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Targeting the von Hippel–Lindau E3 Ubiquitin Ligase Using Small Molecules To Disrupt the VHL/HIF-1α Interaction

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Supporting Information

ABSTRACT: E3 ubiquitin ligases, which bind protein targets, leading to their ubiquitination and subsequent degradation, are attractive drug targets due to their exquisite substrate specificity. However, the development of small-molecule inhibitors has proven extraordinarily challenging as modulation of E3 ligase activities requires the targeting of protein–protein interactions. Using rational design, we have generated the first small molecule targeting the von Hippel–Lindau protein (VHL), the substrate recognition subunit of an E3 ligase, and an important target in cancer, chronic anemia, and ischemia. We have also obtained the crystal structure of VHL bound to our most potent inhibitor, confirming that the compound mimics the binding mode of the transcription factor HIF-1α, a substrate of VHL. These results have the potential to guide future development of improved lead compounds as therapeutics for the treatment of chronic anemia and ischemia.

E3 ubiquitin ligases (of which over 600 are known in humans)[1] confer substrate specificity for ubiquitination and are more attractive therapeutic targets than general proteasome inhibitors[2,3] due to their specificity for a small number of protein substrates. Unfortunately, the development of E3 ligase inhibitors has proven challenging, in part due to the fact that they must disrupt protein–protein interactions.[4] These interactions are notoriously difficult to target using small molecules due to their large contact surfaces and the shallow grooves or flat interfaces involved. Conversely, most small-molecule drugs bind enzymes or receptors in tight and well-defined pockets.[5] Since the discovery of nutlins, the first small-molecule E3 ligase inhibitors,[6] a few additional compounds have been reported that target inhibitors of apoptosis proteins (IAPs),[7,8] SCF^{Met30},[9] and SCF^{Cdc4};[10] however, the field remains underdeveloped.

One E3 ubiquitin ligase with exciting therapeutic potential is the von Hippel–Lindau (VHL) complex consisting of VHL, elongins B and C, cullin 2, and ring box protein 1 (Rbx1).[11] The primary substrate of VHL is hypoxia-inducible factor 1α (HIF-1α), a transcription factor that upregulates numerous genes such as the pro-angiogenic growth factor, vascular endothelial growth factor (VEGF), glucose transporter, GLUT1, and the red blood cell inducing cytokine, erythropoietin, in response to low oxygen levels.[12] While HIF-1α is constitutively expressed, its intracellular levels are kept very low under normoxic conditions via its hydroxylation by prolyl hydroxylase domain (PHD) enzymes and subsequent VHL-mediated ubiquitination (Figure 1).[13] Small-molecule inhibition of this pathway therefore would lead to

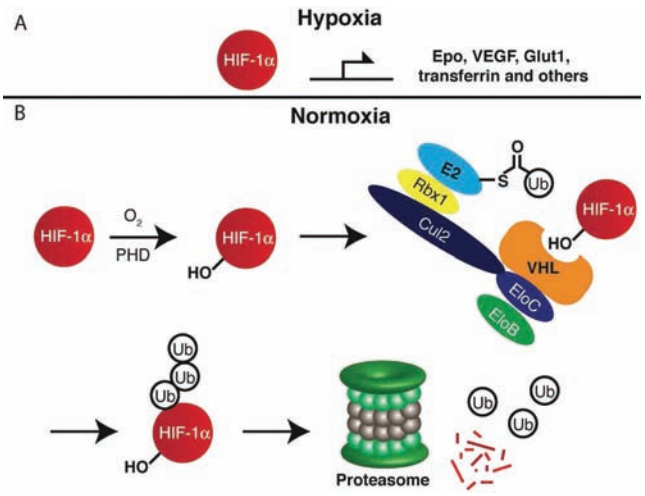

Figure 1. (A) HIF-1α accumulation leads to the transcriptional upregulation of genes involved in the hypoxic response, such as erythropoietin (Epo), VEGF and others. (B) Under normoxic conditions, HIF-1α is hydroxylated, recognized by VHL, ubiquitinated, and degraded by the proteasome, preventing transcriptional upregulation.

