Drug Design

DOI: 10.1002/anie.201206231

Small-Molecule Inhibitors of the Interaction between the E3 Ligase VHL and HIF1α**

*Dennis L. Buckley, Jeffrey L. Gustafson, Inge Van Molle, Anke G. Roth, Hyun Seop Tae, Peter C. Gareiss, William L. Jorgensen, Alessio Ciulli, and Craig M. Crews**

Protein–protein interactions (PPIs) are vital to most biological processes, yet despite recent advances they remain notoriously difficult to target due their relatively large surfaces lacking the deep pockets of more tractable targets.[1] While targeting these interactions with large α-helical mimics[2–5] has been relatively successful, developing druglike small-molecule inhibitors of PPIs remains highly challenging. Recently, some success has resulted from the use of virtual screening,[6] fragment-based approaches,[7] and the targeting of "hot spots";[8] however the hit rates for protein interfaces remain low.[1c]

One class of PPIs with promising therapeutic potential is that of E3 ligases with their substrates. E3 ligases bind to their protein substrates, allowing E2 enzymes to transfer ubiquitin subunits to the target protein. Because of their control of widespread biological systems, E3 ligases are highly desirable drug targets.[9] However, since the discovery of the nutlins, the first small-molecule E3 ligase inhibitors,[10] only a handful of E3 ligases have been successfully targeted.[11–13]

The von Hippel–Lindau protein (VHL) is a component of a multi-subunit E3 ligase that recognizes the prolyl-hydroxylated transcription factor HIF1α and tags it for degradation by the proteasome (Figure 1).[14] However, under hypoxic conditions, the prolyl hydroxylase domain enzymes (PHDs) are unable to hydroxylate HIF1α, resulting in the accumulation of HIF1α and subsequent upregulation of the genes involved in the hypoxic response, including GLUT1, VEGF, and erythropoietin. HIF1α stabilization, through the use of PHD inhibitors,[15] is being investigated in the clinic as a possible treatment for chronic anemia.[16] Alternatively, the inhibition

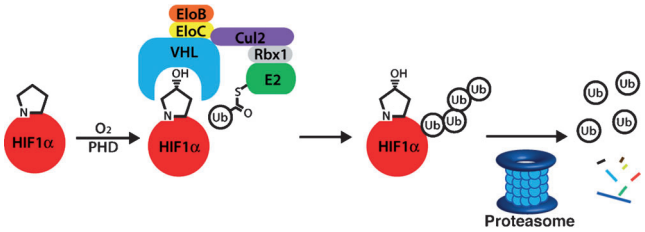

Figure 1. HIF1α is hydroxylated under normoxic conditions, leading to recognition by VHL followed by ubiquitination and degradation by the proteasome.

of the VHL–HIF1α interaction with peptidic inhibitors fused to the tat translocation domain has been shown to stabilize HIF1α,[17] illustrating that inhibition of this interaction is an alternative or complementary strategy to PHD inhibitors for the treatment of anemia.

Recently, we reported a series of VHL ligands, including **1** (see Table 1) capable of competitively inhibiting the binding of a fluorescent peptide derived from HIF1α to VHL.[18] These inhibitors contain a hydroxyproline residue, which is crucial for binding to VHL,[19] and an isoxazolylacetamide fragment, which was designed to interact with a water molecule previously identified as an important part of the hydrogen-bonding network between VHL and HIF1α.[20] However, these molecules bound with limited potency and only a small number of analogues were made, hindering the ability to draw conclusions about structure–activity relationships (SARs). Herein we report a detailed study of VHL ligand SAR, including the discovery of N-terminal fragments with an alternative binding mode, as shown by X-ray crystallography. The optimization of both the C- and N-terminal fragments, followed by their combination, yielded our most potent ligand to date, which binds with an IC_{50} value in the sub-micromolar range.

