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2-Oxoglutarate Analogue Inhibitors of HIF Prolyl Hydroxylase

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Abstract—Hydroxylation of hypoxia-inducible factor, a nuclear transcription factor, is catalysed by iron and 2-oxoglutarate dependent hydroxylases. Various analogues of the 2-oxoglutarate cosubstrate were synthesised and shown to inhibit the activity of human hypoxia-inducible factor-1α prolyl hydroxylases in cell-free extracts.

The hypoxic response in higher organisms, which upregulates genes such as those encoding for vascular endothelial growth factor and erythropoietin, is regulated by hypoxia-inducible factor (HIF),[1] an α,β heterodimeric transcription factor. The β-subunit of HIF is constitutively present, but levels of the HIF α-subunit are directly regulated by dioxygen tension.

Under normoxic conditions, the α-subunit of HIF is continuously degraded in a dioxygen dependent process. Prolyl-4-hydroxylase isoforms (PHD1, 2 and 3)[2,3] catalyse the modification of conserved prolines in two oxygen dependent degradation domains (ODDD) in HIF-α.[4–7] Hydroxylation of the prolines mediates recognition of HIF by the von Hippel-Lindau tumour suppressor protein (pVHL)[8,9] resulting in ubiquitinylation of HIF-α and signalling for its destruction in the proteasome (Fig. 1).[10,11]

HIF-α is also modified in an oxygen-dependent manner at an asparagine in its C-terminal transactivation domain (CTAD)[12] in a reaction catalysed by Factor Inhibiting HIF (FIH);[13,14] hydroxylation of this asparagine directly mediates the hypoxic response by blocking the interaction between HIF-α and the nuclear protein p300.[15,16]

Both the HIF prolyl and asparaginyl hydroxylases belong to the superfamily of iron (II) and 2-oxoglutarate (2OG, **1**) dependent oxygenases[17]. Modulation of their activity could promote either anti-angiogenic responses, useful in the treatment of cancer, or pro-angiogenic responses, useful in the treatment of, for example, ischaemic disease.

N-Oxalyl amino acid derivatives, designed to act as analogues of 2OG, were found to inhibit a related Fe(II) and 2OG-dependent enzyme, procollagen prolyl-4-hydroxylase, which was targeted in attempts to generate anti-fibrotic agents.[18] More recently, *N*-oxalylglycine (**2**) and *N*-oxalylalanine have been reported to inhibit both human and *Caenorhabditis elegans* HIF prolyl hydroxylases and the human HIF asparaginyl hydroxylase.[3,19] In whole-cell experiments, esters of the oxalyl amino acid derivatives were more active than the corresponding acids, presumably due to increased membrane permeability; it is likely that the esters are hydrolysed prior to interaction with the HIF hydroxylases. Crystallographic analyses with the human HIF asparaginyl hydroxylase have revealed that **2** binds to iron in a near identical fashion to 2OG in that the 2-oxo and 1-carboxylate groups act as iron ligands.[19] **2** presumably acts as an inhibitor since the presence of an amide hinders nucleophilic attack at its 2-carbonyl. Cyclic hydroxamates, based upon a naturally occurring compound alahopcin, have also been shown to inhibit the PHD.[20] Here we report the preparation of a range of 2OG analogues[4–12] and their analysis as HIF prolyl hydroxylase inhibitors using a cell-free assay system. Inhibitors were generally selected for their potential to chelate the iron in a bidentate fashion and to bind to a basic residue (proposed to be arginine-367 in human PHD1[21]) involved in 2OG binding (Fig. 2).

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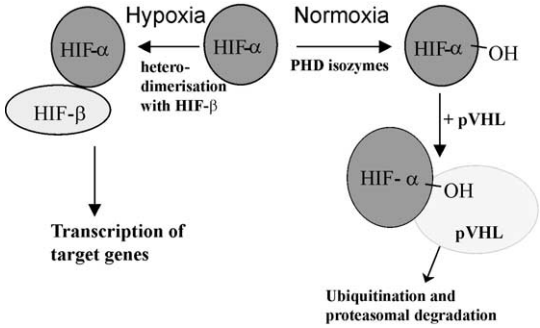


Figure 1. HIF-α is hydroxylated by the PHD isozymes under normoxic conditions and is targeted for proteasomal destruction by pVHL. In the absence of oxygen, HIF-α and HIF-β interact in the nucleus and activate transcription of target genes.

