The inhibition of factor inhibiting hypoxia-inducible factor (FIH) by β-oxocarboxylic acids†

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Cyclic β-oxocarboxylic acids inhibit factor inhibiting hypoxia-inducible factor *via* ligation to the active site iron.

Recent studies have identified a mechanism by which the hypoxic response in metazoans is regulated by the availability of molecular oxygen.[1] Post-translational hydroxylation of either of two conserved proline residues in the α-subunit of an αβ-transcription factor named hypoxia-inducible factor (HIF) (Fig. 1), enables binding of HIF-α to the von Hippel-Lindau protein (pVHL).[2] The latter acts as a targeting protein for a ubiquitin E3 ligase complex that enables proteasomal destruction of HIF-α. The dependence of HIF-α prolyl-4-hydroxylation on oxygen provides a link between hypoxia and levels of HIF-α[3] The transcriptional activity of human HIF-α is also inhibited by hydroxylation at the *pro-S* β-position of Asn-803 in one of its transcriptional activation domains (Fig. 1).[4]

The hydroxylases comprise three prolyl-4-hydroxylases (PHDs1-3)[3,5] and an asparaginyl-hydroxylase, factor inhibiting HIF (FIH).[6] Inhibition of the HIF hydroxylases is of current interest from the perspective of upregulating hypoxically driven transcripts for the treatment of ischemia and anaemia. Sequence and activity analyses, coupled to crystallographic data for FIH, have revealed that all of the HIF hydroxylases belong to the family of ferrous iron and 2-oxoglutarate (2OG) dependent oxygenases (Fig. 2). Spectroscopic and crystallographic analyses have revealed that many of these enzymes, including FIH, bind their iron cofactor *via* a conserved 2His–1Asp/Glu triad of residues.[7]

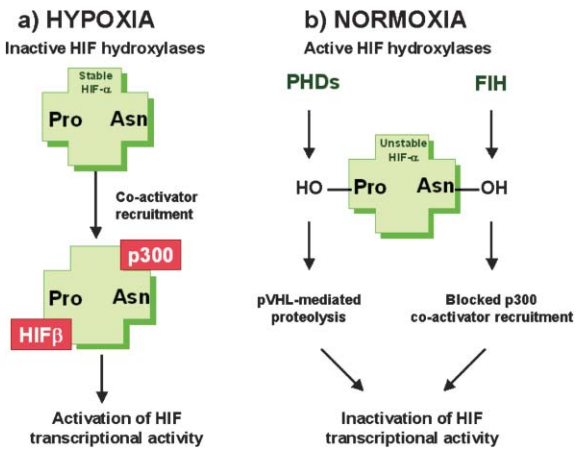

Fig. 1 (a) Role of post-translational hydroxylation in the hypoxic response; (b) the reactions catalysed by the HIF prolyl-4-hydroxylases (PHDs 1–3) and the HIF asparaginyl-hydroxylase (factor inhibiting HIF, FIH).

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The hypoxic response can be induced in cells by reagents including cobaltous ions and non-specific iron chelators (*e.g.* desferrioxamine);[2] these reagents may effect their activity by competing with iron (cobaltous ions) or depriving the HIF hydroxylases of ferrous iron (desferrioxamine), though other mechanisms are possible. Prior to the identification of the role of prolyl-hydroxylation in the hypoxic response, procollagen prolyl-hydroxylase had been targeted for therapeutic intervention owing to its role in diseases associated with an over proliferation of fibrotic tissue. This work led to the identification of procollagen prolyl-hydroxylase inhibitors some of which mimic the 2OG cosubstrate.[8] *N*-Oxalylglycine inhibits both procollagen prolyl-hydroxylase and the HIF hydroxylases and has been shown to bind to the active site iron of FIH in an analogous manner to 2-oxoglutarate,[6] as do derivatives of *N*-oxalylglycine which have been recently reported to enable selective inhibition of FIH over the PHDs.[9] 4-Oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (**4a**), another compound first identified as a procollagen prolyl-hydroxylase inhibitor[5,10] has also been reported as an inhibitor of the PHDs both with purified enzyme (apparent K_i for PHD2: 10 μM;[11] IC_{50} for procollagen prolyl-hydroxylase: 2 μM[5]) and in cells. In addition to possible inhibition of the HIF hydroxylases by chelation of iron in solution, **4a** has the potential to ligate to the active site iron *via* its 1,10-phenanthroline group

