Structural insights into human heme oxygenase-1 inhibition by potent and selectiveazole-based compounds

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The development of heme oxygenase (HO) inhibitors, especially those that are isozyme-selective, promises powerful pharmacological tools to elucidate the regulatory characteristics of the HO system. It is already known that HO has cytoprotective properties and may play a role in several disease states, making it an enticing therapeutic target. Traditionally, the metalloporphins have been used as competitive HO inhibitors owing to their structural similarity with the substrate, heme. However, given heme’s important role in several other proteins (e.g. cytochromes P450, nitric oxide synthase), non-selectivity is an unfortunate side-effect. Reports that azalanstat and other non-porphyrin molecules inhibited HO led to a multi-faceted effort to develop novel compounds as potent, selective inhibitors of HO. This resulted in the creation of non-competitive inhibitors with selectivity for HO, including a subset with isozyme selectivity for HO-1. Using X-ray crystallography, the structures of several complexes of HO-1 with novel inhibitors have been elucidated, which provided insightful information regarding the salient features required for inhibitor binding. This included the structural basis for non-competitive inhibition, flexibility and adaptability of the inhibitor binding pocket, and multiple, potential interaction subsites, all of which can be exploited in future drug-design strategies.

1. Introduction

The heme oxygenase (HO) system comprises two active isozymes, HO-1 and HO-2, and is responsible for the regioselective, oxidative cleavage of heme at the alpha-meso carbon. The degradation of heme produces equimolar amounts of carbon monoxide (CO), ferrous iron (Fe2+) and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (figure 1) [1–4]. While these products were originally viewed as ‘waste’ products, increasing evidence has shown that all three are biologically active, and have contributing as well as complementary roles to provide significant cytoprotection (reviewed in [5]). Studies in knock-out mice have shown that HO-1 deficiency is characterized by intrauterine mortality and chronic inflammation; over 95 per cent of HO-1−/− knock-out mice die in utero [6,7]. In the only two human cases of HO-1 deficiency reported to date [8,9], numerous anomalies were observed, including hemolysis, inflammation, nephritis, asplenia and early death [10]. Thus, HO-1 appears to play a critical role in normal cellular function in both laboratory animals and humans, largely due to conversion of a toxic molecule, heme, to cytoprotective molecules. The pro-oxidative, pro-inflammatory effects of excess free heme, which lead to fibrotic events, can be countered by its degradation by the HO system as well as the cytoprotective and anti-inflammatory effects of its by-products—namely CO, biliverdin (bilirubin) and Fe2+—making them novel targets to alleviate tissue inflammation, oxidative stress and fibrosis (reviewed in [11]).

Endogenously formed CO, of which the HO system produces approximately 85 per cent, has been shown to be an important gasotransmitter, with a regulatory role in a variety of cellular functions, including anti-inflammatory,
antiapoptotic, antiproliferative, as well as vasodilatory effects [12–15]. Many of these activities contribute to the cytoprotective characteristics of HO. In many cases, the mechanisms underlying these effects involve an increase in the activity of a pathway such as: synthesis of cyclic guanosine monophosphate via activation of soluble guanylyl cyclase (sGC) [16,17], stimulation of calcium-dependent potassium channels [18] and activation of mitogen-activated protein kinase signalling pathways [19–22]. In other instances, CO may be inhibitory through its interaction with a heme moiety, as has been reported for haemoglobin, myoglobin, prostaglandin endoperoxide synthase, nitric oxide synthase (NOS), catalase, peroxidases, respiratory burst oxidase, pyrrolases, cytochrome c oxidase, cytochrome P450 and tryptophan dioxygenase. This is further complicated by cross-talk between the NOS and HO systems via a common interaction of nitric oxide (NO) and CO with sGC [22].

In keeping with the cytoprotective role of HO, both bilirubin and its proximal product, bilirubin, have antioxidant properties, and are important scavengers for free radicals, such as superoxide, peroxides, hydroxides, hypochlorous acid, singlet oxygen, nitroxides and peroxynitrite [23–27]. Although seemingly counterintuitive, free iron, which promotes production of intracellular reactive oxygen species (ROS) [28], ultimately triggers the activation of redox-sensitive signalling pathways to result in cytoprotective benefits with respect to inflammation, mitochondrial biogenesis, apoptosis and cell survival [29–31]. Moreover, the increase in free intracellular iron via heme degradation results in an augmentation of synthesis of ferritin, a protein involved in iron sequestration [32,33]. Indeed, the binding of free iron to the cytoplasmic ‘iron-sensing’ RNA-binding proteins, iron-regulatory protein-1 and -2 (IRP1 and IRP2), causes the coordination of events to modify mRNA stability, through binding to iron-regulatory elements of proteins such as H- and L-ferritin, transferrin receptor 1, and ferroportin1, all of which are critical for iron processing and trafficking [34,35].

1.1. Heme oxygenase in disease: important, yet ambiguous and conflicting, roles

The protective role of the HO/CO system has been reported in several disease conditions, including diabetes, heart disease, hypertension, neurological disorders (Alzheimer’s disease) and endotoxemia as well as organ transplantation, fibrosis and inflammation [11,36,37]. There have also been some studies that support a protective role of HO-1 against the development of some types of cancers, i.e. breast and lymph node, which may also involve its role in protection against oxidative stress [38,39]. By contrast, the observation of substantial HO-1 activity in a variety cancers, including pancreatic and prostate, is consistent with the idea that the cytoprotective properties of HO can be disadvantageous for the host through conferring an advantage to the tumour by contributing to cellular resistance to standard cancer treatments. In this type of situation, HO activity becomes a potential therapeutic target for selective HO inhibitors. Indeed, HO-1 activity has been found to be upregulated in response to several therapeutic treatments and implicated in promoting tumour growth; its inhibition has been found to increase responsiveness of pancreatic cancer cells to chemo- and radiotherapy [40–43]. Further implications can be made regarding tumour angiogenesis and subsequent tumour growth as HO-1-derived CO has also been associated with angiogenesis, inducing vascular endothelial growth factor (VEGF) synthesis [44,45], and stimulating the proliferation of endothelial cells [46,47], as well as being implicated in the promotion of angiogenesis by stromal-cell-derived factor 1 [48]. There are also conflicting reports regarding the role HO-1 may have in neuronal cells; its overexpression has been implicated in protection from oxidative injury, while lack of or inhibition of HO-1 activity may attenuate neuronal death (reviewed in [37]). Furthermore, studies have shown that gene deletion of HO-2, which predominates in the brain, is cytoprotective. Thus, the precise role of the HO/CO system in neuronal complications, particularly that of HO-1, though important, is still not clear.