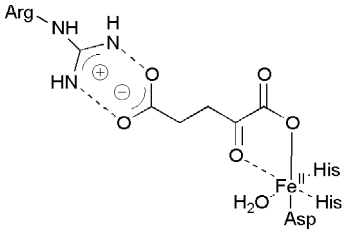
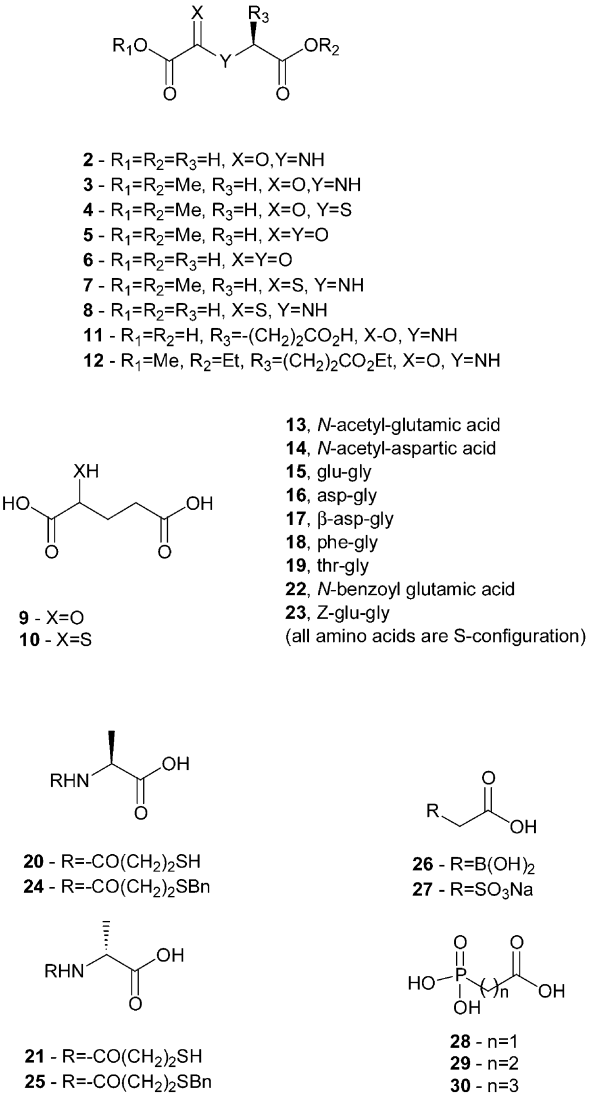


Figure 2. Possible binding of 2OG **1** to the iron(II) and a basic residue in the active site of the PHD enzymes. The arginine may be replaced by other basic residues.

Synthesis of various 2OG analogues was by literature procedures or modifications thereof. Thus, methyl esters of *N*-oxalyl amino acid derivatives were prepared as described by Cunliffe et al.,[22] via reaction of the amino acid with methyl oxalylchloride, followed by saponification (2 N NaOH). The thionoamide analogue of *N*-oxalylglycine (**8**) was obtained by reaction of dimethyl *N*-oxalylglycine with Lawesson's reagent[23] to give (**7**), followed by saponification. (**9**) and (**10**) were made according to Danielli et al.[24] and Battersby et al.,[25] respectively. Enantiomeric thiols (**20**) and (**21**) were prepared via $Na/NH_3(l)$ mediated deprotection of S-benzylated intermediates (**24**) and (**25**), respectively.[26] Boronic acid (**26**) and sulphonate (**27**) analogues were synthesised according to Kinder and Ames[27] and Doi et al.,[28] respectively. Other amino acids and derivatives (Scheme 1) were from commercial sources (**13**–**19** and **22**–**23**, Bachem, **28**–**30**, Sigma-Aldrich).

Inhibition of PHD isozymes present in a crude mammalian renal cell carcinoma extract (RCC4) was assayed as previously reported.[20] A fragment of HIF-1α (residues 549–582), containing the more C-terminal of the two prolyl residues susceptible to hydroxylation, Pro-564, and an N-terminal affinity tag (Gal) that enabled immobilisation of the peptide on agarose beads. Beads were treated with RCC4 cytoplasmic extract (made by lysing cells in 20 mM Tris buffer pH 7.5; 5 mM KCl; 1.5 mM $MgCl_2$; 1 mM dithiothrietol at 4 °C using a Dounce homogeniser, followed by clarification at 13,000g for 15 min) in the presence of 100 μM $FeCl_2$, both in the presence or the absence of candidate inhibitors at 2 mM. The beads were then treated with [^{35}S]-radiolabelled pVHL and washed extensively. The amount of radioactive



Scheme 1. Structures of potential inhibitors assayed.

pVHL captured by the hydroxylated product was analysed by denaturing SDS-PAGE electrophoresis and quantified by autoradiography (Fig. 3). Note that similar levels of activity were observed for **2** and **3** implying the presence of esterase activity in the cell extracts.

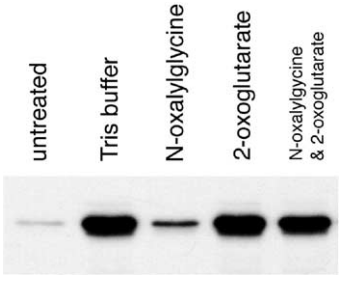


Figure 3. Autoradiograph of an SDS-PAGE gel of an inhibition assay. A decrease in band intensity indicates decreased capture of pVHL, indicative of HIF hydroxylation. The inhibition by NOG was antagonised by additional 2OG. Tris buffer = 50 mM tris(hydroxymethyl)aminomethane.