While optimizing the C- and N-terminal fragments for affinity, we sought to minimize differences in ligand solubility by testing binding affinity in a fluorescence polarization competition assay using 10% DMSO, as opposed to the more physiologically relevant 1% DMSO.[18] While general trends in affinity were similar under both sets of conditions, we found that in cases where solubility was not an issue, ligands had lower IC_{50} values in 1% DMSO.

After the discovery of **1**,[18] we sought to systematically investigate other analogues with five-membered heteroaromatic substituents (Table 1). After examining compounds with various oxazolyl (**1**, **2**, **3**) and thiazolyl substituents (**4**, **5**, **6**, **7**), we found that the original substitution at the 5-position

[*] D. L. Buckley, Dr. J. L. Gustafson, Dr. A. G. Roth, Dr. H. S. Tae, Dr. P. C. Gareiss, Prof. W. L. Jorgensen, Prof. C. M. Crews
Departments of Chemistry, Molecular, Cellular & Developmental Biology and Pharmacology
and Center for Molecular Discovery, Yale University
New Haven, CT 06511 (USA)
E-mail: craig.crews@yale.edu
Dr. I. Van Molle, Dr. A. Ciulli
Department of Chemistry, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)

[**] This research was supported in part by the NIH (AI084140, GM032136), BBSRC (BB/G023123/1), and EC (PIEF-GA-2010-275683). J.L.G. thanks the NIH for a postdoctoral fellowship (F32GM10052101). A.G.R. is a Leopoldina-Nationale Akademie der Wissenschaften Postdoctoral Fellow. We are grateful to the beamline scientists of the Proxima-1 beamline at the Soleil synchrotron facility for their assistance.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201206231.

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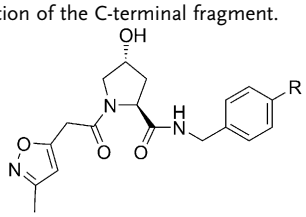
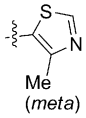
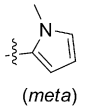
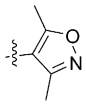
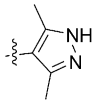
Table 1: Optimization of the C-terminal fragment.



Compd.	R (*para*)	IC_{50} [μM][a] (10% DMSO)	IC_{50} [μM][a] (1% DMSO)
1		7.0 ± 0.5	4.1 ± 0.4[b]
2		11 ± 1	N.D.
3		5.1 ± 0.2	12.7 ± 0.7
4		17 ± 1	14.0 ± 0.5
5		119 ± 2	77 ± 3
6		3.8 ± 0.3	3.2 ± 0.4
7		17.0 ± 0.4	19 ± 1
8		16.4 ± 0.6	32 ± 4
9		17.8 ± 0.3	33 ± 9
10		36 ± 12	19 ± 2
11		270 ± 20	180 ± 10
12		12.1 ± 0.6	8.97 ± 0.07
13		50 ± 10	43 ± 2
14		120 ± 30	70 ± 10
15		18 ± 2	32 ± 3

[a] IC_{50} values were determined by the displacement of FAM-DEALA-Hyp-YIPD from VCB, with the standard error of the mean (SEM) reported. [b] Literature value.[18] N.D. = not determined.

of the heteroaromatic substituent and at the *para* position of the aryl ring was optimal.

Preliminary molecular modeling suggested that substitution of a methyl group at the N position of a pyrrole or 4-position of an azole would increase hydrophobic interactions with Pro99 of VHL.. While the *N*-methylpyrrolyl analogues had only moderate activity, the 4-methyloxazolyl compound **3** was slightly less potent than **1**. In contrast, the 4-methylthiazolyl analogue **6** was not only more potent than **4** but also more potent than the oxazolyl compound **1.**

Given the apparent role of the oxazole C–H donation to the Pro99 carbonyl group,[18] we sought to increase the potency of the ligands by using a better H-bond donor. Unfortunately, we were disappointed to find that the imidazole **11** had poor binding affinity and that the pyrazoles **12** and **13** had moderate affinity but were inferior to **1**. However, the direct comparison of pyrazole **15** and isoxazole **14**, which cannot act as an H-bond donor, was informative, showing that the H-bond can lead to a significant increase in potency.