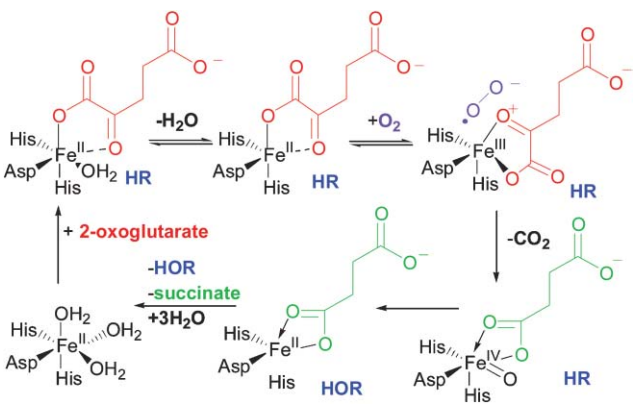


Fig. 2 Outline mechanism for the 2-oxoglutarate dependent oxygenases.

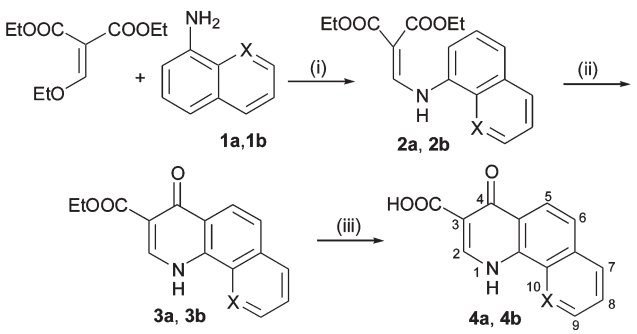
and/or *via* its 4-oxo-3-carboxylic acid group. Here we report a study on the mechanism of action of **4a**, and related compounds, which reveals that **4a** inhibits both FIH and PHD2 and likely binds to the active site iron *via* its 4-oxo-3-carboxylic acid group.

4a was synthesised *via* bicyclic (**2a**) and tricyclic compounds (**3a**) as reported[12] (Scheme 1) and analysed as an inhibitor of a highly purified *N*-terminally truncated form of PHD2 (ntPHD2) and FIH.[9] Consistent with the literature reports using crude full length PHD2, **4a** was found to inhibit ntPHD2. However, it was also found to inhibit FIH with an IC_{50} of 60 μM, in a fluorescence assay based on derivatisation of 2OG.[13] Thus, **4a** is unlikely to be a highly selective inhibitor of the PHDs and its hypoxic activation in cell lines may be the result of both PHD and FIH inhibition. Soft ionisation mass spectrometric studies (see supplementary information)† demonstrated that **4a** bound directly to the FIH-Fe(II) complex as well as to the ntPHD2-Fe(II) complex. Interestingly 1,10-phenanthroline itself also formed an analogous 1 : 1 complex with ntPHD2-Fe(II) but under the conditions used 1,10-phenanthroline was not seen to bind FIH. Together, with the tight binding constant of PHD2 for ferrous iron (<1 μM), it is thus concluded that at least part of the inhibitory effect of **4a** in cells arises from binding at the active site of the HIF hydroxylases rather than chelation of iron in solution.

To investigate the possible binding modes of **4a** to a HIF hydroxylase it was docked (Autodock 3.0)[14] into the active site of FIH using the crystal structure of the FIH·Fe(II)·2OG·HIF fragment complex[7,15] with all heteroatoms except for Fe(II) removed from the coordinate file. Despite the presence of the *o*-phenanthroline moiety, an Fe(II) chelator, in **4a**, the studies predicted bidentate iron ligation by **4a** *via* its 4-oxo-3-carboxylic acid moiety with its carboxylate better positioned for coordination (Fig. 3).

In the binding mode observed, the aromatic rings of **4a** appear well suited to fit in a predominantly hydrophobic pocket of FIH formed by residues Tyr-93, Tyr-145, Gln-147, Leu-188, Leu-186, Tyr-102, Phe-100 and Thr-196 (Fig. 3). Significantly, the HIF substrate does not interact with most of these residues in the reported crystal structures, suggesting that this pocket may be further exploited in inhibitor design.

