Communication

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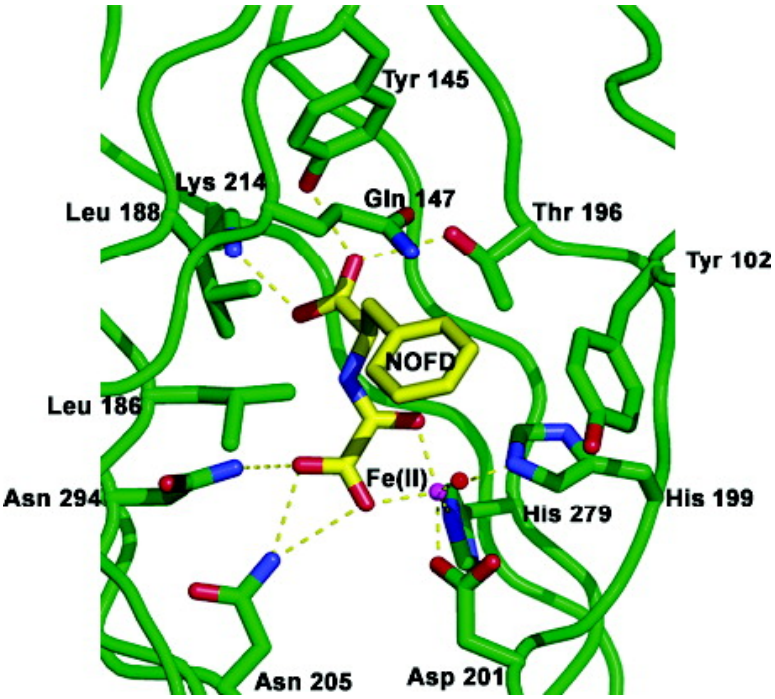

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Selective Inhibition of Factor Inhibiting Hypoxia-Inducible Factor

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In many animals, cellular responses to reduced oxygen concentration (hypoxia) are mediated by an α/β-heterodimeric transcription factor, hypoxia-inducible factor (HIF).[1] Under hypoxia, HIF binds to response elements linked to an array of genes associated with the hypoxic response including, in humans, those associated with angiogenesis (vascular endothelial growth factor, VEGF) and erythropoiesis (erythropoietin, EPO). HIF-β is a constitutive nuclear protein, but levels of HIF-α are low under normal oxygen concentrations (normoxia) but increase in response to hypoxia.

Recent studies have identified the mechanism by which both the levels and transcriptional activity of HIF-α are regulated by molecular oxygen. Post-translational trans-4-hydroxylation of either of two proline residues that form part of consensus motifs located in the oxygen-dependent degradation domain of HIF-α is sufficient to target HIF-α to the von Hippel Lindau protein (pVHL),[2] which in turn recruits a ubiquitin ligase that mediates proteasomal destruction. The transcriptional activity of HIF is more directly inhibited by hydroxylation at the β-position of Asn803 in human HIF-1α that blocks its interaction with the transcriptional coactivator p300.

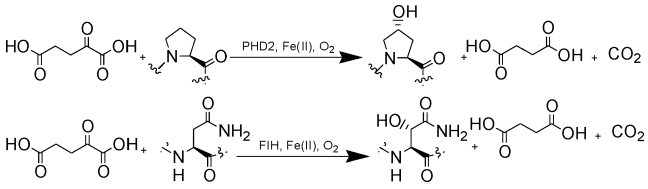
Four hydroxylases have been identified that catalyze the post-translational modification of human HIF-α: three prolyl hydroxylases (PHD1-3)[3] and an asparaginyl hydroxylase, factor inhibiting HIF (FIH).[4] All belong to the family of ferrous iron and 2-oxoglutarate (2OG, **1**)-dependent dioxygenases; most of these enzymes bind their metal cofactor via a conserved 2His−1Asp/Glu triad of residues; structural studies, however, reveal significant variation in the 2OG binding site.[5]

Regulation of transcription by small molecules has the potential for significant medical application, with respect to both the HIF and other signaling pathways. One approach is via small molecules or oligomers that interact directly with nucleic acids.[6] Another is to employ compounds that alter the activity of transcription factors either directly or indirectly. We have been exploring HIF hydroxylase inhibitors as a means of up-regulating hypoxically driven transcripts.

