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Inhibition of Hypoxia Inducible Factor 1—Transcription Coactivator Interaction by a Hydrogen Bond Surrogate α-Helix

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Interactions between transcription factors and coactivator proteins induce chromatin remodeling and facilitate assembly of the RNA polymerase II machinery on target promoters by establishing stable preinitiation complexes.[1] Synthetic ligands that modulate these protein−protein interactions would serve as probes for mechanistic studies related to transcription and, potentially, as leads for drug discovery efforts.[2,3] Transcription of hypoxia-inducible genes, which are important contributors in cancer growth and metastasis,[4,5] is mediated by binding of the cysteine-histidine rich 1 (CH1) region of coactivator protein p300 (or the homologous CREB binding protein, CBP) and the C-terminal transactivation domain (C-TAD$_{786-826}$) of hypoxia-inducible factor 1α (HIF-1α) (Figure 1a).[6] Structural analysis of this transcription factor−coactivator interaction reveals two short α-helical domains from HIF-1α C-TAD as key determinants for its recognition by CBP/p300 (Figure 1b).[6] Synthetic mimics of these domains could potentially inhibit HIF-1α/p300 or HIF-1α/CBP complex formation and downregulate the expression of genes encoding vascular endothelial growth factor (VEGF) and its receptor VEGFR2, which are involved in the induction of new blood vessels (angiogenesis) in solid tumors.[5] This communication describes a synthetic α-helix that modulates the interaction between HIF-1α and CBP/p300 and inhibits transcription of HIF-1α inducible genes in cell culture.

Short peptides typically require stabilizing moieties to retain their folded conformation once excised away from the protein environment.[7] We utilized the hydrogen bond surrogate (HBS) approach to design stabilized α-helical peptides derived from the HIF-1α C-TAD region (Figure 1c).[8] The HBS approach is based on the helix−coil transition theory in peptides, which suggests that the energetically demanding organization of three consecutive amino acids into the helical orientation inherently limits the stability of short α-helices. The HBS strategy affords preorganized α-turns to overcome this intrinsic nucleation barrier and initiate helix formation.[8] The preorganized α-turns are obtained by replacing the N-terminal main chain hydrogen bond between the C═O of the i^{th} amino acid residue and the NH of the i+4th amino acid residue with a carbon−carbon bond through a ring-closing metathesis reaction.[9]

We began our studies by mimicking the α-helical conformation of residues $_{799}$DCEVNA$_{804}$ which are reported to be critical for the interaction between the CBP/p300 CH1 domain and HIF-1α (Figure 1b).[6] Specifically, mutagenesis data suggest that Cys-800 and Asn-803 play critical roles in HIF-1α and p300/CBP complex formation and signal transduction in hypoxia.[10,11] HBS **1** was designed to feature the key residues outside of the HBS macrocycle (Table 1). The *N*-terminal residues Ser-797 and Tyr-798 do not directly contact the coactivator surface. We placed alanine residues at these positions to facilitate the macrocycle synthesis (Supporting Information). Isothermal titration microcalorimetry data revealed that this helix bound GST-p300 with submicromolar affinity. We next analyzed the potential of this compound to downregulate HIF-1α induced transcription of the *VEGF* gene in HeLa cells, under hypoxic conditions, by employing real-time quantitative RT-PCR (qRT-PCR) assays, as described in the Supporting Information.[12] An iron

