitivity relative to the wild-type RCC4/VHL cell line, although not to the extent of the VHL-deficient RCC4 cell line (Fig. 3A).

Western blot analysis was used to examine the ability of the VHL mutants Y98H and Y112H to regulate HIF-α subunits relative to wild-type VHL function (Fig. 3B). HIF-1α and HIF-2α protein levels were evaluated with immunoblot detection in each of the cell lines. We observed high levels of both HIF-1α and HIF-2α in the native RCC4 cells and low levels in the wild-type RCC4/VHL cells. The Y98H VHL mutation suppressed normoxic HIF-1α stabilization, although not to the extent of wild-type VHL, and the Y112H mutation had no apparent suppressive effect on HIF-1α.

As tumors originate from a single transformed cell in the midst of a population normal untransformed tissue, effective genotype-selective antitumor agents should then be able to discriminately eradicate transformed tissue that coexists side by side with normal tissue. We used coculture experiments of RCC4 cells, representative of transformed cells, and RCC4/VHL cells, representative of untransformed tissue, to simulate the tumor microenvironment where cancerous tissue is intermingled with normal tissue. Genetically, these cells differ only with respect to VHL expression and experiments have shown no effect of VHL expression on in vitro proliferation rates (4). This coculture model was used to determine if ChA3 could discriminate between the VHL-deficient renal carcinoma tumor cell and the VHL-positive cell. Enhanced cyan fluorescent protein (ECFP) fluorescently labeled RCC4 cells and enhanced yellow fluorescent protein (EYFP) fluorescently labeled RCC4/VHL cells were mixed in equal proportions. This mixed population of cells was then treated with vehicle or increasing concentrations of ChA3 for 4 h. At the conclusion of the treatment, the cells were rinsed with PBS and refed with fresh medium and allowed to grow for an additional 4 days.

**Figure 2.** ChA3 targets VHL-deficient cells. A, clonogenic assay of 786-O and 786/VHL cells. Cells (300, 3000, and 30,000) were plated in triplicate in 6-cm dishes. The following day, ChA3 was added. At the conclusion of a 9- to 11-d incubation period, the colonies were stained with crystal violet and counted. The average of the three plates is graphed as percentage survival relative to the untreated plates. Bars, SE. Photograph of plates with 3,000 cells treated with vehicle or increasing concentrations of ChA3 for 4 h. At the conclusion of the treatment, the cells were rinsed with PBS and refed with normal medium. Four days later, the cells were fixed and photographed.

Cell lines and their VHL-positive counterparts with clonogenic survival assays (Fig. 2A and B). Both 786-O and RCC4 cell lines deficient in VHL were more sensitive to ChA3, whereas the VHL-positive matched counterparts were only sensitive at much higher drug concentrations. These results are consistent with the XTT assays and indicate that ChA3 is a genotype-selective agent targeting cells deficient in VHL activity.
Both of the VHL mutants had less detectable HIF-2α protein than the RCC4 cells, indicating that both of these mutants retain the partial ability to regulate HIF-2α in vivo. The Y98H mutant suppressed normoxic stabilization of the HIF-2α subunit to lower levels than the Y112H VHL mutant. Correspondingly, lower levels of Glut1 protein, a HIF target, were observed in the Y98H mutant relative to the Y112H mutant. Immunoblots were also done for VHL protein, and α-tubulin served as a loading control.

Y112H VHL mutant cells are more sensitive to ChA3 than the Y98H VHL mutant (Fig. 3C and D). The increased sensitivity of the Y112H mutant relative to the Y98H mutant to ChA3 correlates with the relative levels of the HIF-α subunits and Glut1 protein levels. These results suggest that it is the diminished ability to suppress the normoxic stabilization of the HIF-α subunit and the subsequent transcription directed by HIF that accounts for the increased sensitivity to ChA3.

