

# Evaluation of aspirin metabolites as inhibitors of hypoxia-inducible factor hydroxylases†

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Known and potential aspirin metabolites were evaluated as inhibitors of oxygen-sensing hypoxia-inducible transcription factor (HIF) hydroxylases; some of the metabolites were found to stabilise HIF- $\alpha$  in cells.

The  $\alpha$ , $\beta$ -hypoxia-inducible transcription factor (HIF) regulates the transcription of a gene array whose products work to counteract the effects of hypoxia and establish oxygen homeostasis in animals. Prolyl-4-hydroxylation signals for HIF- $\alpha$  degradation, and asparaginyl-3-hydroxylation reduces the interaction between HIF- $\alpha$  and a transcriptional co-activator. In the presence of oxygen, the hydroxylation of human HIF- $\alpha$  is catalysed by 2-oxoglutarate- (2OG, **1**; Fig. 1) dependent Pro-hydroxylases (PHD1–3) and an Asn-hydroxylase (FIH). The reduction of HIF hydroxylase activity in hypoxia causes the accumulation of transcriptionally active HIF- $\alpha$ , so enabling the hypoxic response. PHD2 has been shown to be the most important HIF hydroxylase for down-regulating the hypoxic response during normoxia in healthy cells.<sup>1–3</sup>

During our efforts to identify HIF hydroxylase inhibitors, we considered known and potential metabolites of acetyl salicylic acid (aspirin, **2**; Fig. 1). As well as being an anti-inflammatory, **2** is used for the prevention of heart disease and cerebral thrombosis.<sup>4</sup> Metabolites of **2** and salicylic acid (**3**; Fig. 1) may also have biological activity; 2,3-dihydroxybenzoic acid (**4**; Fig. 1), a known siderophore,<sup>5</sup> and 2,5-dihydroxybenzoic acid (**5**; Fig. 1) and the potential metabolite 2,6-dihydroxybenzoic acid (**6**; Fig. 1) are proposed to impact on a variety of biochemical systems.<sup>6–8</sup>

Hippuric acid (**7**; Fig. 1) and salicyluric acid (**8**; Fig. 1) are known major metabolites of aspirin. Related glycine derivatives of hydroxyaryl compounds, such as **9–11** (Fig. 1), are reported to be collagen prolyl-4-hydroxylase (C-P4H) and PHD2 inhibitors.<sup>9,10</sup> Benzoic acid (**12**; Fig. 1) can be metabolised *via* the benzoate degradation/hydroxylation pathway into 3,4-dihydroxybenzoic acid (**13**; ESI, Fig. S1†), which is a

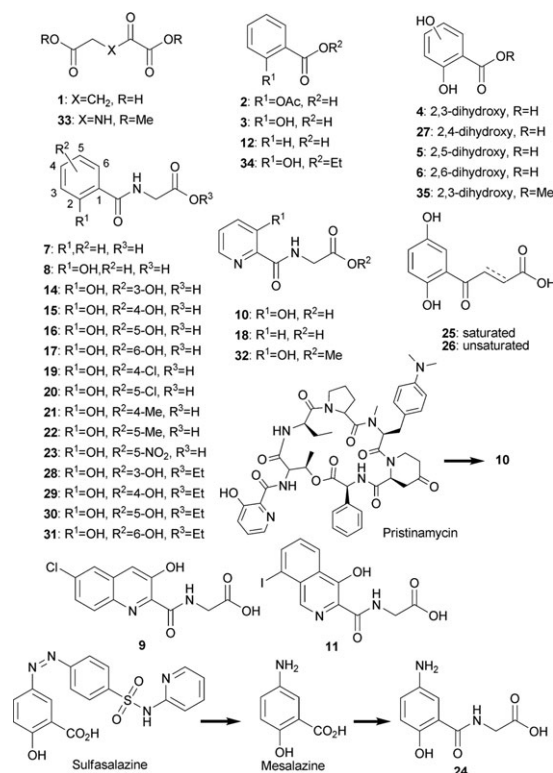


Fig. 1 Potential inhibitors and related compounds used in the study.