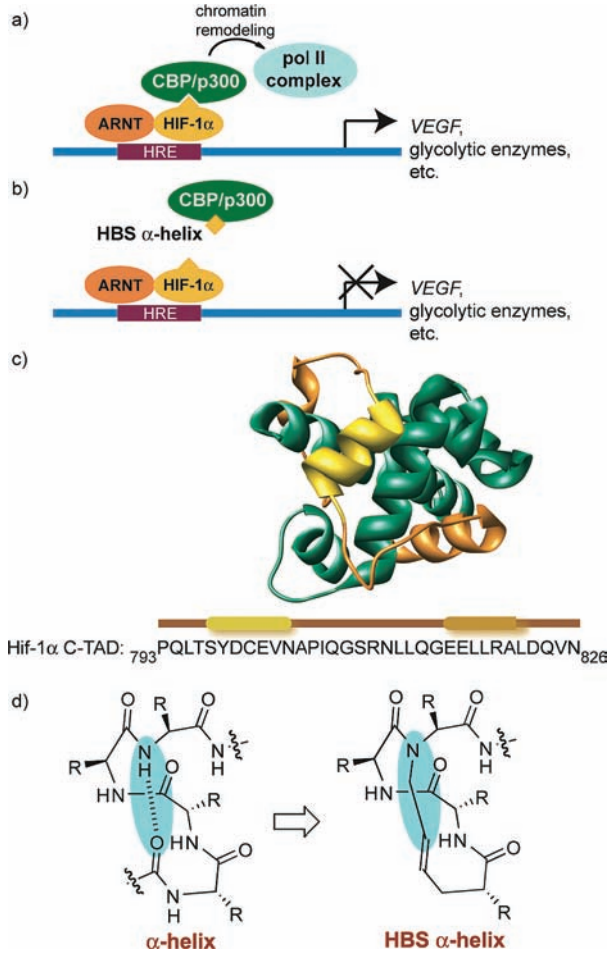

Figure 1. (a) Transcription of hypoxia inducible genes is controlled by the interaction of DNA-bound HIF-1α/ARNT heterodimer with transcription coactivator CBP/p300. (b) Competitive inhibition of the HIF-1α C-TAD complex with CBP/p300 CH1 domain leads to downregulation of *VEGF* transcription. (c) α-Helices from the C-TAD$_{793-826}$ domain of HIF-1α bind to the cysteine-histidine rich 1 (CH1) region of CBP/p300. HIF-1α is shown in orange and yellow colors, and CBP/p300 in green (PDB code 1L8C). (d) HBS α-helices feature a carbon−carbon bond in place of an *N*-terminal *i* and *i*+4 hydrogen bond.

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Table 1. Summary of Key Biophysical and *in Vitro* Data for Peptides Designed To Target HIF 1α—p300 Interactions

compound	sequence[a]	% helicity[b]	K_d (nM)[c]	transcription inhibition[d]
1	XTAADCEYNA	40	950 ± 90	0 ± 3
2	XTAADCEYNAR	53	420 ± 35	45 ± 8
3	XTAADREYNAR	51	≫2200	2 ± 7
4	AcTAADCEYNAR	15	825 ± 50	8 ± 3
chetomin	–	–	120 ± 25	50 ± 5

[a] X denotes pentenoic acid residue in the HBS macrocycle. [b] Values obtained from circular dichroism spectroscopy studies. [c] From isothermal titration microcalorimetry analysis. [d] % Inhibition of *VEGF* gene measured by real-time qRT-PCR assays in HeLa cells with 1 μM peptide or 200 nM chetomin.

chelator, deferoxamine mesylate (DFO), was used to mimic hypoxia in cell culture.[13] HBS **1** did not show measurable inhibition of transcription in this cell-based assay after 12 and 24 h incubation periods, potentially indicating poor cellular uptake of **1**. (We chose not to pursue confocal microscopy studies with fluorescently labeled peptides to evaluate uptake due to the concern about the effect of the dye on the uptake properties of these very short oligomers.[14])

Based on our conjecture that uptake of **1** was hindering its activity in cell-based assays, we designed HBS **2** with an extra arginine at the C-terminus. Polyarginines are well-known to assist transport of attached peptides and proteins;[15] however, recent studies suggest that in short peptides incorporation of one or two cationic residues might be sufficient to enhance uptake in certain cell lines.[16] We also prepared and tested HBS helix **3** and an unconstrained analogue **4**. HBS **3** was designed to be a negative control in which the key Cys-800 residue that targets a hydrophobic pocket is mutated to an arginine.

The solution conformation of the constrained and unconstrained peptides was investigated by circular dichroism spectroscopy. CD studies were performed in 10% trifluoroethanol in phosphate buffered saline (pH 6.3). CD spectra of **1**−**3** are consistent with those observed for canonical α-helices (Figure 2a). The relative percent helicity of peptides was estimated by the mean residue ellipticity at 222 nm, as previously described.[17] HBS **2** is more helical than HBS **1**, likely because of the potential *i* and *i*+4 ionic interactions between the glutamic acid residue and the C-terminal arginine (Table 1). As expected, the short unconstrained peptide **4** is largely unstructured (Figure 2a). Isothermal titration microcalorimetry analysis shows that addition of the C-terminal arginine group does not have a detrimental effect on the ability of HBS **2** to bind GST-p300 (Figure 2b and Table 1). The unconstrained derivative **4** targets p300 CH1 domain with a 2-fold lower affinity, and as predicted, the negative control HBS **3** is a weaker binder.