We simultaneously sought to optimize the N-terminal fragment, while keeping the *p*-chlorobenzylamino fragment of **16** constant because of its small size and the availability of the starting material for its synthesis. We originally explored aryl and heteroaryl acetamides. Disappointingly, each was significantly worse than the isoxazolyl analogue. This included the pyrazolyl **22** and imidazolyl compounds **19** and **21**, which were designed to interact more strongly with the structural water molecule owing to their increased capacity as H-bond acceptors.[21] Testing more diverse fragments led to more success (Table 2), as we found that the chlorobenzamide derivative **24** approached the activity of the corresponding isoxazoleacetamide **16**.

To elucidate the mode of binding of the chlorobenzamide fragment, a crystal structure of **24** with the pVHL-ElonginB-ElonginC complex (VCB) was obtained (Figure 2). The structure showed that the hydroxyproline core reorients slightly, maintaining many of the previously highlighted interactions.[18] In particular, the hydroxy group H-bonds to both S111 and H115, while the amide H-bonds to the H110 backbone and the Y98 side chain. Interestingly, the N-terminal fragment clearly binds to VHL in an alternative fashion. While the isoxazolyl moiety interacted with the structural water previously identified[20] that binds to N67,

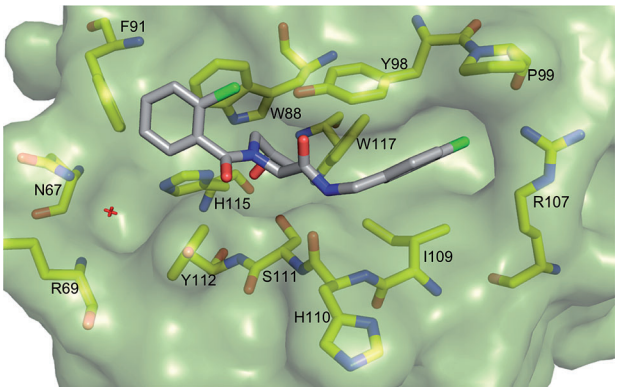


Figure 2. Crystal structure of VCB in complex with **24**. pVHL is shown as a pale green surface, the ligand as a gray stick representation, and the pVHL residues forming the binding pocket as yellow stick representations.

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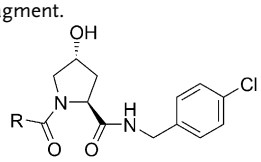
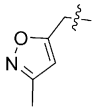
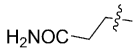
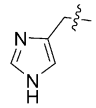
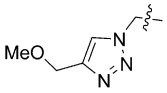
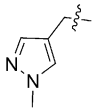
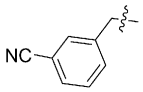
Table 2: Exploration of diverse N-terminal fragments and the discovery of the benzamide fragment.



Compd.	R	IC_{50} [μM][a] (10%DMSO)
16		38 ± 3
17		330 ± 10
18		> 1000
19		> 1000
20		280 ± 40
21		> 1000
22		372 ± 7
23		300 ± 50
24		52 ± 2

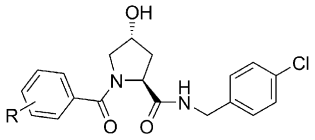
[a] IC_{50} values determined by the displacement of FAM-DEALA-Hyp-YIPD from VCB, with SEM reported.

R69, and H115, the benzamide instead is oriented away from the water pocket, and lies adjacent to the side chain of W88.