**HIF-2α increases sensitivity to ChA3.** Perturbation of the HIF-regulatory function of VHL correlates with an increased susceptibility to ChA3-mediated toxicity. This is supported by our experiments with VHL mutants with diminished HIF-regulatory function. We sought to determine if the drugs caused any appreciable effects on HIF protein levels through Western blot analysis (Fig. 4A). Pyrazoloacridine caused a slight increase in HIF-1α and HIF-2α levels at the 4- and 8-h treatment points. Echinomycin treatment conversely resulted in a modest decrease in HIF-1α and HIF-2α levels particularly at the later time points. Interestingly, HIF-1α levels remained stable during the duration of ChA3 treatment, but a time-dependent increase in HIF-2α levels was observed beginning at ~2 h and continued through the duration of the 20-h treatment. α-Tubulin protein levels served as the loading control. Echinomycin treatment resulted in a modest decrease in HIF-α subunit levels, whereas ChA3 paradoxically increases the levels of the HIF-2α protein.

A means of directly examining the effects of HIF activity on ChA3-mediated toxicity is to study mutants of the HIF-α subunit that escape regulation by the VHL protein. RCC4 cells contain both HIF-1α and HIF-2α, whereas 786-O cells only have HIF-2α. Thus, we focused on HIF-2α as the mediator that sensitizes RCC cells to ChA3. Hydroxylation of Pro531 of HIF-2α is essential for VHL binding (7, 13). Thus, mutation of HIF-2α P531 to alanine abrogates binding to VHL and the subsequent degradation. Using site-directed mutagenesis, we mutated P531 and N847 to alanines; these two residues are critical to HIF degradation and transcriptional activity, respectively.

Individual clones of HIF-2α degradation-resistant mutant RCC4/VHL cells were screened for normoxic expression of HIF-2α by Western blot (Fig. 4B). RCC4/VHL HIF-2α P531A/N847A clones 3 and 17 have levels of HIF-2α comparable with that of the VHL-deficient RCC4 cells. HIF-1α levels were also examined. RCC4 cells

![Figure 3](https://example.com/figure3.png)

**Figure 3.** VHL mutants with diminished ability to regulate HIF only partially reduce ChA3 sensitivity. A, cells were treated with increasing concentrations of ChA3 for 4 d and then assayed for viability with the XTT assay. ChA3 IC50: RCC4, 8.8 ng/mL; RCC4/VHL, 41.6 ng/mL; RCC4/VHL Y98H, 10.63 ng/mL; and RCC4/VHL Y112H, 11.6 ng/mL. B, Western blot. Cells were lysed in urea lysis buffer, and an immunoblot was done for HIF-1α, HIF-2α, α-tubulin, Glut1, and VHL. On VHL blot, solid arrowhead indicates FLAG-tagged VHL and open arrowhead indicates VHL. C, clonogenic assay. Cells were plated in a 6-cm dish and treated with 12.5, 25, and 50 ng/mL of ChA3. Cells were incubated for 12 to 14 d. Colonies were then stained and quantified. Bars, SE. D, representative plates were photographed, and 3,000 cells were treated with 25 ng/mL ChA3.
had the highest levels of HIF-1α protein, whereas the RCC4/VHL cells had the least. Clones 3 and 17 had HIF-1α levels slightly elevated relative to RCC4/VHL but much less than RCC4 cells. Glut1 protein levels were used to assess the level of HIF transcriptional activity in the HIF-2α P531A/N847A clones. RCC4 cells had the highest level of Glut1 followed by clone 3, clone 17, and then the RCC4/VHL control cell line. Clones 3 and 17 were chosen for further evaluation about enhanced ChA3-mediated cytotoxicity based on the elevated HIF-2α levels and elevated Glut1 levels. Levels of VHL are also shown in Fig. 4B, as well as α-tubulin, which serves as the loading control.