We evaluated the ability of compounds **2**−**4** to modulate HIF-1α mediated transcription in HeLa cells, as described above. Gratifyingly, treatment of the culture with 1 μM HBS **2** for 12 h downregulates *VEGF* transcription to roughly the same levels as those for 200 nM chetomin, a known potent inhibitor of *VEGF* transcription (Table 1 and Figure 3a).[18] As expected, control compounds **3** and **4** show reduced activity in cell culture (Table 1). Similar results were obtained after a 24 h incubation period (Supporting Information). The reduced activity of the peptide **4** potentially reflects the proteolytic instability of this unconstrained peptide, as stabilization of peptides in α-helical conformation is expected to enhance their resistance to proteases. We have previously reported improved proteolytic stability and cell culture activity of HBS α-helices as compared to their unconstrained counterparts.[19]

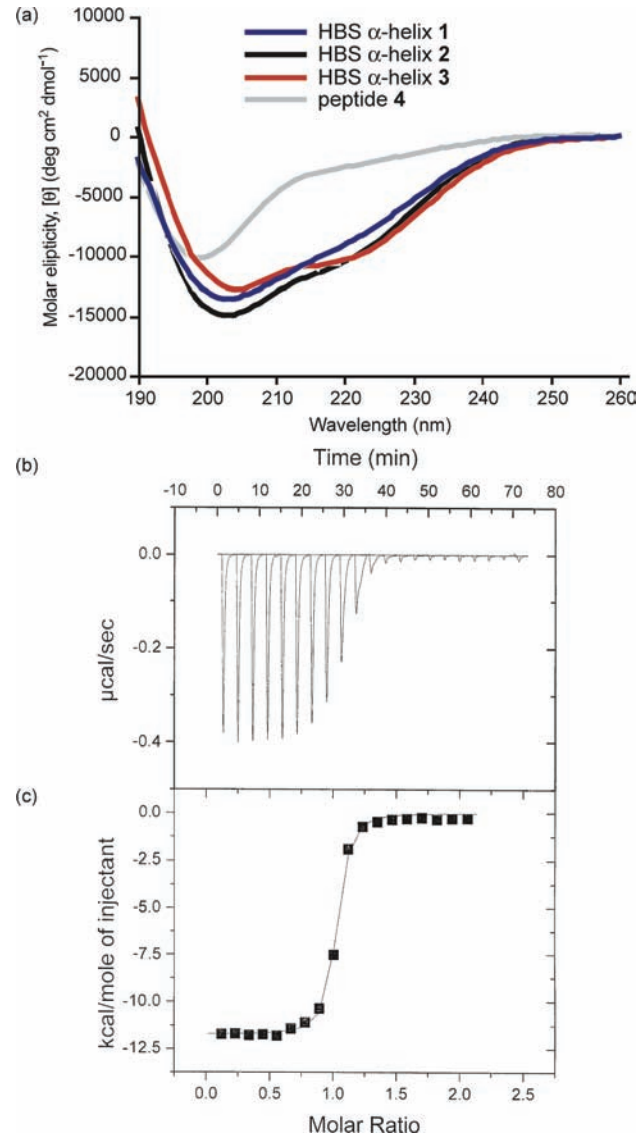

Figure 2. (a) Circular dichroism spectra of HBS helices **1**−**3** and unconstrained peptide **4** in 10% TFE in phosphate buffered saline (pH 6.3). (b, c) Isothermal titration microcalorimetry analysis of HBS **2** binding to GST-p300. (b) Baseline-subtracted raw ITC data for injections of HBS **2** into a solution of the GST-p300 in Tris buffer at 25 °C. (c) Integration of titration signals (squares), caused by the binding of HBS **2** with GST-p300, fit to a single-site binding model (line).