1.2. Heme oxygenase inhibitors

The development of isozyme-selective HO inhibitors would serve as powerful pharmacological tools to dissect the HO/CO system, especially in clarifying situations in which conflicting observations have been reported such as in neuronal and cancer cells, as well as the mechanisms underlying its physiological effects in conjunction with specific roles for each isozyme. Moreover, this avenue may provide novel therapeutic strategies in dealing with various disease states. For example, in the treatment of hemolytic disease such as hyperbilirubinemia, in which the Hmox1 gene is significantly induced, it may be beneficial to inhibit the inducible HO-1 isozyme selectively without any deleterious effect to the ‘housekeeping’ isozyme, HO-2 [49]. Inhibition of HO-1, using pegylated zinc protoporphyrin, has already been associated with anti-tumour activity [50] and has been shown to enhance the effects of contemporary chemotherapeutic agents, particularly those that generate ROS [51]. Traditionally, the metalloporphyrins (e.g. tin/zinc/chromium protoporphyrin) have been used as inhibitors in this field, on the basis of their structure being similar to the heme moiety, which makes for useful competitive inhibitors for HOs. Interestingly, a recent comprehensive study looked at the ability of a variety of metalloporphyrins (i.e. iron/zinc/tin/chromium bis glycol/deuteron/meso protoporphyrin) to inhibit the HO isozymes [49]. Tin mesoporphyrin was found to be the most potent inhibitor for both isozymes, with HO-2 selectivity being greatest for tin protoporphyrin while zinc compounds were the least inhibitory towards HO-2. However, none of the metalloporphyrins tested were selective for HO-1.

Figure 1. The oxidative degradation of heme in the heme oxygenase/carbon monoxide (HO/CO) pathway results in the release of equimolar amounts of carbon monoxide, ferrous iron and biliverdin, the latter of which is converted to bilirubin by biliverdin reductase.

\[ \text{Heme} \rightarrow \text{CO} + \text{Fe}^{2+} + \text{biliverdin} \]
Unfortunately, the strong inhibitory efficacy of the metalloporphyrins, due to their structural similarity to heme, is marred by undesired side-effects, notably non-selectivity, given the significant role heme has in a number of physiologically relevant enzymes such as cytochrome P450, NOS and sGC. As such, there have been concerns raised regarding the validity of interpretations and conclusions derived from studies using these compounds [52–54]. However, it has also been demonstrated that some of these metalloporphyrins, when used over a specific concentration range, do maintain selectivity against HO in vitro; notably, chromium mesoporphyrin IX is the most useful of the metalloporphyrins, selectively inhibiting HO but not NOS or sGC at concentrations restricted to 5 μM or less [55]. Thus, there is a recognizable and urgent need to cultivate isozyme-selective HO inhibitors in order to develop pharmacological tools and explore novel therapeutics in the HO field. One of the means to explore this potential is to look at the differences between the three-dimensional structures of the isozymes, which can be exploited in this regard.

1.3. Structural conservation of heme oxygenase isozymes

The HO system comprises two active isozymes [4]. HO-1 is an approximately 32 kDa protein that is predominantly expressed in the spleen and can be induced by a variety of stimuli, including heat-shock, heavy metals, heme and ROS. Thus, it is known as the inducible HO. By contrast, HO-2, the constitutive enzyme, is an approximately 36.5 kDa protein that has a wider distribution in the body, but with its highest concentration being in the brain and testes. A third isozyme, HO-3, has been demarcated as a pseudogene and is inactive, despite sharing approximately 90 per cent sequence identity with HO-2 [56]. Sequence alignment between HO-1 and HO-2 shows 45 per cent identity between the full-length sequences, with 55 per cent identity in the conserved core region, especially that of the conserved heme pocket regions (figure 2a) [58]. Structural alignment of the crystal structures of the truncated, heme-conjugated human HO-1 and HO-2 shows remarkable structural conservation.
with r.m.s.d. of the α-carbons ranging from 0.697 to 0.862 Å, for the various conformations determined thus far (i.e. open and closed conformations of the human HO-1 holoenzyme) [58].

The three-dimensional structures of both HOs are predominantly α-helical with the heme sandwiched between the distal and proximal helices (figure 2c) [58–61]. A number of conserved glycines in the distal helix provide flexibility to accommodate substrate binding and product release. The active site of the apoprotein is generally more open than the holoenzyme. In the holoenzyme, the heme iron is coordinated by His45 in HO-1 (His45 in HO-2) on the proximal side with a water molecule serving as the sixth ligand on the distal side, and the distal and proximal helices are positioned closer together to form a more ‘closed’ conformation. As elucidated by the crystal structure of the HO-1 holoenzyme, there is still an inherent flexibility of the distal helix, which allowed it to be crystallized in both ‘open’ and ‘closed’ conformations. Both apo- and holoenzymes contain a hydrogen-bond network involving Asn210, Arg136, as well as a second level of residues, which includes Tyr58 and Tyr114 to stabilize the position of the catalytically critical Asp140 residue (Asp160 in HO-2). In the high-resolution structure of the HO-1 holoenzyme [61], it was revealed that the distal and proximal helices ‘clamp down’ to tighten the structure and allow coordination of the heme moiety with His25 as well as interaction of its propionate groups with basic side chains without perturbation of the Asp140 side chain. The tightened structure due to heme conjugation also traps critical water molecules in the distal cavity, which form part of a well-ordered hydrogen-bond network involving Asp140. This network may serve as a proton shuttle to anchor the catalytically critical distal water ligand of heme required for oxygen activation [61]. Although the analogous network was not fully resolved in the heme-conjugated HO-2 crystal structure, the conformation of Asp160 is similar to that of Asp140 in HO-1; thus, it is presumed to follow the same underlying mechanism of catalysis [58].

The major point of sequence divergence between HO-1 and HO-2 is in the C-terminal region (figure 2a), as well as the presence of three heme regulatory motifs (HRMs) in HO-2 with a characteristic Cys-Pro signature; while HRM3 is centred around Cys127, HMR1 and HMR2 reside in the C-terminus [58]. There is some controversy with respect to the precise role of these HRMs. Early studies indicated that these HRMs may bind heme directly [62,63]. However, recent reports have indicated that the HRMs affect catalytic efficiency without affecting heme affinity and do not result in additional heme-binding sites [64,65]. Rather, HMR1 and HMR2 form a thiol/redox switch that modulates HO-2 affinity, on the basis of the redox environment. Reducing conditions release the Cys265 thiol group from a disulphide bond with Cys282, allowing it to form an alternative axial heme ligand with lower affinity than His45 [66]. Thus, only one heme moiety conjugates with HO-2, coordinated by either His45 or the lower affinity Cys265.