Normoxic stable expression of HIF-2α mutant protein was tested for its ability to sensitize VHL-positive cells to the toxic effects of drugs. HIF-2α transcriptional activity has no influence on the sensitivity of RCC4/VHL cells to pyrazoloacridine (Fig. 4C). HIF-2α normoxic stabilization and transcriptional activity, however, sensitizes the cells to ChA3-mediated toxicity as measured by XTT assay (Fig. 4C). Clones 3 and 17 overexpressing HIF-2α are more

---

**Figure 4.** HIF-2α restores ChA3 sensitivity in VHL-positive cells. A, RCC4 and RCC4/VHL cells were treated with the following agents for 20 h: pyrazoloacridine, 1,000 ng/mL; ChA3, 50 ng/mL; and echinomycin, 10 ng/mL. Levels of HIF-1α and HIF-2α were examined through immunoblot. B, HIF-2α double-mutant P531A/N847A was stably introduced into RCC4/VHL cells by retroviral infection. Western blot shows the HIF-1α, HIF-2α, Glut1, and VHL levels. α-Tubulin serves as the loading control. C, cell viability was examined with the XTT assay following 4 d of incubation with pyrazoloacridine or ChA3. Pyrazoloacridine IC50: RCC4, 216 ng/mL; RCC4/VHL, 192 ng/mL; RCC4/VHL HIF2 clone 3, 159 ng/mL; and RCC4/VHL HIF2 clone 17, 150 ng/mL. ChA3 IC50: RCC4, 31.5 ng/mL; RCC4/VHL, >50 ng/mL; RCC4/VHL HIF2 clone 3, 39.3 ng/mL; and RCC4/VHL HIF2 clone 17, 38.8 ng/mL. D, clonogenic assay. Cells were plated in 6-cm dishes, treated with ChA3, and incubated for 12 to 14 d, after which colonies were stained and quantified. Bars, SE. Representative plates were photographed, and 3,000 cells were treated with 50 ng/mL ChA3.
sensitive to ChA3 than is the control RCC4/VHL cell line, indicating that normoxic HIF-2α transcriptional activity is sufficient to sensitize cells to ChA3. Results from the XTT assays were further confirmed through clonogenic assays (Fig. 4D). Both the XTT and clonogenic studies indicate that normoxic HIF-2α activity augments the cytotoxic effects of ChA3. These results indicate not only that we have identified an agent that selectively targets VHL-deficient cells. More importantly, this agent is a HIF-dependent cytotoxin.

<table>
<thead>
<tr>
<th>NSC#</th>
<th>Compound Name</th>
<th>Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>58514</td>
<td>ChA3</td>
<td>k16.1</td>
</tr>
<tr>
<td>3053</td>
<td>Actinomycin D</td>
<td>k16.1</td>
</tr>
<tr>
<td>526417</td>
<td>Echinomycin</td>
<td>k16.1</td>
</tr>
<tr>
<td>13502</td>
<td>Echinomycin</td>
<td>k16.1</td>
</tr>
<tr>
<td>36002</td>
<td>Asiatoside</td>
<td>k16.1</td>
</tr>
<tr>
<td>38270</td>
<td>Olivetin</td>
<td>k16.1</td>
</tr>
<tr>
<td>77471</td>
<td>Mitomycin</td>
<td>k16.1</td>
</tr>
<tr>
<td>131547</td>
<td>Tubulosine</td>
<td>k16.1</td>
</tr>
<tr>
<td>135015</td>
<td>SABIN</td>
<td>k16.1</td>
</tr>
<tr>
<td>143020</td>
<td>Methotrexate Mg salt</td>
<td>k16.1</td>
</tr>
<tr>
<td>146396</td>
<td>Nitidine pseudomethanol</td>
<td>k16.1</td>
</tr>
<tr>
<td>227262</td>
<td>Bouvardia ternifolia</td>
<td>k16.1</td>
</tr>
<tr>
<td>325319</td>
<td>Didemnin B</td>
<td>k16.1</td>
</tr>
<tr>
<td>265450</td>
<td>Nogalamycin</td>
<td>k16.1</td>
</tr>
<tr>
<td>122023</td>
<td>Valomycin</td>
<td>k16.1</td>
</tr>
<tr>
<td>286193</td>
<td>Tiapulin</td>
<td>k28.3</td>
</tr>
<tr>
<td>280584</td>
<td>Triclabidine phosphate</td>
<td>k14.20</td>
</tr>
<tr>
<td>375575</td>
<td>Cyclodentyl cytosine</td>
<td>k24.3</td>
</tr>
<tr>
<td>127718</td>
<td>5-Aza-2-deoxycytidine</td>
<td>k11.21</td>
</tr>
<tr>
<td>4728</td>
<td>Aminothiadiazole</td>
<td>k16.3</td>
</tr>
</tbody>
</table>

