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HIF-1α peptide derivatives with modifications at the hydroxyproline residue as activators of HIF-1α

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ABSTRACT

Hypoxia-inducible factor (HIF)-1α undergoes degradation under normoxia, which involves its proline hydroxylation and subsequent binding of proline-hydroxylated HIF-1α to the von Hippel-Lindau protein–Elongin B–Elongin C (VBC) complex. In this study, we designed and synthesized a series of peptides containing 556–575 residues of HIF-1α with modifications at the Pro-564 residue to inhibit the interaction between proline-hydroxylated HIF-1α and VBC. Employing a fluorescence polarization-based interaction assay, we evaluated inhibitory potency of these peptides and selected potent inhibitors. We then analyzed their effects in the cell level to show that the selected inhibitors induced HIF-1α stabilization in normoxic cells. Considering that proline hydroxylation of HIF-1α is routinely targeted for modulating the HIF pathway, our approach of using inhibitors against the interactions between HIF-1α and VBC would provide an alternative way of upregulating HIF-1 activity.

Hypoxia-inducible factor (HIF)-1 mediates oxygen homeostasis by controlling expression of genes involved in various biological processes including angiogenesis, energy metabolism, erythropoiesis, and apoptosis.[1] HIF-1 is a heterodimer consisting of an oxygen-regulated α subunit and a constitutively expressed β subunit. Under normoxic conditions, HIF-1α undergoes oxygen-dependent degradation via hydroxylation by prolyl-4-hydroxylases (PHDs) on two conserved proline residues, Pro-402 and Pro-564, within the C-terminal oxygen-dependent degradation domain (CODD) of human HIF-1α. The proline-hydroxylated HIF-1α then interacts with the von Hippel-Lindau protein (VHL) in the VHL–Elongin B–Elongin C (VBC) complex, which leads to ubiquitination by the action of E3 ubiquitin ligase and degradation in proteosome. On the other hand, HIF-1α accumulates as proline hydroxylation diminishes in hypoxic conditions and acts as a transcription factor in a cell type-specific fashion to activate the expression of many angiogenic genes. Accordingly, mechanisms of HIF-1α stability and activation have gained considerable attention for the development of angiogenic therapies. Most work has primarily focused on targeting PHD activity to prevent the degradation pathway of HIF-1α and to augment its downstream signals with enhanced transcription of angiogenic factors,[2] which might be beneficial for treatment of ischemic diseases in brain and heart. Another way of increasing the expression of angiogenic factors through the stabilization of HIF-1α would be to suppress the degradation pathway for proline-hydroxylated HIF-1α. This could be achieved simply by intervening the degradation pathway of HIF-1α itself such as by treating with proteasome inhibitors or by activating deubiquitinating enzymes.[3] Such strategy, however, is not HIF-specific but may affect ubiquitination in general. Thus, we made an attempt to specifically inhibit the HIF-1α–VBC interaction without disturbing other protein regulatory pathways involved in ubiquitination. Here we designed and synthesized a series of 20-residue peptides derived from CODD of HIF-1α, and further examined their inhibitory effects in molecular and cellular levels.

According to a recent report on the structure of the complex, the 4-hydroxyl group of HIF-1α hydroxyproline (HyP)-564 interacts through hydrogen bonds with His-115 and Ser-111 residues of VHL in the VBC complex.[4] Therefore, we substituted the Pro-564 residue of the CODD region with amino acids having various side chains, with an expectation that the resulting peptide inhibitors would bind VBC competitively with the HyP-564 CODD peptide. We used an Fmoc protection-based standard protocol to synthesize 20-residue peptides containing 556–575 residues of human HIF-1α with modifications at the proline residue. As shown in Figure 1, a series of amino acids or amino acid derivatives were incorporated at the modified position **X**.