Our research group is involved in a multi-disciplinary programme comprising investigators specializing in medicinal chemistry, pharmacology and toxicology, and biochemistry/structural biology, and focuses upon the design and development of novel and potent, isozyme-selective inhibitors of HO. Our initial investigations were stimulated by reports of some non-porphyrin-based compounds that were found to inhibit HO. Novel synthetic peptides were identified as potent inhibitors of HO-1 activity and shown to have the ability to prolong heterotopic heart graft survival in rats [67]. The most notable HO-1 inhibitors included peptide RESLRNLGNY, having an IC₅₀ of 200 μM, and peptide RNLLeNleRNLLeNleG-ConH₂ (RPD1258), with an IC₅₀ of 20 μM, against rat spleen HO-1 in vitro. However, this effect of HO-1 inhibitory activity was compromised by a secondary effect of inducing HO-1 expression. A series of imidazopyridinelines and substituted prolines were also reported to inhibit both the HO from the parasite Plasmodium yoelli and the HO of the host mice [68]. Several compounds were identified in this study, most notably 2-[3-(2-piperidin-1-yl)ethoxyphenyl]imidazo[1,2-a]pyridine (100% inhibition of mouse HO at 100 μM) and one stereoisomer of 5-(2-hydroxyphenyl)-2-methylpyrrolidine-2,3,4-tricarboxylic acid 3,4-diethyl ester (100% inhibition of mouse HO at 100 μM). Initial work by our group on these compounds did not give favourable results as potential lead compounds. However, an imidazolide-based compound, namely (25,45S)-2-[2-(4-chlorophenyl)ethyl]-2-{[1(1H-imidazol-1-yl)methyl]-4-[[4-(aminophenyl)thio]methyl]-1,3-dioxolane (azalanstat, QC-1, figure 3), originally designed by Syntex as an inhibitor of mammalian lanosterol 14α-demethylase (14-DM), gave promising results in its ability to inhibit HO [3]. The non-porphyrin-based structure of azalanstat was a key initial criterion because it would minimize cross-reactivity with other heme-binding proteins. A medicinal chemistry programme was initiated based on the lead compound, QC-1, and the topological analysis shown in figure 3. The resultant analogues were screened by an in vitro CO formation assay, using microsomal preparations from rat spleen and brain as physiological sources of HO-1 and HO-2, respectively [69]. The in vivo efficacies of some select compounds were also evaluated in mice and rats, as well as in cultured renal proximal tubule epithelial cells [70]. Initial studies identified a number of compounds that were selective for the inducible HO-1 isozyme [69]. Indeed, kinetic analyses of QC-1 and its analogues confirmed a non-competitive mode of inhibition, unlike the metalloporphyrins that compete for the heme-binding site, thus putatively minimizing the chances of cross-reactivity with other heme-binding proteins such as NOS and sGC, and increasing selectivity for the HO isozymes.

The initial structure–activity relationship (SAR) study [69] investigated the ortho-, meta- and para-amino derivatives of each of the four diastereomeric analogues of QC-1. Although no pattern in potency/selectivity for HO-1 was observed with respect to the position of the amino functionality, it was found that the (2S,4S) and the (2R,4S) diastereomeric configurations produced analogues with greater potency and selectivity for HO-1. In a further study, candidate compounds lacking the aminothiophenol moiety of azalanstat were synthesized and evaluated; the specific compounds synthesized were the four diastereomeric analogues having a methyl group in place of the methylene(aminothiophenol) moiety attached to the 1,3-dioxolane ring, as well as the compound (QC-15) in which this moiety was replaced by a hydrogen...
atom. The most potent and selective compound towards HO-1 inhibition was \((2R,4R)-2-[2-(4\text{-chlorophenyl})\text{ethyl}]-2-[\{(1H\text{-imidazol-1-yl})\text{methyl}\}-4\text{-methyl}-1,3\text{-dioxolane hydrochloride. QC-15}\) exhibited similar potency/selectivity for HO-1, albeit without containing any stereogenic centres, an attractive feature for synthesis. The results of the study thus far would suggest that structural differences between HO-1 and HO-2 exist, differences that may be exploited to introduce selectivity. The compounds resulting from the second study were also found to be selective for HO, with little or no effect on neuronal NOS and sGC, although they still showed potent inhibitory activity towards cytochromes P450 (i.e. CYP3A1/3A2, CYP2E1) [71,72].

The structures of the novel imidazole–dioxolane-based HO inhibitors are similar to the known azole-based antifungal compounds, particularly ketoconazole (KTZ), which has been shown also to have anti-tumour effects in prostate cancer [73]. Given the increase in HO-1 protein expression in tumours, such as hyperplastic and undifferentiated malignant prostate tissue [40], as well as the requirement of HO-1 for many solid tumours [43], it was hypothesized that perhaps the anti-tumour effect of KTZ may be mediated by HO-1 inhibition [74]. Testing of a series of antifungal agents demonstrated that the structures containing diazoles or triazoles are potent inhibitors of both HO isozymes. It was hypothesized that the azole moiety of these compounds may interfere with HO activity by binding the heme iron and forming a complex that is inaccessible to the catalytic site. However, it was found that KTZ did not affect the heme absorbance spectrum at the low concentrations resulting in inhibition. Indeed, analyses showed that KTZ inhibition was non-competitive and that it was also not due to interference with cytochrome P450 reductase or destruction of the HO protein. Moreover, KTZ showed weak inhibition towards NOS, yet no inhibition towards NADPH cytochrome P450 reductase.

In continuation of our medicinal chemistry programme based on the azalanstat lead, a series of 2-oxy-substituted 1-(1H-imidazol-1-yl)-4-phenylbutanes were synthesized having halogens in the phenyl ring and replacement of the dioxolane moiety by a hydroxyl or carbonyl functional group [75]. The entire library of compounds was found to be highly active, with the bromine- and iodine-substituted derivatives being the most potent. The imidazole–dioxolanes were all selective for HO-1, and exhibited substantially lower activity towards HO-2. The corresponding imidazole–ketones and imidazole–alcohols showed selectivity towards HO-1 to a lesser degree than the similarly substituted imidazole–dioxolanes.

