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Authors: Naasson M. Mbenza, Nawal Nasarudin, Praveen G. Vadakkedath, Kamal Patel, Ahmad Zaidi Ismail, Muhammad Hanif, Leonard James Wright, Vijayalekshmi Sarojini, Christian G. Hartinger, and Ivanhoe K. H. Leung

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COMMUNICATION

Carbon monoxide is an inhibitor of HIF prolyl hydroxylase domain 2

Naasson M. Mbenza,[a, b] Nawal Nasarudin,[a] Praveen G. Vadakkedath,[a, c] Kamal Patel,[a] A. Z. Ismail,†[a] Muhammad Hanif,[a, d] L. James Wright,[a] Vijayalekshmi Sarojini,[a, c] Christian G. Hartinger,[a, d] and Ivanhoe K. H. Leung* [a, d, e, f]

Abstract: Hypoxia-inducible factor prolyl hydroxylase domain 2 (PHD2) is an important oxygen sensor in animals. By using the CO-releasing molecule-2 (CORM-2) as an *in situ* CO donor, we demonstrate that CO is an inhibitor of PHD2. This report provides further evidence about the emerging role of CO in oxygen sensing and homeostasis.

Carbon monoxide (CO) is a colourless and odourless gaseous molecule. Although most commonly known as an acute toxin that outcompetes molecular oxygen for the binding of haemoglobin at high concentrations,[1] CO is produced endogenously in humans by haem oxygenases as a co-product of haem catabolism.[2–5] At physiologically relevant concentrations, CO is a signalling molecule that is involved in a number of important physiological functions.[5–9] It has been shown to mediate signalling processes in the brain,[10,11] induce vasorelaxation,[12,13] suppress inflammation,[14] modulate mitochondrial functions,[15–17] and affect cell proliferation and apoptosis in tissue- and cell-specific manners.[18–22]

One of the most intriguing aspects of CO is its relationship with cellular oxygen homeostasis. Not only is the endogenous production of CO oxygen-dependent (as haem oxygenases require molecular oxygen as co-substrate),[2–5] there is also increasing evidence that shows CO may play critical roles in cellular oxygen sensing and signalling.[23,24] Of particular interest is the observation that, in normoxia, physiologically-relevant concentrations of CO may activate and stabilise hypoxia-inducible factor α (HIFα).[18,25] In hypoxia, in contrast, it has been shown that CO may actually promote the degradation of HIFα.[17,26,27] HIFα is a master regulator of hypoxic responses to maintain oxygen homeostasis.[28–34] During hypoxia, HIFα dimerises with HIFβ and other co-activators to transcriptionally activate a number of genes, such as those that are involved in erythropoiesis and angiogenesis, to counteract the effect of low oxygenation.[28–34]

The activity of HIF is regulated by a complex network of multiple intertwined pathways. One of these pathways regulates HIFα at the level of protein stability.[28–34] HIFα and HIFβ are constitutively produced regardless of cellular oxygen concentrations. HIFα is subjected to oxygen-dependent proteolytic degradation. This process is regulated by HIF prolyl hydroxylase domain-containing proteins (PHDs), which, in humans, exist in three isoforms.[28–34] PHDs belong to the Fe(II) and 2-oxoglutarate (2OG)-dependent oxygenase superfamily.[28–34] Members of this superfamily typically require Fe(II), 2OG, molecular oxygen and ascorbate for optimal activity.[35–38] PHDs catalyse the hydroxylation of HIFα in the presence of oxygen.[28–34] The resultant hydroxyl group(s) on HIFα acts as a signal for its degradation via the ubiquitin-proteasome pathway through binding to the von Hippel-Lindau protein.[28–34]

In human HIF1α, two prolyl residues, Pro-402 and Pro-564, are involved in this pathway.[28–34] The two hydroxylation sites are termed the N-terminal oxygen-dependent degradation domain (NODD, where Pro-402 is present) and C-terminal oxygen-dependent degradation domain (CODD, where Pro-564 is present). It has been shown that the hydroxylation of either CODD or NODD may lead to the degradation of HIF1α.[39,40] Although NODD hydroxylation is more sensitive to changes in oxygen concentration than CODD hydroxylation,[41] kinetics and binding studies showed that CODD is the preferred substrate and is a stronger binder of PHDs than NODD, which is also supported by structural studies.[42–45]

Given the central position that PHDs play in regulating hypoxic response, the modulation of PHDs by endogenously produced compounds is of significant interest from both therapeutic and basic biology perspectives. CO, in general, has a high affinity for ferrous Fe(II). For example, the affinity of CO to the reduced iron-haem in haemoglobin is about 220 times stronger than that of molecular oxygen.[46] In addition, it has also been shown that CO could bind to a range of metalloproteins, including iron-dependent enzymes such as iron and nickel iron hydrogenases,[47-51] as well as copper-dependent proteins including tyrosinases and haemocyanins.[52-54] Given that PHDs