We then synthesized a number of benzamide inhibitors and found a wide variety of compounds with improved binding potency (Table 3). In particular, we found that the 3-amino-2-methylbenzamide compound **28**, the *m*-cyanobenzamide compound **33**, and the 2-bromo-4-chlorobenzamide compound **36**, were more potent than the isoxazoleacetamide inhibitor **16**. Additionally, we found that we were able to add large substituents at the *meta* position while maintaining potency.

After optimizing both outer fragments independently, we sought to optimize potency of the overall molecule in a combinatorial fashion (Table 4). We found that while in some cases, such as the ethoxybenzamide class, the trend of increasing activity of C-terminal aryl substituents from chloro to oxazolyl to methylthiazolyl remained similar to the original isoxazolylacetamide, in many cases the oxazolyl compound was worse than both the chloro and methylthiazolyl. In most

Table 3: Optimization of the benzamide N-terminal fragment.



Compd.	R	IC_{50} [μM][a] (10%DMSO)
24	2-Cl	52 ± 2
25	H	110 ± 3
26	3,4-dimethoxy	24 ± 2
27	2-NH_2	38 ± 1
28	3-NH_2,2-Me	10.4 ± 0.8
29	4-NH_2	29 ± 3
30	4-Cl	70 ± 10
31	3-F	38.5 ± 0.5
32	3-Br	19.6 ± 0.8
33	3-CN	8.9 ± 0.1
34	3-OMe	26.3 ± 0.4
35	3-OH	17 ± 0.6
36	2-Br,4-Cl	15.5 ± 0.4
37	3-Ph	33 ± 4
38	isonicotinamide	32 ± 1

[a] IC_{50} values determined by the displacement of FAM-DEALA-Hyp-YIPD from VCB, with SEM reported.

cases, the methylthiazolyl analogue remained the most potent compound. We were also pleased to find that **51** bound to VHL with an IC_{50} of 0.9 μM in 1% DMSO, our most potent compound to date, and the only small-molecule compound thus far that inhibits the interaction between VHL and the HIF1α peptide at concentrations below 1 μM.

We next obtained a crystal structure of the most potent ligand, **51**, in complex with VCB (Figure 3). The optimized methylthiazolyl moiety of the C-terminal fragment binds in a similar conformation to the oxazolyl fragment of **1** that had been previously crystallized.[18] While the methyl group is solvent exposed, it is possible that the larger and more hydrophobic aromatic sulfur atom is better able to fill the small hydrophobic pocket underneath P99 than the corresponding oxygen in **1**. The aryl ring of the N-terminal

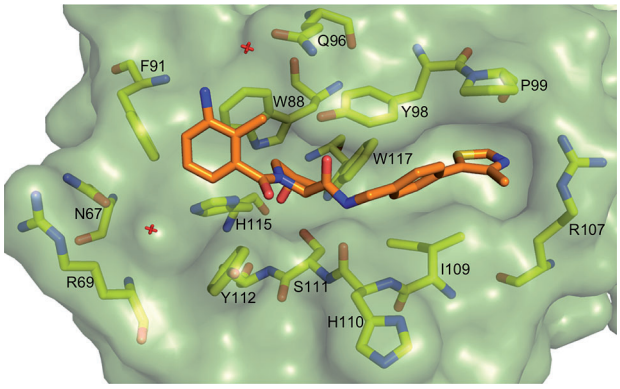


Figure 3. Crystal structure of VCB with **51**. pVHL is shown as a pale green surface, the ligand as an orange stick representation, and the pVHL residues forming the binding pocket as yellow stick representations.

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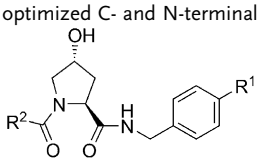
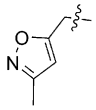
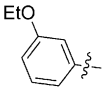
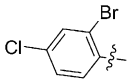
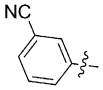
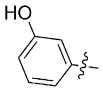
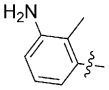
Table 4: VHL ligands with optimized C- and N-terminal fragments.