The SAR study has involved the design and synthesis of many analogues in addition to the types described earlier, for example: imidazole–dioxolane compounds having a

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**Figure 3.** Structures of representative QC-inhibitors used for X-ray crystallographic studies, including azalanstat (QC-1) depicting regions of interest for SAR studies.
variety of substituents at the 4-position of the dioxolane ring, 2-oxo-substituted 1-azoyl-4-phenylbutanes to study the effect of the variation of the azole moiety, a series of 1-aryl-2-(1H-imidazol-1-yl)-4-triazol-1-yl)ethanones and their derivatives, and a series of α-(1H-imidazol-1-yl)-a-phenylalkanes to study the effect of introduction of heteroatoms in the alkyl linker. Reference to these analogues is made later in the discussions of the X-ray crystallographic structural analyses.

2.1. Enhancing structural knowledge of inhibition with X-ray crystallography

As the number of HO inhibitors increased, functional analyses identified a number of potent inhibitors, including several deemed isozyme-selective for HO-1. With the knowledge obtained from the three-dimensional structure of a truncated human HO-1 derivative based on previous X-ray crystallography studies (figure 2b) [59–61], a structural study of complexes formed between recombinant human HO-1 in complex with some of our potent and representative inhibitors was initiated. While the previous approach gave valuable information regarding SARs, and the iterative and systematic methodology was successful in identifying salient features for inhibition, what was missing was the ‘why’—i.e. the underlying mechanism leading to inhibition as well as isozyme selectivity. The inhibitors for which crystal structures were determined in complex with HO-1 were: 2-[2-(4-chlorophenyl)ethyl]-2-[1H-imidazol-1-yl] methyl-1,3-dioxolane (QC-15), 1-(adamantan-1-yl)-2-(1H-imidazol-1-yl)ethanone (QC-82), 4-phenyl-1(2,4,1H-triazol-1-yl)butan-2-one (QC-86), (2R,4S)-2-[2-(4-chlorophenyl)ethyl]-2-[1H-imidazol-1-y]methyl)-4-[(5-trifluoromethylpyridin-2-yl)thio)methyl]-1,3-dioxolane (QC-80), and 1-(1H-imidazol-1-yl)-4,4-diphenyl-2-butane (QC-308) (figure 3). Structural analyses and comparisons of the various complexes enabled us to definitively identify structure–functional relationships to understand the mode of binding to HO-1 and the mechanism underlying the inhibition of heme degradation. Moreover, identification of essential structural features critical for inhibition, both within the inhibitor as well as structural changes within the protein, provided valuable insights that enhanced the design of more effective HO-1 inhibitors. During the course of our study, the X-ray crystal structure of a truncated, human HO-2 derivative containing a Cys127Ala mutation was also determined [58], which provided more insights regarding potential structural differences that may be used in inhibitor design to enhance isozyme selectivity (figure 2c).

2.2. A common binding mode for imidazole-based heme oxygenase-1 inhibitors

One of the early, potent inhibitors found to be isozyme-selective for HO-1 was QC-15, with IC50’s of 4 ± 2 μM for HO-1 and greater than 100 μM for HO-2 as determined with CO-formation assays using rat spleen and brain microsomal fractions, respectively [72]. The potency of this compound was very similar to that of QC-1 (6 ± 1 μM) for HO-1, but removal of the bulky 4-aminothiophenyl groups in the northeastern region resulted in decreased activity towards HO-2 in comparison with QC-1 whose IC50 was 28 ± 18 μM. This compound was also the first to be crystallized, as reported by Sugishima et al. [76], in complex with rat HO-1 (rHO-1, 2.70 Å). Soon thereafter, we successfully crystallized QC-82, also an isozyme-selective and potent inhibitor of HO-1 with an IC50 of 7 ± 1 μM, in complex with human HO-1 (hHO-1) at a resolution of 1.54 Å [77]. The similarity in inhibitory effect was quite interesting, given the structural differences between these two compounds. While QC-15 is similar to QC-1 except in the absence of the bulky northeastern substituent, the structure of QC-82 diverges even further. The central dioxolane group was replaced by a ketone, the 4-chlorophenyl group in the western region was replaced by a bulky adamantanyl group and the central linker region between the two had been shortened by two carbon atoms. The only commonality was the eastern imidazole moiety. Interestingly, spectral analyses of both inhibitor–enzyme complexes also showed a red-shift in the Soret peak implying that, while the heme was still conjugated to the enzyme, its environment had been modified [76,77].

Comparison of the structures of the two inhibitor–HO-1 complexes revealed a common binding mode for these seemingly structurally distinct, imidazole-based compounds (figure 4a,b) [76,77]. While it appears that the inhibitors bind within the heme-binding pocket, they do not displace and rather require the heme moiety for binding, which explains the non-competitive nature of the inhibition as determined through kinetic analyses. This observation has been confirmed recently by Sorrenti et al. [78], who reported that the substrate was required for binding to membrane-free full-length hHO-1. The inhibitor binds to the distal side of heme, with the imidazolyl group serving as an anchor with the nitrogen at position 3 coordinating with the heme iron to form a true hexacoordinated heme, replacing the critical distal water molecule as the sixth coordinating ligand. The western region extends back into the distal side of the heme-binding pocket where it fits into a hydrophobic pocket. Comparison of the structures of inhibitors–HO-1 complexes with the respective holoenzymes revealed that there is very little change in the overall structure to accommodate the inhibitors. Alignment of the hHO-1–QC-82 complex with the ‘closed’, active conformation of the native holoenzyme gave a Cα r.m.s.d. of only 0.74 Å. Both the heme and proximal helix shift back to expand the distal pocket to some extent. In the structure of rHO-1 with QC-15, the heme and proximal helix are shifted approximately 0.8 Å towards the α-meso carbon along the α–γ axis of heme; in that of hHO-1 with QC-82, the coordinating nitrogen of His25 and the Fe atom are shifted by 0.91 and 0.85 Å, respectively. However, it is the inherent flexibility of the distal helix that allows the heme-binding pocket to open up to allow the inhibitor to slip in and also expands the distal pocket to accommodate the inhibitor. Moreover, the extent of expansion is dependent upon the size of the inhibitor with the helix being shifted to a greater extent to accommodate the bulkier adamantanyl group of QC-82, resulting in a distal pocket which is more open relative to either the native holoenzyme or rHO-1 in complex with QC-15. Indeed, accommodation of the bulky adamantanyl group of QC-82 resulted from a significant conformational change in the distal helix (Ser142 to Ile150) with a maximal displacement of 3.92 Å corresponding to Gly144 (figure 5i). In the complex of HO-1 with QC-15, the mainchain conformation of Ser142 and Gly143 changed along with the bending angle so as to open up the distal helix to such an extent that the helical structure at the bending point (Gly143–Gln143) was disrupted. By contrast, the
greater shift to accommodate the adamantanyl group of QC-82 in the hHO-1 complex resulted in an apparent helical break from Ser142 to Gly144. Thus, the adamantanyl group may represent the upper limit of what can be accommodated by shifts in the distal helix.