[a] Dr N. M. Mbenza, N. Nasarudin, Dr P. G. Vadakkedath, Dr K. Patel, Dr A. Z. Ismail, Dr M. Hanif, Prof. L. J. Wright, A/Prof. V. Sarojini, Prof. C. G. Hartinger, Dr I. K. H. Leung
School of Chemical Sciences
The University of Auckland
Private Bag 92019, Victoria Street West
Auckland 1142 (New Zealand)
[b] Dr N. M. Mbenza
School of Biological Sciences
Victoria University of Wellington
PO Box 600, Wellington 6140 (New Zealand)
[c] Dr P. G. Vadakkedath, A/Prof. V. Sarojini
The MacDiarmid Institute for Advanced Materials and Nanotechnology
Victoria University of Wellington
PO Box 600, Wellington 6140 (New Zealand)
[d] Dr M. Hanif, Prof. C. G. Hartinger, Dr I. K. H. Leung
Maurice Wilkins Centre for Molecular Biodiscovery
The University of Auckland
Private Bag 92019, Victoria Street West
Auckland 1142 (New Zealand)
[e] Dr I. K. H. Leung
School of Chemistry
The University of Melbourne
Parkville, VIC 3010 (Australia)
Email: ivanhoe.leung@unimelb.edu.au
[f] Dr I. K. H. Leung
Bio21 Molecular Science and Biotechnology Institute
The University of Melbourne
Parkville, VIC 3010 (Australia)
† Current address: Department of Chemistry, Universiti Putra Malaysia, 43400, Serdang, Selangor (Malaysia)

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are Fe(II)-dependent metalloenzymes, we speculate that CO may inhibit PHDs by competing with molecular oxygen for binding at the active site Fe(II). Herein, by using the catalytic domain of PHD2 ($PHD2_{181-426}$), the PHD isoform that is considered to be the most important oxygen sensor,[55–57] as a model system, we provide evidence that demonstrates CO is an inhibitor of PHDs.

As CO is a gaseous molecule, CO-releasing molecule-2 (CORM-2; $[RuCl_2(CO)_3]_2$) was used as an *in situ* CO donor. The use of CORMs (including CORM-2) as CO donors in biological systems is well established.[58–60] Nonetheless, experiments were first conducted to investigate the CO-release kinetics under our reaction condition. In our reactions, CORM-2 was first dissolved in dimethyl sulfoxide (DMSO), which was then immediately added to the aqueous buffer containing the enzyme mixture. In agreement with a previous study conducted by Motterlini and co-workers,[59] we found that CORM-2, when freshly dissolved in DMSO, decomposed mainly into *fac*-$[RuCl_2(CO)_3(DMSO)]$ and *cis*,*cis*,*trans*-$[RuCl_2(CO)_2(DMSO)_2]$, indicating that there was minimal liberation of CO (at least) in the first few minutes (Figure S1). We then measured the kinetics and the amount of CO that was released after the CORM-2/DMSO stock solution was added to aqueous buffer. A CO-trapping assay that monitors the conversion of deoxymyoglobin to carboxymyoglobin by ultraviolet/visible (UV/vis) absorption spectroscopy was applied.[61] 2OG and ascorbate were also added to the aqueous solution to mimic the condition for PHD2-catalysed reactions. Upon addition of the freshly prepared CORM-2/DMSO solution (to give 100 μM CORM-2 final concentration) to a buffer solution containing 50 μM deoxymyoglobin, full conversion of deoxymyoglobin to carboxymyoglobin was observed in the first UV/vis spectral measurement (ten seconds after mixing) (Figure S2), indicating liberation of CO from CORM-2 in aqueous buffer. Titration of CORM-2 to deoxymyoglobin (50 μM) showed that all myoglobin was saturated with CO at ~1:1 CORM-2 to myoglobin ratio (Figure S3), indicating that, for every CORM-2, roughly one CO was released. This is in line with a previous study that showed around 0.7 moles of CO was liberated from each mole of CORM-2.[59] The difference is likely due to handling of the CORM-2 stock solution and buffer conditions. These experiments showed that CORM-2 is an efficient source of CO in aqueous buffer.

Another potential concern about the use of CORM-2 to study PHD2-catalysed reactions is the possibility for decomposed or non-decomposed CORM-2 (rather than CO) to inhibit PHD2. This concern is particularly valid for metalloenzymes such as PHD2 as it has been reported that some transition metals may inhibit the enzyme by displacing the active site Fe(II).[62,63] As $RuCl_3$[17,64,65] and $RuCl_2(DMSO)_4$[64,66,67] have been used as negative controls for biological experiments using CORMs, the ability of these compounds to inhibit PHD2 was investigated. By using a matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS)-based assay that measures peptide hydroxylation, and 19-mer HIF1α CODD/NODD peptides as substrates (see Table 1 for peptide sequences),[24] we found that $RuCl_3$ is an inhibitor of PHD2 under the standard assay condition with 1 μM PHD2 and 10 μM Fe(II) (Figure S4). However, this effect can be alleviated by supplementing the enzymatic reaction mixture with excess Fe(II) (for example, >200 μM; Figure S4). Similarly, under the same reaction condition with 200 μM Fe(II), $RuCl_2(DMSO)_4$ did not inhibit PHD2 at up to 1 mM concentration (Figure S5).