C-P4H inhibitor.<sup>9</sup> **3** and **12** are present in the human diet, and can be metabolised into **4–6** under inflammatory conditions (ESI, Fig. S1†).<sup>11</sup> Aspirin metabolite **4** occurs in humans *via* tryptophan metabolism.<sup>12</sup>

Derivatives of **2** and **3** were synthesised (see ESI†) to test the possibility that they might be HIF hydroxylase inhibitors (**8**, **10** and **14–26**; Fig. 1). **10** and **24** were of interest, as **10** is a metabolite of depsipeptide antibiotic pristnamycin P1,<sup>13,14</sup> and **24** is a metabolite of anti-inflammatory compounds mesalazine and sulfasalazine.

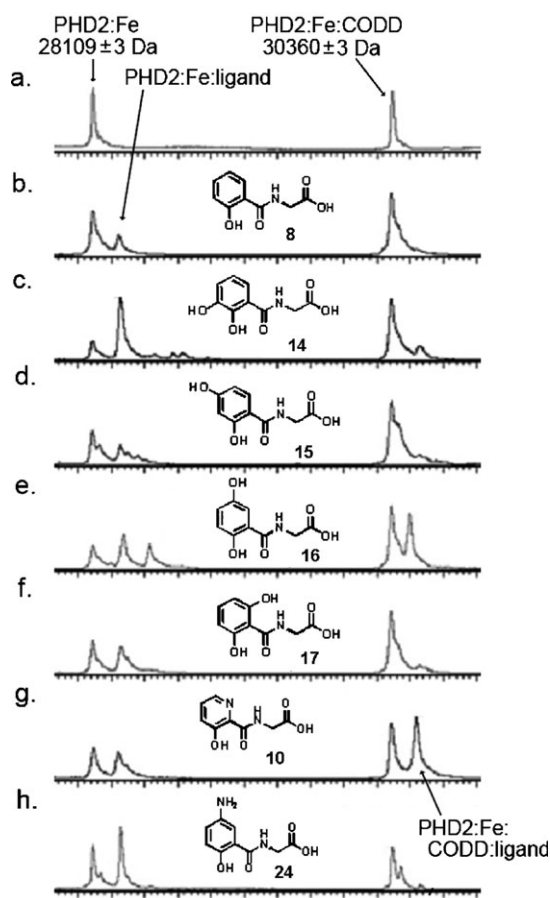
Using a non-denaturing electrospray ionisation mass spectrometry (ESI-MS) assay (Fig. 2), **3–6**, **8**, **10** and **14–27**, but not their corresponding esters or compounds without the aromatic  $\alpha$ -hydroxyl group, were found to bind to tPHD2-Fe (with different affinities) and to compete for binding with 2OG; aspirin itself was not observed to bind in this assay (tPHD2 is a truncated form of PHD2 that can be efficiently produced in a soluble form).<sup>15</sup> In the presence of a 19-residue peptide substrate representing the HIF-1 $\alpha$  C-terminal oxygen-dependent

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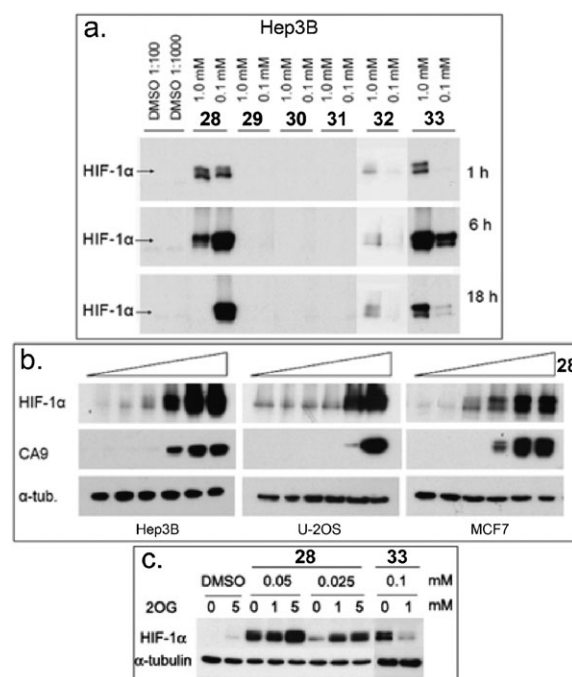