increased endogenous erythropoietin production and could supplant the current use of recombinant erythropoietin to treat chronic anemia associated with chronic kidney disease and cancer chemotherapy.[14] To this end, PHD inhibitors are under examination in clinical trials; however, a possible alternative would be the development of an inhibitor of the VHL/HIF-1α interaction. Such an inhibitor may avoid the HIF-independent off-target effects observed with PHD inhibitors,[15] which have already proven immensely useful as biological probes.[16,17]

While VHL also has HIF-1α-independent functions such as binding to and stabilizing p53 and acting as an adaptor for the phosphorylation of CARD9,[12] these proteins likely bind VHL differently than HIF-1α. In fact, previous work by Willam et al. has shown that polypeptides containing the HIF-1α oxygen-dependent degradation domains (ODDs) linked to the cell-permeable tat

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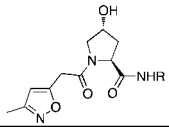
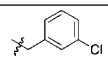
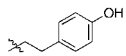
translocation domain stabilize HIF and induce an angiogenic response, suggesting that competitive inhibition of VHL is capable of producing a downstream biological response.[18]

We hypothesized that small-molecule inhibitors of the VHL/HIF-1α interaction could be rationally designed using hydroxyproline (Hyp) as a starting point, since residue Hyp564 on HIF-1α makes key interactions with VHL[19,20] and is crucial for HIF-1α binding.[21] We used the *de novo* design software BOMB to guide the selection of plausible hydroxyproline analogues.[22] **1** and **2** were synthesized to test a promising design featuring an isoxazole moiety positioned to interact with a crystallographic water observed in the structure of VHL bound to the HIF-1α peptide (549−582)[19] and a benzyl group stacked along the side chain of Tyr98.

The compounds' ability to bind to VHL was measured by the competition of a fluorescent HIF-1α peptide, FAM-DEALA-Hyp-YIPD (K_d = 560 nM).[23] Fluorescence polarization (FP) of the peptide was measured in the presence and in the absence of the VCB complex consisting of VHL, elongin B, and elongin C, to get the maximum and minimum values for polarization, respectively. The polarization was then measured in the presence of VCB and serial dilutions of the small-molecule ligands in order to observe the competition of the fluorescent peptide. These values were then normalized to the maximum and minimum polarization values to calculate percent inhibition of the interaction, from which an IC_{50} was determined. Both compounds were able to displace the fluorescent peptide albeit at high concentrations (Table 1). In addition, the nonfluorescent DEALA-Hyp-YIPD

Table 1. Binding of Initial Ligands to VHL



	R	IC_{50} (μM)[a]	SEM (μM)
1		117	10
2		120.1	7.1
3	CH_3	> 250	N/A

[a]Average IC_{50} values were determined from three independent trials, each in triplicate.

was used as a positive control and found to bind with IC_{50} = 0.91 μM and K_d = 180 nM by ITC. While the smaller **3** was unable to fully displace the fluorescent peptide under the same conditions, we observed binding to VHL through the use of WaterLOGSY NMR spectroscopy,[24] as demonstrated by the positive ligand signals due to cross-relaxation from water in the presence of protein (Figure 2). As no binding was observed with smaller hydroxyproline fragments, this suggested that we identified a minimal pharmacophore.

Encouraged by these initial results, we sought to increase the affinity of our VHL ligands by modifying the benzylamine moiety of **1** while maintaining the methyl-isoxazole fragment. In order to generate analogues rapidly, we developed a solid-phase synthesis that involved the attachment of Fmoc-Hyp-OAllyl to Wang resin.[25] Fmoc deprotection, coupling with 3-methyl-5-isoxazoleacetic acid followed by allyl ester deprotection, subsequent coupling with various amines, and cleavage with trifluoroacetic acid led to the rapid generation of VHL