Results

Initially, we investigated the importance of the amide group in hydroxylase inhibition by **2** and dimethyl oxalylglycine **3**. Substitution of the amide nitrogen in **3** with a sulfur to give thiolester **4**, led to a similar level of activity as for **3** whilst thionoamides **7**/**8** were less active. Substitution of the amide nitrogen with oxygen also led to active compounds **5**/**6**, but the deprotected analogue **6** was less active, possibly reflecting instability (Fig. 4). Inhibitors in which the 2-keto group of 2OG was replaced with a thiol or an alcohol were then tested. As these compounds lack the carbonyl group that reacts with dioxygen they are less likely to undergo nucleophilic attack by an activated dioxygen molecule. There was a clear difference in activity between the (±)-2-hydroxyglutarate **9**, which was barely if at all active, and the (±)-2-mercaptoglutarate **10**, which was active, derivatives of glutaric acid.

Although care should be taken in interpreting the results of these assays using crude extracts, inhibition by thiol **10** is notable, since thiols are present in a number of medicinally significant inhibitors of metalloenzymes. A similar difference in potency between **9** and **10** has been observed for inhibition of AlkB, a 2OG oxygenase involved in DNA repair;[29] (±)-2-mercaptoglutarate had the lowest IC_{50} (0.12 mM) of the 2OG analogues tested; in contrast the C-2 alcohol showed no inhibition up to 4 mM. The difference in potency between alcohol **9** and thiol **10** as inhibitors may reflect the lower pK_a of thiols.

In the hope of generating inhibitors that might bind iron in a tripodal manner, *N*-oxalyl-(*S*)-glutamate (**11**) and its triester derivative (**12**) were tested. Neither of these compounds was significantly active, possibly reflecting largely hydrophobic interactions formed between the enzyme and the methylenes of 2OG. However, various other derivatives of glutamate and aspartate were active, including *N*-acetyl-(*S*)-glutamate (**13**), *N*-acetyl-(*S*)-aspartate (**14**), and dipeptides (*S*)-glu-gly (**15**), (*S*)-asp-gly (**16**) and β-(*S*)-asp-gly (**17**). Due to the spacing of their carboxylates, these compounds can all potentially act as analogues of 2OG and/or succinate. Replacement of the (*S*)-glutamate of **15** with other amino acids [(*S*)-phe-gly (**18**) or (*S*)-thr-gly (**19**)] led to loss of activity. However, moderate inhibition was observed by the *N*-(3-thiopropionyl)alanine enantiomers **20** and **21** (Fig. 5).

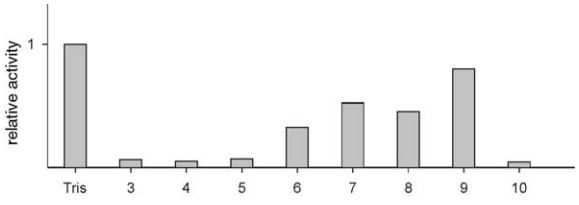


Figure 4. This chart shows the effect of modifying the amide function in compounds **2** and **3**. Numbers on the horizontal axis refer to compounds in Scheme 1.

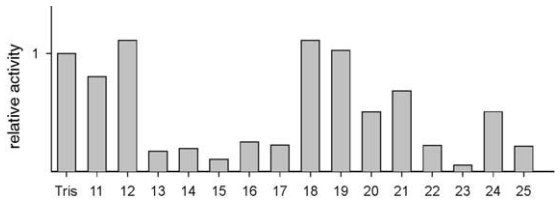


Figure 5. Inhibition of HIF prolyl hydroxylases by amino acids and derivatives. Numbers on the horizontal axis refer to compounds in Scheme 1.

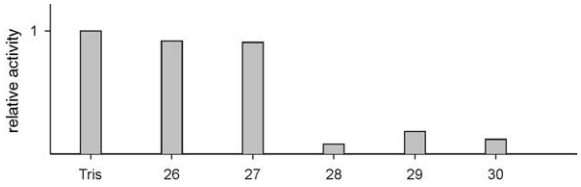


Figure 6. Inhibitory potential of different acids towards the HIF prolyl hydroxylase. Numbers on the horizontal axis refer to compounds in Scheme 1.

The activity displayed by certain derivatives with hydrophobic groups, for example *N*-benzoyl-glutamate (**22**) and Z-(*S*)-glu-gly (**23**), and the *S*-benzyl derivatives of **20** and **21**, (**24**) and (**25**), is interesting as they suggest in the presence of a hydrophobic binding pocket at the active site of the PHD enzymes.

Phosphonic, boronic and sulphonic acid derivatives of carboxylic acids were also analysed. Whilst the boronic (**26**) and sulfonic acid (**27**) derivatives of acetic acid were inactive, the phosphonic acid derivatives of acetic (**28**), propionic (**29**) and butyric acid (**30**), inhibited hydroxylase activity (Fig. 6). Since this work utilised crude cell extracts, it cannot be certain that all the active compounds exert their effect via direct interaction with PHD isoforms. Nonetheless, 2OG analogues may be useful templates from which to build selective and potent inhibitors of the HIF hydroxylases.

The 2OG analogues described here may also inhibit other human oxygenases; however, modifications to the template structures may be useful in the development of selective pro-angiogenetic agents.

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