The studies also predicted that analogues of **4a**, lacking one nitrogen (**4b**) or one pyridine ring (**8a**) could inhibit FIH (Fig. 4); if a binding mode in which the phenanthroline nitrogens ligate to the iron had been observed, it would be expected that they would not be good inhibitors. To test this prediction, **4b** and **8a** were synthesised.



Scheme 1 *Reagents and conditions:* (i) heat, 1 h, 80–85%; (ii) Ph_2O, reflux, 1–1.5 h, 80%; (iii) 10% KOH, reflux, 0.5–1 h, 95%. X = N for **1a**, **2a**, **3a** and **4a** and CH for **1b**, **2b**, **3b** and **4b**.

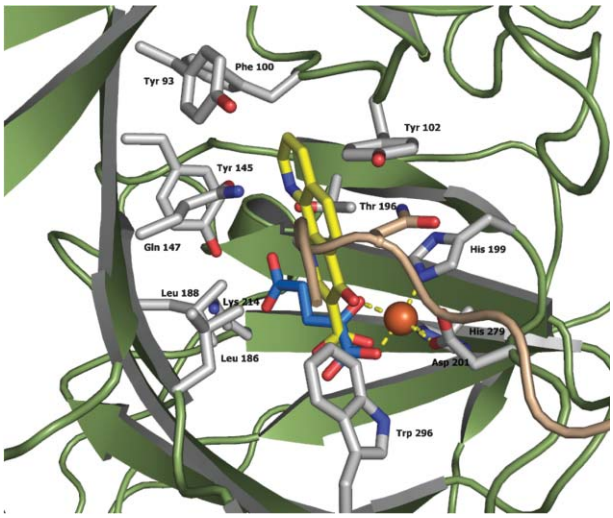


Fig. 3 The structure of FIH complexed with Fe(II) (orange), HIF substrate (beige), and 2OG (blue). Selected side chains are shown as grey stick representation. The docked inhibitor, **4a** (yellow), is shown to display its predicted binding mode.

Diethylethoxymethylene malonate was coupled with 1-aminonaphthaline (**1b**) or aniline (**5a**) to give intermediates **2b** or **6a**, respectively; which were then heated (Ph_2O reflux) to yield the corresponding cyclic compounds **3b** or **7a**. Alkaline hydrolysis of **3b** or **7a** yielded **4b** or **8a** in near quantitative yields (Schemes 1 and 2).

4b and **8a** inhibit FIH with IC_{50} values of 170 μM and 180 μM, respectively. Although these values are lower than the value obtained for **4a**, they indicate a significant degree of inhibition by **4b** and **8a**, which supports the observed ligation of **4a** to the active site Fe(II) of FIH *via* its 4-oxo-3-carboxylic acid moiety rather than its phenanthroline nitrogens. The lower activity of **4b** *versus* **4a** may reflect the effect of the N-10 on the ligation ability of the oxocarboxylic acid or the better ability of **4a** to chelate iron in solution.

We then examined the utility of the quinoline **8** as a template for further modification for FIH inhibition. C-7 of the phenyl ring in **8** was chosen for derivatisation in the hope of obtaining hydrophobic interactions with Tyr-93 and Phe-100 at the rear part of the pocket proposed to bind **4a** as shown for compound **8a** in Fig. 4.

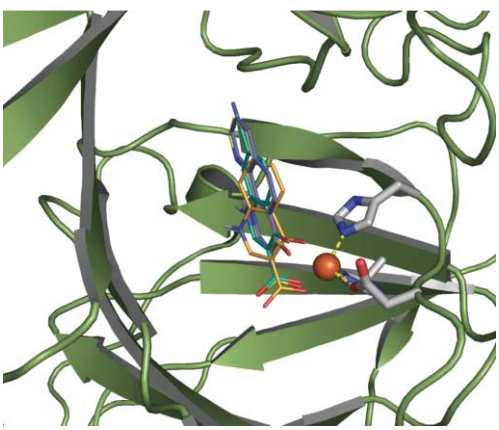
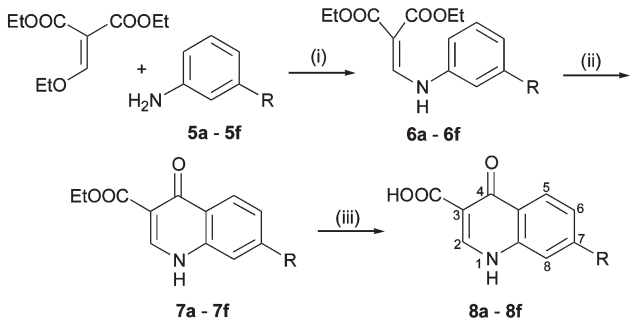