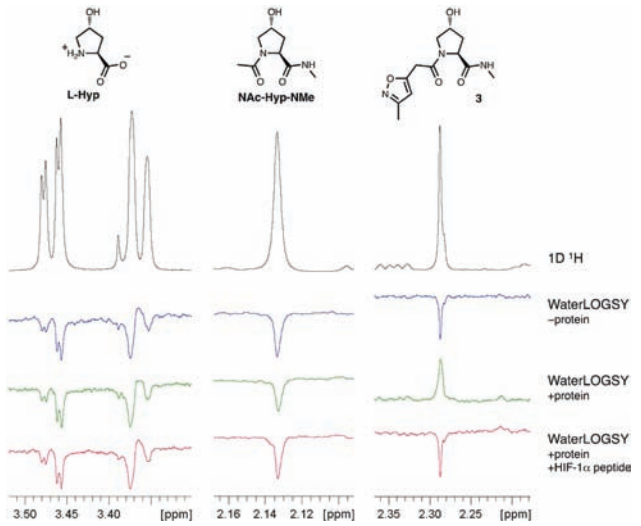
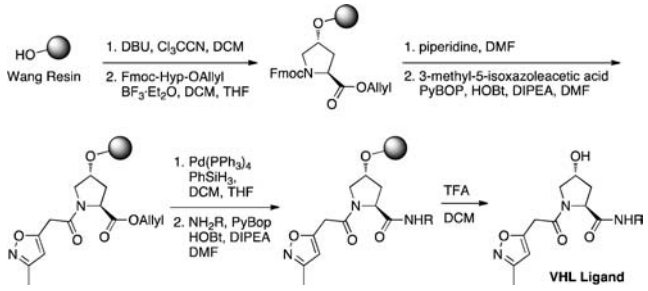


Figure 2. WaterLOGSY NMR spectroscopy shows binding of **3**, but not L-Hyp or NAc-Hyp-NMe, to VHL.

ligands (Scheme 1).[26,27] These ligands were then tested for their ability to bind VHL using the HIF-1α peptide FP displacement assay.

Scheme 1. Solid-Phase Synthesis of VHL Ligands



Incorporation of various halogenated benzylamines showed that para substitution yielded the highest affinity and that there were only slight differences of affinity between chlorides and bromides, although the corresponding fluoride was less potent. We also found that substitution with electron-withdrawing groups such as esters, nitro groups, nitriles, and ketones led to more potent ligands than substitution with electron-donating methoxy and *tert*-butyl substituents. We then considered larger heterocyclic substituents at the para position of the benzylamine moiety and synthesized **15**, which was found to bind with a 4.1 μM IC_{50} value (Table 2). The K_d of **15** was determined by ITC to be 5.4 ± 0.2 μM (Figure S3 in the Supporting Information).

The cocrystal structure of VHL bound to **15** confirmed that it bound to the same site on VHL as HIF-1α and that the hydroxyproline ring recapitulates the interactions seen in the HIF-1α peptide:VHL complex (Figure 3).[19,20] The hydroxyl group makes contacts with both His115 and Ser111, and the amide NH makes a contact with the His110 carbonyl (Figure 4). Furthermore, the crystal structure shows that the aryl ring is involved in a side-on interaction with the Tyr98 phenol, possibly explaining the higher affinity of ligands containing electron-poor aryl groups. In addition, a hydrogen bond is formed between the oxazole CH and the Pro99 carboxyl

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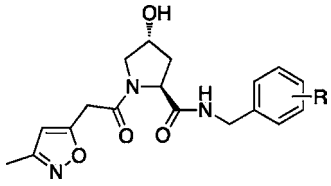
Table 2. Structure–Activity Relationship of VHL Ligands



	R	IC_{50} (μM)[a]	SEM (μM)
1	3-Cl	117	10
4	H	130	10
5	2-Cl	149	13
6	4-Cl	20.5	1.9
7	4-F	149	13
8	4-Br	32.0	3.6
9	4-tBu	>250	N/A
10	4-OMe	106	13
11	4-CO_2Me	39.4	2.2
12	4-NO_2	16.0	0.6
13	4-CN	60.3	5.3
14	4-COMe	22.6	2.0
15	4-(5-oxazoyl)	4.1	0.4

[a]Average IC_{50} values were determined from three independent trials, each in triplicate.