The substrate selectivity of the PHD enzymes for different HIF-α isoforms has recently been investigated.[7] Under normoxic conditions, PHD2 appears to be important in regulating HIF-1α cellular levels, but has a smaller action on HIF-2α levels. In contrast, PHD3 appears to more significantly regulate the effects of HIF-2α. Given the differences in preference of target genes for the HIF-α isoforms, inhibition of the individual HIF hydroxylases may be important in modulating specific gene transcriptional activation to mediate a therapeutic effect.[8]

N-Oxalylglycine (NOG, **2**) and its pro-form dimethyl *N*-oxalylglycine (DMOG) have been reported to inhibit the activity of both the PHDs and FIH. Additionally, DMOG has been shown to activate the HIF pathway in animals.[9] However, **2** is also an inhibitor of other 2OG oxygenases, including human enzymes such as pro-collagen prolyl hydroxylase, for which it was first developed as an inhibitor, and phytanoyl Co-A hydroxylase. Hence, it is unlikely to be suitable for medicinal use.

Scheme 1. Reactions Catalyzed by HIF Hydroxylases



Analysis of crystal structures of FIH−Fe(II)−2OG/NOG complexed with fragments of the C-terminal transactivation domain of HIF1-α suggested that it may be possible to inhibit FIH with *N*-oxalyl amino acids derived from amino acids with hydrophobic side chains.[10] We therefore synthesized a set of *N*-oxalyl amino acids via solution and/or solid-phase methodology (Schemes S1 and S2 in the Supporting Information).

The *N*-oxalyl amino acids were assayed as inhibitors of highly purified recombinant FIH and an N-terminally truncated form of PHD2 using fragments of human HIF-1α as substrates. Truncation of the PHD2 was found to be necessary in order to produce soluble highly active enzyme in *Escherichia coli*. The putative inhibitors (**2**−**12**) were initially screened in a fluorescence-based assay that monitors consumption of **1** by its postincubation derivatization with *o*-phenylenediamine.[11] In the case of PHD2, the results indicated that **2** was the most potent inhibitor, and that with the exception of *N*-oxalyl-L-Ala (**3**), the degree of inhibition decreases significantly as the size of the side chain increased. The increased potency of the L- versus that of the D-*N*-oxalyl-Ala derivatives is consistent with results obtained with crude extracts containing endogenous PHD activity[2] and with that of inhibition of pro-collagen prolyl hydroxylases (see Supporting Information for reference).

The results for FIH were strikingly different. For all of the enantiomeric forms of *N*-oxalyl amino acids tested, the D-enantiomer was more potent (Figure 1). Further, although the *N*-oxalyl-D-Val (**6**) and *N*-oxalyl-D-Leu (**8**) derivatives were of a similar order of potency to that of **2**, the *N*-oxalyl-D-Phe (**10**) was significantly more potent. The selectivity of **10** for FIH over PHD2 was confirmed by assays measuring the release of $^{14}CO_2$ from labeled 2OG (**1**). With increasing concentrations of **2**, the activity of both FIH and PHD2 could be entirely inhibited (Figure S1, apparent K_i for **2** versus FIH was 1.2 ± 0.3 mM). However, with **10**, only FIH was affected in the same manner; **10** was not an inhibitor of PHD2 at 1 mM. An apparent K_i of FIH of 83 ± 18 μM for **10** was obtained by an assay employing a HIF-1α786−826 peptide fused with

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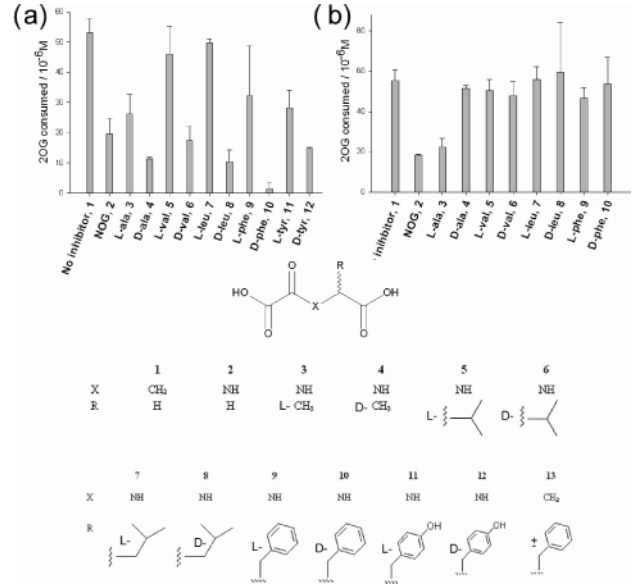

Figure 1. Effect of 1 mM *N*-oxalyl amino acid on the activity of (a) FIH and (b) PHD2 as measured by 2OG (**1**) consumption. For full structures of test compounds, see Supporting Information.