While the imidazolyl group serves as an anchor by coordinating with the heme iron moiety, the binding of the western region is stabilized through hydrophobic interactions involving residues lining the distal hydrophobic pocket, i.e.

Figure 4. Ribbon diagrams depicting the structures of HO-1 in complex with the respective QC-inhibitors. Heme (orange) and inhibitors (purple) are depicted as stick diagrams. Black dashes illustrate metal coordination via the nitrogen atom of the azole group of either His25 or the QC compound. Hydrogen bonds are depicted as yellow dashed lines. Selected residues mentioned in the text are depicted as stick diagrams and labelled for clarity. For simplicity, only water molecules in the distal pocket are shown. Electrostatic surface potentials, as calculated using PyMOL [57], depict positively (blue) and negatively (red) charged areas, and reveal hydrophobic pockets (white) into which the western and northeastern regions of the QC-inhibitors fit. All images were prepared using PyMOL [57]. (a) hHO-1 in complex with QC-15 (PDB #2DY5), (b) hHO-1 in complex with QC-82 (PDB #3CZY—Chain A), (c) hHO-1 in complex with QC-86 (PDB #3K4F), (d) hHO-1 in complex with QC-80 (PDB #3HOK—Chain B), (e) hHO-1 in complex with QC-308 (PDB #3TGM—Chain B). The two distal hydrophobic pockets (1ˢ HP and 2ˢ HP) that stabilize the two phenyl moieties of QC-308 allow a ‘double-clamp’ mode of binding.
region. Further observation revealed that the shorter, central linker of QC-82 resulted in this group not extending as far into the hydrophobic pocket, hence making fewer contacts. Moreover, in addition to hydrophobic contacts, the chlorophenyl group of QC-15 may also be stabilized by \( \pi - \pi \) stacking interactions attributable to the prevalence of aromatic residues in this region. More information regarding the structural basis of inhibitor binding was gleaned by using the known structure data to interpret previous functional data. Previously, a negative correlation was found between potency and electronegativity of the halogen substituent in the western region within a series of alcohol derivatives of 2-oxy-substituted 1-(1H-imidazol-1-yl-4-phenylbutanes) [75,77]. For example, the potency of the alcohol derivative of QC-15 (IC\(_{50} = 0.5 \pm 0.1 \mu M\)) increased upon substitution of the chlorophenyl group by bromophenyl (IC\(_{50} = 0.14 \pm 0.06 \mu M\)) or the more electronegative iodo-phenyl group (IC\(_{50} = 0.06 \pm 0.03 \mu M\)), and decreased when replaced by the less electronegative fluoro-phenyl (IC\(_{50} = 1.4 \pm 1.1 \mu M\)) or a non-substituted phenyl group (IC\(_{50} = 6.2 \pm 0.8 \mu M\)). A close inspection of the hydrophobic pocket revealed the presence of a polar thiol group (Met34), which may explain why electronegative moieties in the western region still give rise to potent inhibitors. Alternatively, the larger halogens may provide more points and better contact with the distal hydrophobic pocket.

One of the key differences between QC-15 and QC-82 is the central linker region, which contains a dioxolane and ketone group, respectively. Analysis of the differences in interaction with HO-1 provided insights into potential features, which could be exploited in future drug design. The dioxolane group of QC-15 is involved in a hydrogen bond network involving a water molecule as well as the carbonyl group of Thr135 [76]. A subsequent structure of hHO-1 in complex with QC-80 (IC\(_{50} = 2.1 \pm 0.6 \mu M\)), which also contains a central dioxolane component, revealed a similar stabilizing interaction [80]. However, this network appears not to be essential for binding the central region of these imidazole-based compounds, as there is no analogous water molecule available for a similar hydrogen-bond interaction with the ketone group of QC-82 in one of the molecules in the asymmetric unit. By contrast, in the crystal structure of hHO-1 in complex with QC-86 (IC\(_{50} = 2.5 \pm 0.4 \mu M\)), another azole-based compound with a central ketone moiety, it appears that the carbonyl group is stabilized by a hydrogen bond involving an active site water molecule that is a part of a potential hydrogen-bond network involving another water molecule and the Thr135 carbonyl group [81]; the water molecule may also be involved in the Asp140 hydrogen-bond network. Given the more open conformation of the distal pocket to accommodate the bulky adamantanyl group of QC-82 relative to the other compounds for which structures have been determined, it is likely that this structural expansion acts to impede its ability to trap water molecules, resulting in greater fluidity of the solvent structure. Further insight into this central region was gained by looking at previous functional data involving the series of 2-oxy-substituted 1-(1H-imidazol-1-yl-4-phenylbutanes), which showed an interesting trend. Comparison of IC\(_{50}\) values showed that, generally, compounds with a central hydroxy group tended to be more potent than their dioxolane or ketone counterparts. Substitution of the central dioxolane group of QC-15 with a ketone group did not affect its IC\(_{50}\) significantly (IC\(_{50} = 4.7 \pm 0.5 \mu M\)), whereas substitution to a central hydroxyl group increased its potency by almost 10-fold (IC\(_{50} = 0.5 \pm 0.1 \mu M\)). This may be due, in part, to a greater potential to form hydrogen bonds with trapped water molecules or neighbouring residues. The increased rotational flexibility of the hydroxyl group, relative to a carbonyl or dioxolane, may also allow interaction with Asp140 to stabilize binding further and/or disrupt the critical Asp140 hydrogen-bond network further. Unfortunately, the increased potency observed for the alcohol derivative of QC-15 is marred by a loss of selectivity (IC\(_{50} = 4.0 \pm 0.6 \mu M\) for HO-2). Thus, culmination of all the structural and functional data reveals that, while
hydrogen-bond networks involving trapped water molecules and neighbouring residues may contribute to the stabilization of the central region of an inhibitor, they are not critical for its binding to HO-1.