To evaluate the influence of modifications at Pro-564 on the interaction of HIF-1α with VBC, we analyzed the competitive binding of the synthesized peptide inhibitors by a fluorescence polarization-based interaction assay.[5] Briefly, recombinant VBC complex was generated from plasmids for human VHL, human Elongin B and human Elongin C by co-expression in *Escherichia coli*, and mixed with a fluorescein-labeled form of the HyP-564 CODD pep-

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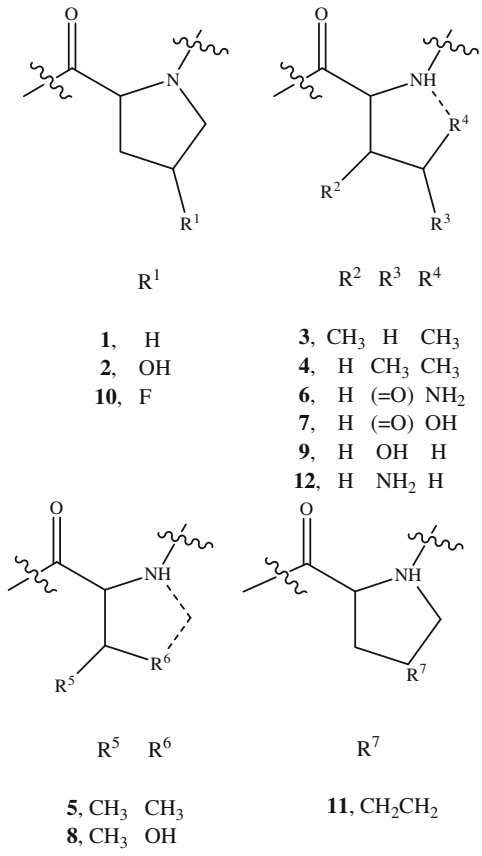

Figure 1. Structures of peptide inhibitors derived from the HIF-1α CODD. Modifications at Pro-564 are shown for the peptides containing amino acids 556–575 of human HIF-1α.

tide (**2**) in the presence of increasing concentrations of the peptide inhibitors in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.5% Nonidet P40). When the mixtures were determined for fluorescence polarization, the values decreased to varying degrees as the concentrations of the inhibitors increased (Fig. 2). The K_i values of the peptide inhibitors were then determined by fitting the curves with the known K_d value of the complex between the fluorescein-labeled form of **2** and VBC (ca. 0.14 μM[5]), and are presented in Table 1. Among the tested peptide inhibitors, the one containing homoserine (Hse) at **X** (**9**) showed the highest inhibitory potency with K_i of 3.4 μM, which was comparable to **2**. Although the hydroxyl group in Hse of **9** has conformational flexibility compared to that in HyP of **2,** it could act as the hydrogen bonding donor and acceptor simultaneously, similarly as the hydroxyl group in HyP. Therefore, the spatial constraint of the pyrrolidine ring that makes multiple van der Waals contacts with hydrophobic residues of VHL in the complex structure[4] does not appear to contribute much to the interaction of the peptide with VBC. These results rather suggest that hydrogen bonding capacity might be critical for the binding of proline-hydroxylated HIF-1α to VBC. Accordingly, without changing the pyrrolidine backbone of HyP, we synthesized a peptide derivative **10** with substitution of the hydroxyl group by a fluoride group, which can serve as a weak hydrogen bonding acceptor but not as a donor. Such substitution gave rise to a slightly higher inhibition constant than that of **2** or **9**, reassuring the importance of the hydrogen bonding capacity of the interacting hydroxyl group. On the other hand, peptide inhibitors containing amino acids with acyclic hydrophobic side chains (**3**–**5**) showed moderate inhibitory effects. The peptide **5** containing a short hydrophobic side chain had a K_i of 16 μM, while **3** and **4** with longer hydrophobic side chains had much higher K_i values of 58 and 50 μM, respectively, implying that bulky methyl groups are not favored and the size the side chain is important for proper interactions. In addition, the peptide inhibitors having amino acids with a neutral amide group (**6**) and with a negatively charged side chain (**7**) in the acyclic backbone showed nearly the same inhibitory potency as **5**. The peptide **8** with a substituted hydroxyl group in the methyl position of **5** also exhibited a similar K_i value. The relatively short stretch of the hydroxyl group from α-carbon of **8** might weaken hydrogen bonding capacity, which likely gave rise to a higher K_i value than the peptides **2** and **9**. Inspired by these observations, we made a peptide derivative **12** with introduction of an amino group replacing the hydroxyl group of Hse to test the effect of a positively charged side chain in the interaction between the CODD peptide and VBC. Unexpectedly, its K_i value decreased by approximately thirty fold compared to that of **9**. This result indicates that the positive charge of the ammonium group can severely affect the electronic environment around the binding site, whereas the neutral or negatively charged groups are favored in the binding site. Interestingly, enlargement of the five-membered ring of pyrrolidine to a six-membered ring of piperidine (Pip) resulted in an increase of the affinity from virtually no binding for **1** to K_i of 77 μM for **11**. The molecular mechanism of such interaction is not apparently clear, but it could be speculated that the piperidine ring in the VBC binding site might resemble the ring