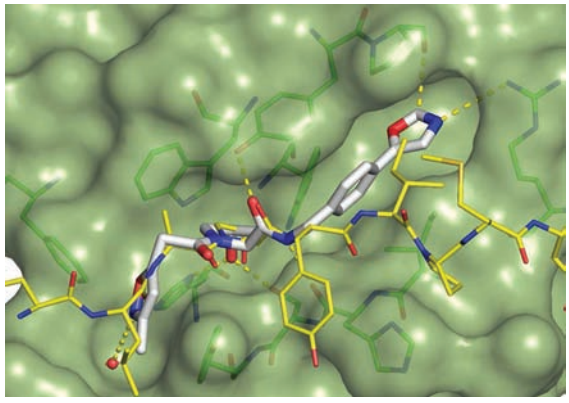


Figure 3. The 2.9 Å cocrystal structure of **15** (white carbons) bound to VHL, indicating that its binding mimics that of the HIF-1α peptide (yellow carbons, PDB 1LM8[20]).

oxygen. Furthermore, the side chain of Arg107 reorients slightly (Figure S2 in the Supporting Information), and hydrogen bonds to the oxazole nitrogen of the inhibitor. These structural data will allow for further rational design to optimize the potency of future inhibitors of the VHL/HIF-1α interaction.

In summary, we have described the design and synthesis of the first small-molecule ligands for VHL, the protein recognition subunit of an E3 ubiquitin ligase. Starting from the minimal hydroxyproline recognition unit and using *in silico* design as well as structure-guided medicinal chemistry, we were able to improve ligand affinity for VHL to single digit micromolar. Furthermore, the most potent inhibitor was cocrystallized with VHL, and shown to bind at the HIF-1α binding site. These small-molecule inhibitors of the VHL/HIF-1α protein–protein interaction have the potential to be developed into cell-penetrant chemical probes that mimic the hypoxic response as well as novel therapeutics to treat disease conditions such as chronic anemia, acute ischemia, and stroke.

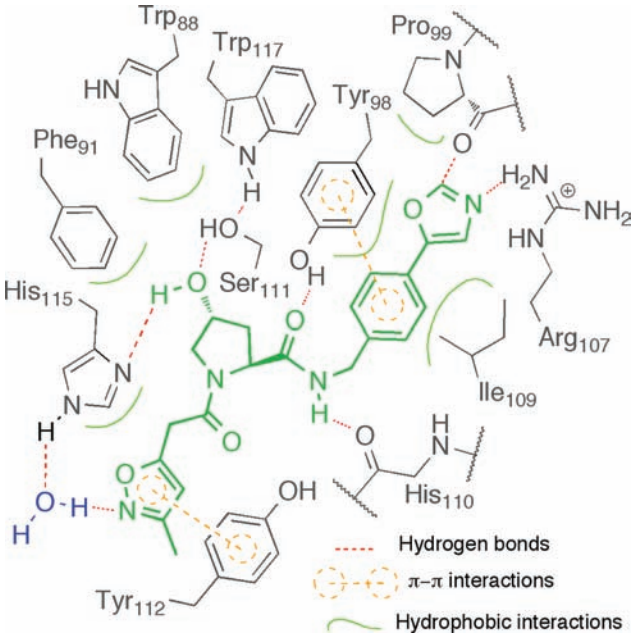


Figure 4. Graphical representation showing the key interactions between **15** and VHL.

■ ASSOCIATED CONTENT

Supporting Information

Chemical and biochemical procedures, characterization of novel compounds, complete refs 7 and 9, and details of the X-ray diffraction data collection and analysis. This information is available free of charge via the Internet at http://pubs.acs.org. The crystal structures of pVHL-Elongin B-Elongin C and pVHL-Elongin B-Elongin C-**15** complex described in this paper have been deposited in the Protein Data Bank (PDB codes 3ZRF and 3ZRC, respectively).

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