Recently, to gain more insights into the structural requirements for the central region of the QC compounds, a series of \( \alpha-(1H\text{-imidazol-1-yl})-\omega\)-phenylalkanes was synthesized to examine the effect of introducing heteroatoms into the central alkyl linker [82]. Moreover, linkers of various lengths were also investigated, which provided information regarding the size limits required for effective binding. Interestingly, introduction of an oxygen atom decreased inhibitory potency against HO-1, while derivatives containing a sulphur atom had increased potency relative to those with a simple alkyl linker. The presence of polar groups within the distal hydrophobic pocket, e.g. Met34 and Met51, may be playing a role in these stabilizing/destabilizing interactions. Increasing linker length from one to five carbon atoms resulted in a progressive increase in inhibitory potency against HO-1, from \(44 \pm 6\) to \(3.5 \pm 0.7\ \mu M\), respectively; the absence of a linker resulted in no activity. Presumably, this trend is due to greater contacts with the distal hydrophobic pocket as the linker is increased, resulting in greater stabilization of the western phenyl moiety. A minimal linker is required for activity, which suggests that stabilization by the distal hydrophobic pocket is in fact crucial for binding of this class of compounds. While a five-carbon linker resulted in the most potent compounds, the most HO-1 selective compounds are those containing a four-atom linker between the phenyl and imidazolyl moieties. Alignment of the crystal structures of the hHO-1 complex with \(2QPP\) shows that a potential difference in the size of the inhibitor binding site for both hHO-1 and hHO-2 (PDB code 3CZY, i.e. our largest inhibitor binding site) with that of hHO-2 (PDB code 2QPP) shows that a potential difference in the size of the western hydrophobic pocket may account for this selectivity. The HO-2 pocket is larger partly due to substitutions of shorter amino acids; for example, the side chains of Met51 and Val50 in HO-1 extend further in towards the western region of the QC compounds than their Thr71 and Ala70 counterparts in HO-2. Thus, a longer linker may be required to allow the proper hydrophobic contacts for stabilization in HO-2.

The common binding mode of the imidazole-based HO-1 inhibitors involves coordination of the heme iron through an eastern imidazolyl anchor and stabilization of the western region by a distal hydrophobic pocket through hydrophobic interactions and potentially \(\pi-\pi\) stacking interactions. Movement of the distal helix allows expansion of the distal hydrophobic pocket to accommodate bulky groups on the western end. The central region is not critical for binding although water-mediated hydrogen-bond networks may contribute to its stabilization and an optimal linker length of four carbons may allow isozyme selectivity towards HO-1 without binding HO-2. It is possible that this central region’s main contribution is to the proper spacing between the eastern and western regions, i.e. the ‘anchor’ and the hydrophobic moiety, so that they may make optimal contacts of heme coordination and within the distal hydrophobic pocket, respectively. Interestingly, binding of inhibitor does not perturb the critical Asp140 residue, nor any of the surrounding residues involved in its critical hydrogen-bond network. Rather, the underlying mechanism by which the imidazole-based compounds inhibit HO is by disrupting the ordered solvent structure and, thus, the hydrogen-bonded network, and ultimately displacing the catalytically critical distal water ligand to inhibit heme oxidation.

2.3. Anchoring inhibition

The elegance of the structure and mechanism underlying heme oxidation has allowed its inhibition by this diverse class of non-porphyrin-based compounds. The pentacoordinate nature of the HO-1 heme, with His25 serving as a proximal coordinating ligand, as well as the immutable positioning of the critical Asp140 and its hydrogen-bond network, allows the precise positioning of the catalytically critical water molecule to serve as a ligand on the distal side upon heme conjugation [60,61,83,84]. It also makes the distal side of heme a prime target in designing inhibitors with a coordinating functional group to serve as an anchor for binding to displace the critical water ligand. Indeed, the main feature for binding of our QC compounds is coordination of the heme iron through the N-3 nitrogen of an unsubstituted imidazolyl moiety. Thus, a potential strategy for improving inhibitor potency is to build a stronger ‘anchor’ by modifying this imidazolyl group.

A series of compounds was designed in which different azole groups were substituted for imidazole in the eastern region [85]. Functional analyses determined that 1,2,4-triazole- and 1H-tetrazole-based inhibitors are potent inhibitors of HO-1, with an improved selectivity over HO-2. A crystal structure for hHO-1 in complex with a compound containing a 1,2,4-triazolyl substituent, namely 4-phenyl-1(1,2,4-1H-triazol-1-yl)butan-2-one (QC-86, \(IC_{50} = 2.5 \pm 0.4\ \mu M\)), was determined to a resolution of 2.20 Å. Analysis of the enzyme–inhibitor interaction revealed that the triazolyl moiety is able to provide an additional point of anchor stabilization as the presence and positioning of the N-2 nitrogen atom is close enough to form a potential hydrogen bond with the amide group of Gly143 (figure 4c). This additional hydrogen bond may explain the slightly increased potency of this compound relative to its imidazolyl counterpart (cf. \(IC_{50} = 4 \pm 2\ \mu M\)). Interestingly, shifting the position of the additional nitrogen atom to form the 1,2,3-triazolyl variant significantly decreases potency (\(IC_{50} = 89 \pm 1\ \mu M\)). Because there was no obvious structural basis for the decrease, this phenomenon was investigated further by calculating the Mulliken charges for the coordinating nitrogens of each of these azole variants. Similar values were determined for both the 1,2,4-triazole ring (\(-0.464\)) and the imidazole ring (\(-0.446\)), while the resultant delocalized electron system of the 1,2,3-triazole ring decreased the electronegativity of the ‘coordinating’ nitrogen (\(-0.281\)) to potentially weaken its coordination with the heme iron. Further stabilization of the anchor may be a result of repulsive forces contributed by the ketone group of Gly139 as the N-2 nitrogen of the 1,2,3-triazole would be located 3.05 Å away from this functional group.

Recent studies have shown that replacing the imidazole with either a 1,2,4-triazole or a tetrazole significantly diminishes the inhibitory effects on the cytochrome P450 family of drug metabolizing enzymes [86]. Thus, strengthening the QC-anchor in this manner would be an efficient strategy to design potent and selective HO-1 inhibitors. It may also be worthwhile to explore other strategies to strengthen the anchor by replacing the coordinating atom itself. It was puzzling, however, to observe that the azole–dioxolanes and the azole–alcohols derived from the active 1,2,4-triazole- and
1H-tetrazole-based ketones were generally less active than their imidazole-based derivatives [81].