Figure 5. Drugs with similar activity profiles to ChA3 also target VHL-deficient cells. A, 15 compounds, including ChA3 from cluster k16.1, are listed as well as five compounds that are inversely correlated with ChA3. B, compounds listed in (A) are plotted against cell lines. Normalized sensitivities are plotted in the colorimetric graph. Blue, sensitive cell lines; red, resistant cell lines. C and D, clonogenic assay of RCC4 and RCC4/VHL cells. Cells were plated in 6-cm dishes. C, the following day, vehicle or echinomycin was added at a concentration of 0.625, 2.5, or 10 ng/mL. D, vehicle or actinomycin D was added at a concentration of 0.39, 1.56, or 6.25 ng/mL. At the conclusion of treatments, colonies were stained with crystal violet and counted. The average of the three plates is graphed as percentage survival normalized to the control plates. Bars, SE. Photographs: 300 cells treated with echinomycin (10 ng/mL) or actinomycin D (1.56 ng/mL).
Drugs in cluster k16.1 with ChA3 are also selectively toxic.
ChA3 is an aureolic acid compound known to bind DNA in the minor groove, thus altering DNA conformation and inhibiting transcription. It is not known mechanistically which, if any, of these characteristics is responsible for the increased toxicity observed in VHL-deficient cells. We hypothesized that we might gain insight into the potential mode of differential toxicity through the analysis of drugs with similar activity profiles to ChA3. Thus, we projected the activity profile of ChA3 (NSC 58514) onto the anticancer maps at the 3D Mind Web site.2 Rabow et al. used self-organizing map-based cluster analysis to classify drugs based on their drug activity profiles into categories based on their cellular activities. ChA3 projected into cluster k16.1. Fifteen of the compounds found in cluster k16.1 are listed in Fig. 5A. The final five compounds listed in Fig. 5A are compounds that have an inverse correlation with ChA3 as examined through COMPARE analysis; each of these drugs is distributed in a different cluster. Each of the drug profiles listed in Fig. 5A is plotted in the colorimetric graph (Fig. 5B) versus the cell lines from the NCI. Mithramycin (an aureolic acid), actinomycin D, and echinomycin are all found in cluster k16.1 in the antimitotic region of the self-organizing map. Based on this analysis, the toxicity of the cluster k16.1 agents echinomycin and actinomycin D was explored more thoroughly through clonogenic survival studies.

ChA3 inhibits tumor growth in vivo. Side-by-side comparison of ChA3-mediated toxicity in VHL-deficient tumors versus VHL-positive tumors is the defining experiment in evaluating this strategy in vivo. Figure 6A and B shows the difficulty in completing a side-by-side in vivo comparison. As VHL is a tumor suppressor and consistent with numerous published reports, introduction of VHL into the 786-O cells dramatically inhibits the ability of the cells to form tumors in nude mice (4). Ten nude mice were injected with 786-O cells in one flank and 786/VHL cells in the opposite flank and followed over the course of 8 weeks (Fig. 6A). Whereas the 786-O cells formed tumors, tumor formation was suppressed in the 786/VHL cells. Figure 6B shows a nude mouse 11 weeks after injection. There is no evidence of 786/VHL tumor in the right flank but large 786-O tumor is obvious in the left flank. Thus, the antitumor effects of ChA3 in vivo were tested only against the VHL-deficient 786-O tumors.

786-O tumors were grown until they reached ~200 mm³, and then the nude mice were randomized into either the vehicle control or the ChA3 treatment groups. Treatment dosage, schedule, and i.p. injection as the route of administration were chosen based on data from the DTP. In addition, Pittillo and Woolley (14) found that relatively high concentrations of ChA3 were maintained in blood following i.p. administration. Tumors were treated every 3 days for a total of five treatments over the course of 14 days. Results are graphed in Fig. 6C and photographs are shown in Fig. 6D.