**Fig. 2** Deconvoluted ESI-MS spectra showing the results of the incubation of tPHD2-Fe with an equimolar amount of **8**, **10**, **14**–**18** or **24** after <5 min incubation at 23 °C and sample cone voltage = 80 V.

degradation domain, which includes a prolyl hydroxylation site (CODD, residues 556–574), only **10** and **16** were observed to form significant amounts of a tPHD2-Fe-CODD-inhibitor complex (ESI, Fig. S2†). Overall, the MS results were highly reproducible (except for **16**, which appeared to undergo partial reaction) and indicate that: (i) the glycine side-chain was not required for binding of the tested compounds to the tPHD2-Fe complex, but that its presence increased the affinity, (ii) compounds without the  $\alpha$ -hydroxyl and glycyl groups did not form complexes, (iii) the hydroxyl group stabilised complex formation (compare **8** and **14**–**17** in Fig. 2), and (iv) the mesalazine metabolite, **24**, could form a complex with tPHD2-Fe (Fig. 2h).

2OG turnover assays, without and with pre-incubation, revealed that **10** and **14** (~95% at 1 mM) inhibited tPHD2 under both conditions (ESI, Fig. S3†). In contrast, only **14** (~90% at 1 mM) displayed significant inhibition under the standard assay conditions with FIH. IC<sub>50</sub> values for tPHD2 inhibition were determined for the reported<sup>9</sup> inhibitors, **10** (113 ± 1.2  $\mu$ M) and **14** (27.4 ± 1.3  $\mu$ M), using a fluorescence-based assay (ESI, Fig. S8†).<sup>16</sup>

To test the ability of the aspirin derivatives to inhibit HIF hydroxylases in cells, an ethyl ester derivative, **28**, of **14** that was predicted to be cell-penetrating was prepared, as were other arylglycinate esters (**29**–**32**; Fig. 1). Ester **28** displayed



**Fig. 3** HIF-1 $\alpha$  stabilisation monitored by immunoblot staining SDS-PAGE analysis. (a) The effect of incubating **28**–**33** with Hep3B cell lines on HIF-1 $\alpha$  levels after 1, 6 and 18 h. (b) The dose-dependence of the activity of **28** (from left to right: 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2 mM) in Hep3B, U-2OS and MCF7 cell lines (18 h incubation); levels of the HIF target gene CA9 were monitored as a measure of transcriptional activation. (c) The effect of varying the 2OG concentration (or DMSO as a control) on **28**- and **33**-mediated HIF-1 $\alpha$  stabilisation.

only very low levels of activity (IC<sub>50</sub> > 1 mM) in assays with isolated tPHD2; the observed low activity may have been due to partial ester hydrolysis under the incubation conditions. **28** and **32** induced HIF-1 $\alpha$  stabilisation in human Hep3B cell lines in a concentration-dependent manner, whereas **29**–**31** had no effect (Fig. 3a). These results are consistent with the inhibition of tPHD2 by **10**<sup>9</sup> and **14** observed in *in vitro* experiments. Interestingly, **28** was more potent in cells than **32** or dimethylxalylglycine (**33**, Fig. 1) in inducing HIF-1 $\alpha$  stabilisation, and maintained a notably high level of HIF-1 $\alpha$ , even after 18 h when used at 100  $\mu$ M. Up-regulation of HIF target genes was observed for **28** above 50  $\mu$ M in different cell lines (Fig. 3b), consistent with the determined IC<sub>50</sub> value for **14** (the acid analog of **28**). The effect of variation in 2OG concentration on the activity of **28** and **33** is notable because 2OG was observed to reduce the activity of **33**, but not **28** (Fig. 3c). Although the reason for this difference is unclear, the *in vivo* mechanism of action may be more complex than simple enzyme inhibition. Despite not being identified as a PHD inhibitor *in vitro*, **30** caused HIF-1 $\alpha$  accumulation (ESI, Fig. S4d†). It cannot be ruled out that the compounds were metabolised to other inhibitors,<sup>11,12</sup> that there were other inhibition modes, or that transport effects account for differences in potency.