2.4. A novel, inducible binding mode
While initial work had concluded that the aminothiophenol moiety in the northeastern region of azalanstat is not essential for HO inhibition, it had been noticed that general HO potency was dependent upon the position of the nitrogen atom and that, more importantly, its removal altogether enhanced HO-1 selectivity [69,71,72]. Thus, further investigation of this region was postulated to be important in determining selectivity for HO-2 using thiophenol and substituted phenol derivatives as well as small functionalized derivatives at the 4-position of the dioxolane ring, i.e. the northeastern region [87]. Unfortunately, most of the compounds synthesized were highly potent inhibitors of both HOs with only moderate selectivity for HO-1. The addition of a second aromatic ring system was well tolerated by HO-1 and enhanced selectivity, although an upper limit to this region was found based on the loss of inhibitory activity when this was extended to a bulky adamantanyl group. Interestingly, within the thiophenol/phenol series, four of the six most selective compounds contained hydrocarbon moieties in the northeastern region, suggesting the importance of non-polar functionality in this region. Replacement of the 4-aminothiophenol moiety with smaller, polar, functional groups also resulted in potent inhibitors but with no to modest selectivity for HO-1. Thus, it was concluded that modifications solely in this northeastern region would not result in HO-2 selectivity and, moreover, may not be an efficient avenue in the development of highly selective HO-1 inhibitors.

From a structural point of view, however, it was not directly obvious as to how these bulky northeastern groups would be accommodated to allow binding to HO-1. None of the previous structures indicated much flexibility within the proximal region, with most of the movement occurring distally. A putative proximal hydrophobic pocket had been observed in the structure of hHO-1 in complex with the bulky adamantanyl derivative, QC-82, which had not been apparent in previous structures (figure 4b). Computational analyses were successful in virtually docking both QC-1 [77] as well as (2R,4S)-2-[2-[4-chlorophenyl]ethyl]-2-[1H-imidazol-1-yl]methyl]-4-[phenylsulphonyl]methyl]-1,3-dioxolane [87] such that the bulky northeastern groups fit into this potential pocket. However, the crystal structure of QC-80 (IC$_{50}$ = 2.1 ± 0.6 µM) in complex with hHO-1 revealed that the binding of this class of compounds is, in fact, a result of a novel, inducible binding mode, which could not be anticipated from any of the structural data obtained previously [80]. The bulky northeastern region is accommodated by shifting residues in the proximal helix to induce the creation of a secondary hydrophobic pocket distinct from that suggested from previous structures (figure 4d).

Despite the bulky northeastern region, QC-80 follows the typical binding mode, interacting with hHO-1 in a manner analogous to the azole-based compounds that lack substituents in this region, with one exception. Previous structures of hHO-1, including the apo- and holoenzymes, as well as in complex with the other QC-inhibitors lacking a northeastern region showed a sharp bend of approximately 60° in the proximal helix (Asn30-Ala31) that precedes another helical segment (Glu32-Lys39). Interaction of the bulky 5-trifluoromethylpyridin-2-yl moiety of QC-80 appeared to push back residues in this region to ‘unkink’ and extend the proximal helix up to Gln38 and induce the formation of a secondary peripheral hydrophobic pocket to accommodate the northeastern substituent. Residues linking this newly formed pocket that may contribute hydrophobic interactions to stabilize the 5-trifluoromethylpyridin-2-yl group include Ala28, Ala31, Arg35, Phe214 and Glu215. This conformational change expands the binding pocket to a greater extent than observed with any of the other inhibitors, shifting Gln38 as much as 11 A from its original position in the native holoenzyme. Moreover, there is a rearrangement of residues between the interior and exterior of the pocket. Binding of the northeastern region of the inhibitor displaces the thiol group of Met34, which shifts to the exterior of the newly formed hydrophobic pocket along with the functional groups of Phe33, Phe37 and Gln38, while the functional groups of both Arg35 and Glu32 shift into the interior where they may be stabilized by electrostatic interactions between the two side chains (figure 5b).

Thus, the crystal structure of the QC-80 complex revealed that the proximal helix of hHO-1 also contains a degree of intrinsic flexibility, although not to the same extent as the distal helix, which allows binding of inhibitors with a bulky northeastern region, such as QC-1, by inducing the formation of a secondary hydrophobic pocket to stabilize this region. While binding by an ‘induced fit’ mechanism would be more energetically costly than binding to a preformed binding site, it has provided further insights into the structural properties of hHO-1 that were not apparent previously, as well as a rationale underlying the potency of inhibitors such as azalanstat which contain bulky northeastern regions. This new information may be helpful in future inhibitor design.

2.5. Structure-aided drug design: a novel, ‘double-clamp’ binding mode
The discovery of the novel, induced formation of the secondary hydrophobic pocket to bind the northeastern region of QC-80 shed new light on the observation of the putative proximal, secondary hydrophobic pocket seen in the structure of hHO-1 with QC-82 [77]. While this proximal pocket is not involved in binding the northeastern region as previously postulated, there was potential in its exploitation to improve inhibitor potency by creating compounds that could occupy both preformed hydrophobic pockets simultaneously. The synthesis and characterization of 1-(1H-imidazol-1-yl)-4,4-diphenyl-2-butanone (QC-308, IC$_{50}$ = 0.27 ± 0.07 µM), which contains an additional phenyl moiety in the western region, in contrast to the usual single hydrophobic group in the QC compounds, demonstrated a approximately 15-fold increase in potency relative to its monophenyl analogue, 4-phenyl-1-(1H-imidazol-1-yl)-2-butanone (QC-65; IC$_{50}$ = 4.06 ± 1.8 µM) [75]. This was quite remarkable given that QC-65 was already quite a potent inhibitor. Determination of the crystal structure of the hHO-1 complex with QC-308 revealed the common elements required for binding the majority of the QC compounds and, further, confirmed the presence of a definitive secondary hydrophobic pocket to accommodate and stabilize the second phenyl moiety in the western region of QC-308 (figure 4e) [88]. Thus, each of the two phenyl moieties of the diphenyl analogue fit into separate hydrophobic pockets, resulting in a ‘double-clamp’ binding mode to provide additional stabilization,
which likely accounts for the increased potency relative to the monophenyl variant. Fortunately, neither QC-65 nor QC-308 are isozyme-selective, being potent inhibitors of both HO isozymes (IC₅₀ = 11.3 ± 4.7 µM [75] and 0.46 ± 0.15 µM [88], respectively, for HO-2 in rat brain microsomes). Replacement of the central ketone moiety of QC-65 with a dioxolane group had conferred isozyme selectivity (QC-57; 2-[2-phenylethyl]-2-[1H-imidazol-1-yl]methyl][1,3-dioxolane) with IC₅₀'s of 0.76 ± 0.4 and greater than 100 µM for HO-1 and HO-2, respectively [75]. Thus, using the ‘double-clamp’ strategy to increase the potency of an HO-1 isozyme-selective derivative such as this would be an avenue worth pursuing.