No.	R^1	$R^{2[b]}$	IC_{50} [μM][a] (10% DMSO)	IC_{50} [μM][a] (1% DMSO)	K_i[b] [μM]
16	Cl		37.8 ± 3.1	20.5 ± 1.9[c]	15.2
1	5-oxazolyl		7.0 ± 0.5	4.1 ± 0.4[c]	3.0
6	5-(4-methyl)thiazolyl		3.8 ± 0.3	3.2 ± 0.4	2.4
39	Cl		62.9 ± 2.0	N.D.	N.D.
40	5-oxazolyl		27.0 ± 0.7	19.5 ± 1.0	14.5
41	5-(4-methyl)thiazolyl		14.1 ± 0.3	11.2 ± 0.9	8.3
32	Cl		19.6 ± 0.8	N.D.	N.D.
42	5-oxazolyl		39.5 ± 3.7	31.5 ± 1.9	23.3
43	5-(4-methyl)thiazolyl		7.7 ± 0.4	26.8 ± 1.8	19.9
36	Cl		17.0 ± 0.6	N.D.	N.D.
44	5-oxazolyl		21.2 ± 0.4	24.1 ± 0.6	17.8
45	5-(4-methyl)thiazolyl		4.9 ± 0.2	11.9 ± 0.7	8.8
33	Cl		8.0 ± 0.1	N.D.	N.D.
46	5-oxazolyl		40.3 ± 3.9	47.3 ± 1.3	35.1
47	5-(4-methyl)thiazolyl		12.0 ± 0.5	11.0 ± 0.8	8.1
35	Cl		17.0 ± 0.6	N.D.	N.D.
48	5-oxazolyl		23.4 ± 0.4	18.0 ± 0.4	13.3
49	5-(4-methyl)thiazolyl		3.5 ± 0.2	3.71 ± 0.03	2.8
28	Cl		10.4 ± 0.8	N.D.	N.D.
50	5-oxazolyl		18.9 ± 2.3	14.0 ± 0.7	10.4
51	5-(4-methyl)thiazolyl		1.76 ± 0.06	0.90 ± 0.03	0.67

[a] IC_{50} values determined by the displacement of FAM-DEALA-Hyp-YIPD from complex of VCB, with SEM reported. [b] K_i values calculated from IC_{50} values (1% DMSO) as described by Huang.[22] [c] Literature value.[18]

fragment is oriented similarly to **24**, but we were surprised at the orientation of the aniline. Rather than interacting with the structural water in the pocket bound by N67, R69 and H115 (as the earlier isoxazolyl fragment does), the aniline appears to make a completely novel water-mediated H-bond to the side chain of Q96.

In summary, we report the structure-based design and synthesis of novel VHL ligands, illustrating structure–activity relationships and improving potency. We individually optimized two fragments of the ligands, arriving at a new class of N-terminal benzamide fragments. The interactions made by this new class were then elucidated through X-ray crystallography of the protein–ligand complex. Structural studies of this ligand class revealed both a new binding mode as well as a novel water-mediated hydrogen bond. Finally, using these optimized fragments, we assembled new VHL ligands in a combinatorial fashion, arriving at **51**, the first small-molecule submicromolar inhibitor of the interaction between VHL and a peptide derived from HIF1α. While no cell-based activity has yet been observed, we hope to take advantage of the interactions highlighted by the crystal structure of **51** to design more drug-like inhibitors of VHL with improved cell permeability. These ligands and the novel interactions and binding modes highlighted have the potential to guide the development of future chemical probes that can target the E3 ligase VHL or stabilize HIF1α proteins.

Received: August 3, 2012
Published online: October 12, 2012

Keywords: drug design · E3 ubiquitin ligases · protein crystallography · protein–protein interactions · structure-based design

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