Figure 6. ChA3 retards tumor growth in vivo. A, 786-O and 786/VHL cells were injected s.c. into the flanks of nude mice and followed weekly for ~ 9 wks. Tumor size is represented by tumor volume (mm³). B, photograph of a mouse injected with 786-O cells (left flank) and 786/VHL cells (right flank) 11 wks after injection. C, 786-O tumors were matched for tumor size (~200 mm³) and treated with either vehicle or ChA3. ChA3 was given i.p. every 3 d for five doses. Tumor volumes are graphed on days 0 and 14 following initiation of treatment. D, a vehicle-treated mouse and a ChA3 mouse are shown with their respective tumors. Bars, SE.

\[\text{ChA3 inhibits tumor growth in vivo.}\]

\[\text{ChA3 retards tumor growth in vivo.}\]

\[\text{ChA3 retards tumor growth in vivo.}\]
Fig. 6D. Over the 14-day course of treatment, significant growth was observed in the control group \((n = 8)\), whereas growth in the ChA3 treatment group \((n = 4)\) remained static. The antitumor effects of ChA3 support the concept that loss of VHL can be targeted in vivo.

**Discussion**

In this study, we identify a potential new avenue of treatment to address the dismal outcomes associated with late-stage CC-RCC, focusing on the lack of an effective and reliable systemic therapy. Inactivation of the VHL gene is a frequent and critical event in an estimated 70% of patients with nonhereditary clear cell tumors. Accordingly, VHL status should weigh heavily in the rational design of therapies to combat late-stage clear cell disease. Our strategy was thus to validate the concept of specifically targeting the loss of the VHL tumor suppressor gene through the pharmacologic means (15–18).

We specifically focused on the identification of an agent that showed genotype-selective toxicity against the VHL-deficient CC-RCC using publicly available data and analytic tools. This approach enabled us to rapidly and inexpensively identify several candidate drugs, four of which were examined more closely for the ability to preferentially target VHL-deficient cells. Both ChA3 and echinomycin were found to be significantly more toxic to VHL-deficient cells than to VHL-positive cells. Moreover, simulation of the native tumor microenvironment through coculture experiments of VHL-deficient and VHL-positive cells labeled with ECFP and EYFP, respectively, showed that ChA3 discriminated between the two cell lines as they grew alongside each other with little, if any, bystander toxicity. ChA3 also showed activity against VHL-deficient 786-O tumors in vivo. Successful systemic therapeutic strategies, likewise, will eradicate VHL-deficient CC-RCC tumor cells while sparing normal VHL-positive tissue, thus producing fewer therapy-related adverse effects.

Mechanistically, ChA3 is known to bind DNA at the minor groove and interfere with both cellular replication and transcription. Interestingly, ChA3 had little effect on HIF-1α protein levels and increased the levels of HIF-2α protein. Thus, ChA3 does not seem to mimic the action of VHL with respect to HIF-α subunit stability. Although the mechanism of action is distinct from VHL-replacement, we define a role for VHL status in modifying the toxicity of ChA3 with loss of VHL, leading to a greater susceptibility to ChA3. Thomas et al. have previously shown a role for loss of VHL in modifying the sensitivity of cells to mammalian target of rapamycin (mTOR) inhibition. In the context of mTOR inhibition, HIF overexpression rescued the VHL-associated sensitivity to mTOR inhibition (19). In this study, stable overexpression of a HIF-2α mutant was able to phenocopy the loss of VHL with respect to ChA3 toxicity. Thus, ChA3 induces cell death through a HIF-dependent mechanism (16, 18). Importantly, we also found that drugs with similar NCI60 activity profiles to ChA3, specifically echinomycin and Actinomycin D, showed a genotype-selective toxicity against the VHL-deficient CC-RCC cells. Echinomycin has recently been found to inhibit the sequence-specific DNA binding of the HIF-1 heterodimeric transcription factor (20).