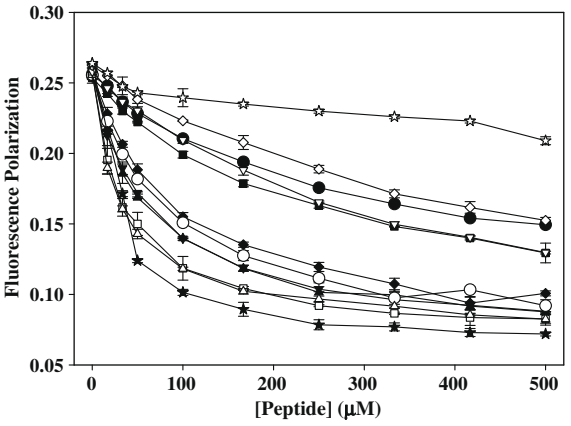

Figure 2. Effects of peptide inhibitors on the HIF-1α–VBC interaction. The interactions were determined by measuring fluorescence polarization values for mixtures of 100 nM (final concentration) of the fluorescein-labeled form of **2** and 300 nM (final concentration) of GST-VBC in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.5% Nonidet P40) in the presence of increasing concentrations of **1** (☆), **2** (★), **3** (●), **4** (■), **5** (▲), **6** (▼), **7** (♦), **8** (○), **9** (□), **10** (△), **11** (▽), and **12** (◇).

Table 1
K_i values of the peptide inhibitors against the interaction between the fluorescein-labeled form of **2** and VBC

Peptide	**X**	K_i (μM)
*DLDLEALA**X**YIPADDDFQLR-NH$_2$*		
1	Pro	>500
2	HyP	3.7 (±3.5)
3	Leu	58 (±7)
4	Ile	50 (±17)
5	Val	16 (±5)
6	Asn	13 (±1)
7	Asp	15 (±2)
8	Thr	18 (±8)
9	Hse	3.4 (±0.9)
10	4FP	5.8 (±1.3)
11	Pip	77 (±7)
12	Ama	109 (±5)

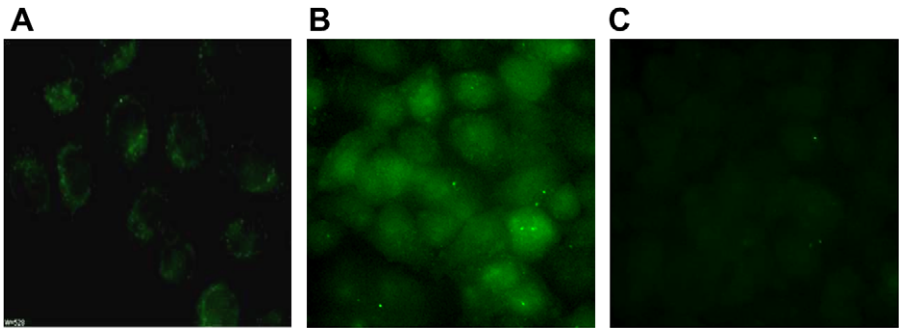

Figure 3. Fluorescence images of HeLa cells transfected with the fluorescein-labeled peptide of **2** using (A) Chariot™ or (B) electroporation, (C) cells were treated with DMSO as a negative control.

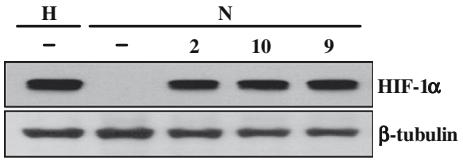

Figure 4. Effects of peptide inhibitors on stabilization of HIF-1α. HeLa cells were transfected with peptide inhibitors (**2**, **9** or **10**) at 1 mM by electroporation and incubated in normoxic conditions for 6 h. Whole cell extracts were loaded on 10% SDS–PAGE, and levels of HIF-1α and β-tubulin were detected by Western analysis along with the samples obtained from cells treated in 1% O_2 hypoxic conditions. N and H represent normoxia and hypoxia, respectively.

conformer adopted by the hydroxylated proline which is likely different from the prevalent conformer of the proline residue. On the other hand, it should be noted that all of the peptide inhibitors did not affect the activity of PHD2 (data not shown).