Fig. 4 Superposition of the docked conformations of **4a** (yellow), **4b** (purple), **8a** (gold) and **8f** (cyan) in the active site of FIH. Iron binding ligands are shown in grey stick representation and iron is represented by an orange sphere.



Scheme 2 *Reagents and conditions:* (i) heat, 1 h, 80–85%; (ii) Ph_2O, reflux, 1–1.5 h, 70–75%; (iii) 10% KOH, reflux, 0.5–1 h, 95%. R = H for **5a–8a**; NHCOMe for **5b–7b**; NH_2 for **8b**; NHCOPh for **5c–8c**; CONHPh for **5d–8d**; CONHBn for **5e–8e** and CN for **5f–8f**.

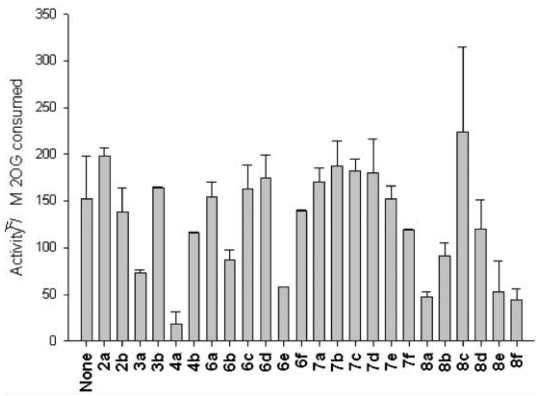


Fig. 5 The effect of 1 mM test compounds **2–4** and **6–8** on the activity of FIH.

The 4-oxo-7-substituted-quinoline-3-carboxylic acid derivatives were synthesized following the same general route (Scheme 2) employing an addition–elimination reaction of diethylethoxymethylene malonate with aniline derivatives (for their syntheses see supplementary information)† to give enamines (**6b** to **6f**) followed by cyclisation to the corresponding quinolines (**7b** to **7f**) and saponification to give acids **8b** to **8f**. In the hydrolysis of **7f**, a trace amount of di-acid was also observed resulting from the hydrolysis of the nitrile group. Compounds **6** to **8** were then screened alongside **4** in assays against FIH (Fig. 5) using a fluorescence based assay.[13]

As expected, the ester derivatives **3** and **7** displayed little activity against FIH, as in general did the diesters (**2** and **6**); the low levels of activity observed for some of these compounds (*e.g.* **6b** and **6e**) may have resulted from hydrolysis under the incubation conditions. For all the 7-substituted quinoline-3-carboxylic acids (**8b** to **8f**), only the cyanide **8f** showed a better activity than the 7-unsubstituted quinoline **8a**, with an IC_{50} of 150 μM.

Docking studies revealed that, as for **4a**, **4b** and **8a**, only one binding mode was found for **8f** (Fig. 4). In contrast, similar analysis for compounds **8c**, **8d** and **8e** implied more than one binding mode; most of which predict binding overlapping with the site occupied by Val-802, Asn-803 and Ala-804 of the HIF substrate (see supplementary information).† The reduced activity for **8c** and **8d** may thus reflect competition with the HIF-α substrate used in the assays (as well as with 2OG). The flexibility of the *N*-benzylamide **8e** may allow it to avoid HIF by conforming to the hydrophobic pocket described earlier, as a consequence possibly increasing its inhibitory activity.

In conclusion, the results indicate that **4a** binds the active site metal of the HIF hydroxylase (at least for FIH) *via* its oxo-carboxylate functionality rather than its bipyridyl group. They also indicate that the tricyclic ring system can be reduced in size to achieve a quinoline structure, suitable for further modification with a view to obtaining selective inhibitors of individual HIF hydroxylases.

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