A consequence of the loss of VHL function is the normoxic accumulation of the α-subunit of the HIF transcription factor and a subsequent increase in HIF transcriptional activity (21–23). Several groups have identified agents that functionally impair HIF transcriptional activity, although most not with the explicit statement to replace VHL function, likely because the clinical scope of HIF inhibitors exceeds clear cell renal carcinoma and may have applications to all solid tumors (24–28). The benefit of HIF inhibition with regard to tumor growth has also been shown genetically with respect to s.c. tumor implantation in immunodeficient mice. Genetic inhibition of HIF, however, is not robust to all tumor microenvironments (29) and it remains to be seen how effective HIF inhibition is in the context of tumors with long-established blood supplies. Xenograft studies with 786-O clear cell renal carcinoma cells have shown tumor suppression with the substitution of VHL biochemical activity with short hairpin RNA interference to HIF-2α (30, 31).

In contrast to the restoration of tumor suppressor function, we sought to target tumor cells through the concept of genotype-selective toxicity. Induction of synthetic lethality in cancer cells is an emerging paradigm in the treatment of cancer and a potential means of achieving genotype-selective toxicity. Synthetic lethality occurs when the combination of two nonallelic and nonlethal mutations results in cell death. As cancer is a genetic disease that results from an accumulation of gene mutations and deletions, inhibiting a second complementary gene through pharmacologic means could lead to a lethal combination of gene/protein dysfunction or synthetic lethality (17). Because ChA3 inhibits the targeted transcription of genes, it is conceivable that ChA3 inhibits a second allele that, in the combination of loss of VHL, leads to synthetic lethality. Future studies will focus on identifying specific target genes that, when inhibited, lead to cell death in the context of VHL loss.

This strategy is particularly applicable to the loss of tumor suppressor genes. The VHL gene is ubiquitously expressed in normal tissues throughout the body; thus, loss of VHL expression is unique to tumor pathology. The high frequency of VHL inactivation in RCC makes it nearly universal to the diseased state, and tumor suppression following reintroduction of VHL into RCC cells underscores the crucial role of VHL in malignancy. A molecular defect that is crucial to the malignant phenotype, unique to diseased tissue, and nearly universal to the diseased state serves as an ideal target for therapeutic intervention. Pharmacologic agents that are toxic in the context of VHL disruption should then have minimal effect on normal tissue.

Systemic therapies have largely been limited in effectiveness due to adverse effects to normal tissue. Normal tissue toxicity limits both the maximum tolerated dose as well as the total duration of treatment. In this study, we narrowed our focus to clear cell renal carcinoma with nonfunctional VHL. Experiments with ChA3 have been valuable in the course of validating VHL-dependent cytotoxicity and elucidating the HIF-dependent mechanism. Exploitation of tumor suppressor loss will hopefully lead to therapies directed specifically at malignant tissue, reducing normal tissue toxicity in the process, leading to a greater therapeutic index and ultimately patient benefit.

**Acknowledgments**

Received 2/13/2007; revised 4/6/2007; accepted 4/20/2007.

**Grant support:** NCI grants CA-082566 (P.D. Sutphin), CA-123823 (D.A. Chan), CA-088480 (A.J. Krieg), and CA-82566 and CA-088480 (A.J. Giaccia) and Canadian Institutes of Health Research (S. Turcotte).

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.

We thank Dr. Dawn Zinyk for helpful assistance with animal husbandry; Dr. Ester Hammond for technical assistance; Drs. Ivana Cecic, Barbara Bedogni, Scott Welford, Marianne Powell, and other members of the Giaccia lab for careful review of the manuscript and discussion; and Dr. Robert Schultz (DTP) for kindly supplying the compounds.
References

Targeting the Loss of the *von Hippel-Lindau* Tumor Suppressor Gene in Renal Cell Carcinoma Cells

Patrick D. Sutphin, Denise A. Chan, James M. Li, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/67/12/5896

Cited articles

This article cites 31 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/67/12/5896.full#ref-list-1

Citing articles

This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/67/12/5896.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.