Next, we chose three peptide inhibitors including **2**, **9** and **10**, which have shown relatively high inhibition potency in the in vitro assay, and investigated their biological effects by monitoring HIF-1α stabilization in HeLa cells. To deliver the peptide inhibitors into cells, we used a commercially available peptide transfection reagent Chariot™, which resulted in poor transfection of the fluorescently labeled **2** into HeLa cells as examined under a fluorescence microscope (Fig. 3B). In contrast, we successfully delivered the fluorescently labeled **2** into HeLa cells (Fig. 3C) using a microporation-based method according to the manufacturer's instructions (Digital Bio Technology; MP-100). For Western blotting, protein extracts from the cells transfected with the peptide inhibitors were prepared by lysis with sample buffer (6% SDS, 1.4 mM β-mercaptoethanol, 20% glycerol, 0.01% w/v bromophenol blue, and 125 mM Tris–HCl, pH 6.8) after cultivation for 6 h. The lysates were then separated on 10% SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-HIF-1α (BD biosciences). As presented in Figure 4, HIF-1α was not detected from the lysate of cells cultured under normoxic conditions in the absence of the peptide inhibitors, whereas it was readily observed from that of cells transfected with the peptide inhibitors, indicating that HIF-1α was induced as its degradation pathway was stalled by the action of the inhibitors for the interaction between HIF-1α and VBC. The amount of accumulated HIF-1α upon treatment with **2**, **9** or **10** was comparable to the hypoxic control. On the other hand, the peptide **3** which showed low inhibitory potency did not stabilize HIF-1α in HeLa cells (data not shown). Therefore, our data suggest that the designed peptides having relatively high inhibitory effects in molecular levels can effectively inhibit the interactions between HIF-1α and VBC in cellular levels.

In conclusion, we have demonstrated that it is possible to selectively inhibit HIF-1α binding to VBC employing peptide inhibitors, thereby stabilizing HIF-1α. This is a novel method that can be exercised alternatively to the methods of inhibiting prolyl hydroxylation for modulating HIF-1α. Previously, it has been reported that expression of VEGF, an important signaling protein for angiogenesis in the downstream was activated by stabilization of HIF-1α with hydroxylated Pro-564.[6] Accordingly, we also expect that treatment with the peptide inhibitors reported in our study would induce angiogenesis by up-regulation of VEGF similarly as observed with other PHD2 inhibitors.[7] Researches on such inhibitors might thus open an alternative way of drug development for treatment of ischemic diseases.

Acknowledgments

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References and notes

1. Semenza, G. L. *Physiology* **2004**, *19*, 176.
2. (a) Tsukiyama, F.; Nakai, Y.; Yoshida, M.; Tokuhara, T.; Hirota, K.; Sakaid, A.; Hayashib, H.; Katsumata, T. *Biochem. Biophys. Res. Commun.* **2006**, *351*, 234; (b) Thomas, R.; Kim, M. H. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 543; (c) Nytko, K. J.; Spielmann, P.; Camenisch, G.; Wenger, R. H.; Stiehl, D. P. *Anti oxidants Redox Signal.* **2007**, *9*, 1329; (d) Linden, T.; Katschinski, D. M.; Eckhardt, K.; Schield, A.; Pagel, H.; Wenger, R. H. *FASEB* **2003**, *17*, 761.
3. Li, Z.; Wang, D.; Messing, E. M.; Wu, G. *EMBO Rep.* **2005**, *6*, 373.
4. Min, J.-H.; Yang, H.; Ivan, M.; Gertler, F.; Gaelin, W. G., Jr.; Pavletich, N. *Science* **2002**, *296*, 1886.
5. Cho, H.; Park, H.; Yang, E. G. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 275.
6. Mcgrath, K., US patent No. 7,053,046 B2.
7. (a) Choi, S. M.; Choi, K.-O.; Park, Y.-K.; Cho, H.; Yang, E. G.; Park, H. *J. Biol. Chem.* **2006**, *281*, 34056; (b) Cho, H.; Lee, H. Y.; Ahn, D. R.; Kim, S. Y.; Kim, S.; Lee, K. B.; Lee, Y. M.; Park, H.; Yang, E. G. *Mol. Pharmacol.* **2008**, *74*, 70.