The *in vitro* toxicity of HBS helices was analyzed with a cell viability assay. In this assay, the cell density and the rate of population doubling were monitored for up to 72 h. We found that, unlike chetomin, HBS **2** does not display significant cytotoxicity (Figure 3b). Recently reported CD studies from our groups and a report by Cook et al. suggest that chetomin inhibits HIF-1α induced transcription by inducing a structural change in CH1 domains of CBP (or p300) through a zinc ejection mechanism.[12,20] CBP and p300 are pleiotropic, multidomain proteins which interact with different components of the transcriptional machinery and p300/CBP associated factor (PCAF) to regulate expression of multiple genes.[21] Each coactivator contains three zinc-bound cysteine-histidine rich domains (CH1-CH3); unfolding of these domains could lead to nonspecific effects on gene expression. Unlike chetomin, addition of HBS **2** does not alter the CD spectrum of GST-p300 (Supporting Information), suggesting that transcription inhibition is not due to denaturation of the target coactivator.

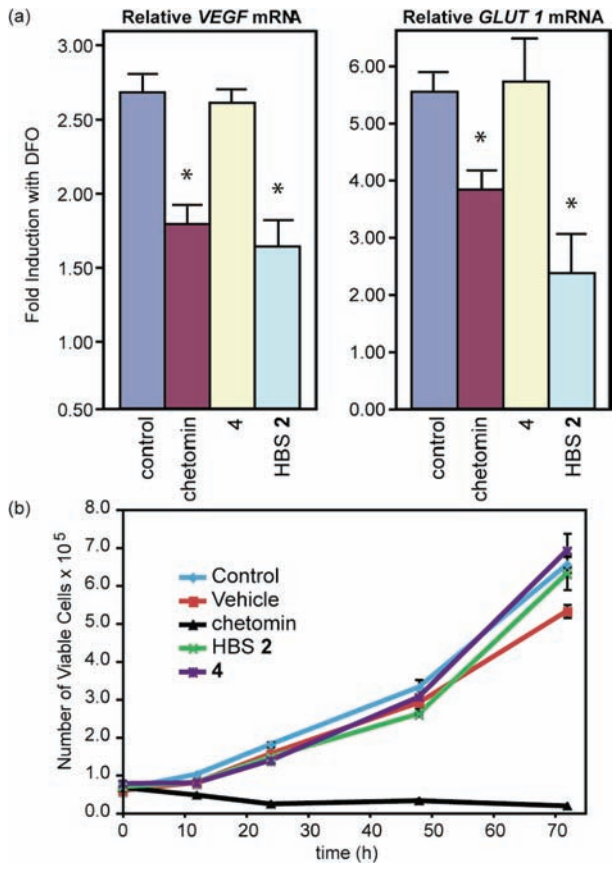

Figure 3. (a) Inhibition of *VEGF* and glucose transporter 1 (*GLUT1*) gene expression with HBS helix **2** and peptide **4**, after 12 h of incubation under hypoxia conditions, as measured by real time qRT-PCR in HeLa cells. *, $P < 0.05$, *t* test. (b) Cell density and population doubling data for cultures treated with chetomin, HBS **2**, and control peptide **4**. 200 nM chetomin and 1 μM peptides (**2** and **4**) were used in the cell culture and cell viability assays. Control: cell culture medium only. Vehicle: 0.1% DMSO in cell culture medium.

The interaction of HIF-1α C-TAD and the CBP/p300 CH1 domain controls expression of over 70 hypoxia-inducible genes.[5] Inhibition of this complex should therefore downregulate multiple genes in a pathway-dependent manner. In these initial studies, we also evaluated the potential of HBS **2** to modulate transcription of another hypoxia-inducible gene, glucose transporter 1 (*GLUT1*). HBS **2** provided similar levels of transcription inhibition for *GLUT1* as for *VEGF* (Figure 3a). This positive control strongly reinforces our design.

In summary, structure-based design of a stabilized helix targeting an important transcription factor−coactivator interaction resulted in an inhibitor that effectively downregulates hypoxia-inducible genes in cell culture. Selective modulation of protein−protein interactions by rationally designed synthetic ligands is a key challenge in chemical biology.[22] Our results underscore the fundamental role played by protein secondary structures, specifically α-helices, in interactions of proteins with other biomolecules,[23] and highlight the potential of such mimetics to offer gene-specific regulators of transcription.[2]

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Supporting Information Available: Synthesis and characterization of HBS α-helices, details of ITC experiment, cell culture, and qRT-PCR and cell viability assays (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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