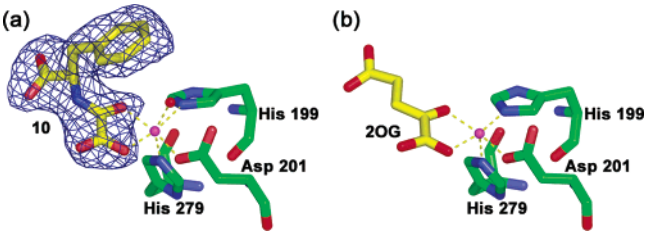

Figure 2. View of the crystal structure of (A) FIH−Fe(II) complexed with **10** (1YCI), (B) FIH−Fe(II) complexed with **1** (1H2N).[10] **10** interacts with iron (pink sphere) via bidentate coordination. Note the poor geometry of the water molecule in (A) completing the hexacoordination of iron along with two histidines, aspartic acid, and **10** due to its steric clash with the phenyl ring of **10**. $|F_o - F_c|$ electron density contoured to 3σ displayed as blue chicken wire representation (PYMOL). The refined structure has an R value of 0.210 and an R_{free} of 0.244.

glutathione-S-transferase as substrate; **10** was a competitive inhibitor with respect to **1**. To examine the mode of binding of **10** at the active site, crystallographic analyses were performed. FIH crystals were grown anaerobically in the presence of Fe(II) and 10 mM **10**, and data were collected to 2.7 Å resolution at a synchrotron radiation source.

N-Oxalyl-D-phenylalanine **10** binds to FIH between the β-sheets of its double-stranded β-helix fold in a manner similar to that observed for **1** and **2**.[10] The oxoacid moiety of **10** binds to the iron(II) with the carbonyl oxygen O2′ trans to Asp201 and its carboxylate oxygen O2 trans to His199 (Figure 2a). The 1′-carboxylate of **10** forms hydrogen bonds to the side chain Nδ2 of Asn205, while its 5′-carboxylate hydrogen bonds to Lys214 Nζ, Tyr145 OH, and Thr196 Oγ1. The phenyl ring of **10** is accommodated into the active site of FIH by forming hydrophobic interactions with Leu186, Leu188, Trp296, and Tyr102. The presence of the phenyl ring results in a slight distortion of the ligand coordination to iron with respect to that of FIH−Fe(II) complexed with 2OG or **2** (1H2L and 1H2N, respectively) (Figure 2b). The vacant coordination site that presumably enables dioxygen binding is partially occupied by the phenyl ring of **10**, which likely also interferes with productive peptide substrate binding. To investigate whether the presence of the phenyl ring in an analogous position in a 2-oxo acid would act as an inhibitor, we then synthesized and tested racemic 4-benzyl-2-oxo-glutarate (**13**). This compound was a less potent inhibitor of FIH than was **2** but did not affect the activity of PHD2 like **10**, nor could it act as a substitute for **1** in FIH assays where peptide hydroxylation was assayed by LC-MS. The decreased potency of **13** can be attributed, in part, to its increased conformational flexibility relative to **10** due to the absence of the amide bond.

The results demonstrate that it is possible to selectively inhibit one of the HIF-α hydroxylases, and so it may help to enable up-regulation of specific genes controlled by the hypoxic response (e.g., EPO or VEGF). More generally, since ester derivatives of *N*-oxalyl amino acids are active as pro-drugs in cell lines and animals, *N*-oxalyl amino acids may be useful for functional assignments of 2OG oxygenases whose biochemical role is unknown, including the JmjC family, some of whom are already known to be involved in transcriptional regulation.[12]

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Supporting Information Available: Details of chemical synthesis, enzymatic assays, crystallographic data collection and refinement. Complete ref 3a. This material is available free of charge via the Internet at http://pubs.acs.org.

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