It was considered that if **14** was an aspirin metabolite, it might be possible to observe some HIF-1 $\alpha$  accumulation by incubating the Hep3B cell lines with aspirin. HIF-1 $\alpha$

accumulation was not observed when Hep3B cell lines were incubated with 0.1–5 mM of **34**, **35**, **2** or **3** (ESI, Fig. S4a–c†), suggesting that **3** (or **34**) and **2** were not significantly metabolised into **14** or other species that caused HIF-1 $\alpha$  stabilisation under the analysed conditions. In contrast, **35** stabilised HIF-1 $\alpha$  under these conditions (ESI, Fig. S4c†).

We then carried out preliminary investigations into whether aspirin could be metabolised into a HIF hydroxylase inhibitor in a human. Aspirin (1 g, single dose) was ingested on two occasions by one of us (B. M. L.), with urine analyses by LC-MS (ESI, Fig. S5–S7†). Together with experiments in which synthetic derivatives were mixed with the biological samples and high resolution MS analyses (<10 ppm mass accuracy), the results revealed that: (i) **3** was only observed in urine after aspirin ingestion (ESI, Fig. S6c and d†), (ii) a significant amount of **7** was present in the urine (ESI, Fig. S6e and f†) and (iii) **8**, reported earlier to be a major aspirin metabolite,<sup>17</sup> was observed only after aspirin intake (ESI, Fig. S6g and h†). Molecules with a mass of 211 Da were consistently present in the urine almost exclusively after aspirin ingestion (ESI, Fig. S7b and c†; a low level of a peak corresponding to **16** was observed without aspirin ingestion), suggesting the formation of dihydroxybenzoylglycine derivatives (e.g. **14**–**17**) via aspirin metabolism. One of these peaks (retention time 2.21 min; ESI, Fig. S7c†) was assigned to **14** on the basis of a comparison with standards, accurate mass and fragmentation data (ESI, Fig. S7c and d†). Peaks (ESI, Fig. S7c†) were also assigned to **16** (2.60 min) and **15** (3.18 min) (MS accuracy <15 ppm). **17** was not observed, consistent with prior work.<sup>8</sup> Although these analyses are preliminary, and the estimated concentration of **14** in the urine in the two analyses varied (~0.05  $\mu$ M and ~0.5  $\mu$ M), they provide evidence for the metabolism of aspirin into **14**.

Several endogenous or diet/drug-derived small organic molecules have been proposed to act as HIF hydroxylase inhibitors, including succinate, fumarate, hydralazine (used as a vasodilator) and flavonoids.<sup>15,18–20</sup> Our data reveal the possibility that aspirin metabolites affect the HIF system via hydroxylase inhibition. Interestingly, aspirin has recently been shown to specifically up-regulate the expression of uPAR (urokinase-type plasminogen activator receptor),<sup>21</sup> which is also strongly induced in a HIF-1 $\alpha$ -dependent manner by hypoxia and the generic 2OG oxygenase inhibitor, **33** (see ESI†).

Analyses of the structures of other pharmaceuticals, including those of mesalazine, sulfasalazine and pristnamycin, suggest that they could give rise to metabolites that are HIF hydroxylase inhibitors. There is no evidence as yet that the concentrations of the relevant metabolites will reach high enough levels to induce physiological effects, nor whether the observed HIF- $\alpha$  induction in cells (Fig. 3) is solely due to

hydroxylase inhibition. However, the possibility that this occurs should be considered, especially for pharmaceuticals like aspirin that are taken on a long term basis or in high doses, and whose physiological effects are mediated via incompletely understood mechanisms.<sup>22</sup> It is possible that the prophylactic properties of aspirin are partly related to the ability of its metabolites to impact on the HIF system, though it is important to state that the present study provides no direct evidence for this proposal.

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