3. Looking forward

Over the past decade, since the discovery of azalanstat as a specific HO inhibitor, we have amassed a large group of azole-based derivatives that exhibit potent inhibitory activity specific for HO, with several showing isozyme selectivity. Through a combination of a medicinal chemistry SAR approach and X-ray crystallographic structural analyses, we have gained valuable insights into the common binding mode of this class of compounds, as well as information regarding the enzyme responsible for its inherent flexibility, which also allows for an ‘induced’ fit to accommodate proximal structural motifs. Each series of derivatives has allowed us to amass more information, which can be used to develop the next generation of inhibitors. As such, we have concluded that this class of azole-based HO-1 inhibitors, originally derived from azalanstat, inhibit heme oxidation through a non-competitive, yet heme-dependent, mechanism by disrupting the ordered solvent structure integral to the critical hydrogen-bond network involving Asp140 and displacing the catalytically critical distal water ligand.

Moreover, from a physiological point of view, this inhibitory mechanism has already demonstrated potential therapeutic applications as some of our imidazole-based HO-1 inhibitors have shown the ability to attenuate the growth of prostate [89] and breast cancer cells in vivo. The relevance of HO in various experimental situations has been explored by other laboratories through administration of these novel imidazole-based drugs to both cultured cells and intact animals. Accordingly, Di Francesco et al. [90] tested the role of HO-1 in the response of human umbilical vein endothelial cells in culture to laminar shear stress. They observed that TNF-α biosynthesis was reduced by shear stress, and that this reduction was reversed in the presence of the selective HO-1 inhibitor QC-15. Similarly, Csongradi et al. [91] used selective HO-1 inhibitors to study the effects of renal HO inhibition on blood pressure in mice. Angiotensin II induced hypertension was exacerbated in the presence of this (QC-13) selective HO-1 inhibitor, as well as the first-generation HO inhibitor tin mesoporphyrin (SnMP). SnMP also induced renal medullary HO-1 protein, while QC-13 was without effect on HO protein levels, which obviated the complication of increasing quantities of the enzyme targeted for inhibition.

The salient features of this class of inhibitors include, first and foremost, an anchor in the eastern region to coordinate with the heme iron atom, usually an imidazole although a 1,2,4-triazole moiety may strengthen the interaction with the added benefit of increasing specificity by decreasing activity against the cytochrome P450 family of enzymes. The anchor is connected by a linker region to a hydrophobic group in the western region, which fits into a distal hydrophobic pocket and is stabilized by both hydrophobic and aromatic stacking interactions. A four-carbon linker is the optimal length to maximize potency without compromising isozyme selectivity for HO-1. A five-carbon linker results in a more potent compound but one that is also inhibitory against HO-2, possibly due to the latter’s larger hydrophobic pocket. The presence of polar residues in the pocket (e.g. Met34) may also accommodate halogenation of the phenyl group as well as the presence of heteroatoms nearby in the linker region, although an alternative explanation may be that the larger halogens provide more points and better contact in the pocket. The distal helix of hHO-1 has a high inherent flexibility, which gives the enzyme the capacity to bind inhibitors with a range of ‘bulkiness’ in the western region, while still maintaining its heme moiety. The maximal limit may be the size of an adamantanyl group, as its binding caused disruption in the helical structure at one point of the distal helix. Moreover, the presence of a putative peripheral secondary hydrophobic pocket can also be exploited to increase potency by introducing diphenyl variants to the western region, which can be stabilized by a ‘double-clamp’ binding mode. A lesser degree of flexibility is associated with the proximal helix, which results in the accommodation of compounds with large substituents in the northeastern region. Interaction of this series of compounds induces a conformational change, essentially resulting in an ‘unkinking and extension’ of the helix and rearrangement of residues from the interior and exterior of the protein, to form a peripheral hydrophobic pocket to accommodate these groups.

It should be noted that all of the structural analyses of HO-1 in complex with the QC-inhibitors to date have been conducted using soluble, truncated derivatives of the protein [76,77,80,81,88]. By contrast, functional characterization of inhibitor potency has been performed using the full-length protein as found in microsomal preparations. A recent comparison of native and recombinant truncated proteins demonstrated differential sensitivity of recombinant and microsomal HO-1 towards inhibitors [92]. Also, this has led to some controversy regarding data interpretation, especially regarding the issue of isozyme selectivity, as some isozyme-selective compounds were found to be equipotent against both isozymes when tested against the recombinant, truncated versions [76,92]. Given the structural and sequence conservation of the HOs within the catalytic core [58], the lack of selectivity found with the truncated purified protein was not surprising. However, the recent characterization of a recombinant full-length HO-1 had indicated that the full-length form exhibits a 2- to 3-fold greater activity relative to that of the truncated, soluble form, which was increased even further in the presence of lipid. Moreover, the C-terminal hydrophobic tail has an essential role with respect to membrane incorporation as well as in formation of a high-affinity complex with cytochrome P450 reductase, deemed essential for maximal catalytic activity [93–95]. The C-terminal domain is also the major point of sequence divergence between the two isozymes, especially in the presence of the HRMs in HO-2 [58]. While current structural information does not imply a role for this hydrophobic tail in the binding of this class of inhibitors, its potential role in influencing conformation...
should not be ignored in future designs, especially in influencing isozyme selectivity. Indeed, the determination of the crystal structure of both full-length hHO-1 and hHO-2 would provide valuable information in this regard.

The quest to build a better inhibitor involves much imagination and constant exploring. While we have gained experience and knowledge regarding this particular class of azole-based non-competitive inhibitors for HO-1, we are always searching for new lead compounds to develop new classes of inhibitors for various applications. The crystal structure of the truncated hHO-2 has already provided us with valuable insights with regard to our complementary goal of developing selective HO-2 inhibitors. Ideally, it would also be useful to develop a series of competitive inhibitors that prevent binding of heme altogether. Theoretically, one would presume that a selective, competitive inhibitor would have powerful therapeutic applications to greatly minimize any HO activity if the substrate is not available for degradation. Recently, caveolin-1 was discovered to competitively inhibit rat HO-1 via the caveolin scaffolding domain (residues 82–101), with a five amino acid sequence (residues 97–101: YWFYR) identified as a minimum sequence for binding. Two aromatic residues, Phe207 and Phe214, in HO-1 are thought to be essential in the association with caveolin-1 through π–π stacking and hydrophobic interactions [96]. Further analysis of this peptide structure may provide insights into new lead compounds to develop a series of potential competitive inhibitors.

We hope that through this exciting process, using both medicinal chemistry SAR and X-ray crystallography, we will be able to develop powerful pharmacological tools for delving deeper into the understanding of the mechanisms by which the HO/CO system exerts its many effects and, in turn, providing a foundation for the development of novel therapeutic approaches for the myriad pathologies with which it is involved.

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