We then tested the inhibition of PHD2 by CORM-2. Under our optimised reaction conditions with 1 μM PHD2 and excess Fe(II) (200 or 400 μM), we found that CORM-2 reduced the hydroxylation of CODD and NODD (Figure 1 and Figure S6). As PHD2-catalysed CODD/NODD hydroxylations were not affected by $RuCl_3$ or $RuCl_2(DMSO)_4$ under these conditions, our results indicated that CO was likely the species that inhibits PHD2. To further confirm the inhibition of PHD2 by CO, competition experiments were performed with deoxymyoglobin. This is because deoxymyoglobin is a good CO binder and it will provide competition with PHD2 for the binding of CO. We found that the addition of 10 μM deoxymyoglobin restores the activity of PHD2 in the presence of 75 μM CORM-2 (Figure S7). As one myoglobin could bind one molecule of CO, the additional protection effect is intriguing. We therefore repeated the experiments in the absence of CORM-2. We found that the addition of deoxymyoglobin led to a slight increase in PHD2-catalysed hydroxylation reactions (Figure S7). As myoglobin is known to react with hydrogen peroxide,[68] the slight increase in PHD2 activity could be due to myoglobin removing the hydrogen peroxide that was produced as a result of PHD2-catalysed reactions. Such effects have been observed with catalase in 2OG-dependent oxygenase-catalysed reactions.[69] Nonetheless, even taking this slight increase of PHD2 activity into account, our results still point towards CO as the main species that inhibits PHD2.

There are reports that some proteins may form adducts with CORM-2 through interactions between surface-exposed histidine residues and the Ru(II) centre of CORM-2.[70,71] As such, it is possible that CORM-2 may react covalently with PHD2 and deactivates PHD2 in a CO-independent manner. However, it was also suggested that the addition of excess imidazole or serum albumin may suppress the formation of the CORM-2-protein covalent adducts as imidazole and serum albumin may act as competing molecular targets to react with CORM-2.[71] We therefore repeated the inhibition experiments with CORM-2 (150 μM) in the presence of 1 mM imidazole or bovine serum albumin (BSA). Our results showed that the inhibition of PHD2 by CORM-2 was not affected by the presence of imidazole (Figure S8). We observed a slight increase of PHD2 activity in the presence of BSA (although the difference could be within error; Figure S8). Hence, although we cannot rule out that some CORM-2 may react covalently with PHD2, our results strongly indicate that the CO-dependent pathway is the predominant inhibition mechanism.

To further evaluate the effect of CO on PHD2 catalytic activity, we also performed PHD2 inhibition experiments by directly bubbling CO into a solution containing PHD2-substrate mixture. Our results showed that the PHD2-catalysed CODD hydroxylation was fully inhibited after bubbling CO through the solution for 10 seconds (Figure S9). However, as the bubbling of CO through the solution may also displace molecular oxygen from the buffer, careful interpretation of the data is required. Binding studies using native mass spectrometry were also attempted as previous studies have shown that it was possible to observe the binding of myoglobin to CO.[72] However, despite numerous attempts, we were unable to directly observe the PHD2-Fe(II)-CO complex.

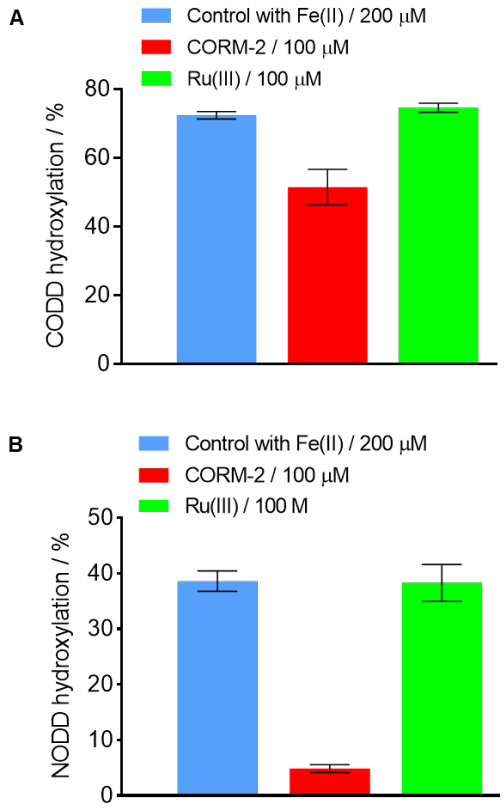

Figure 1. CORM-2 inhibits the hydroxylation of (A) CODD and (B) NODD to different extents. The reaction mixtures contained 1 μM PHD2, 200 μM Fe(II), 60 μM 2OG, 50 μM CODD/NODD, 100 μM CORM-2 or $RuCl_3$ (where applicable), 500 μM ascorbate in 50 mM Tris (pH 7.5). The reaction mixtures were incubated at 37 °C and the concentration of hydroxylated CODD/NODD was measured after 30 minutes of incubation. The results are expressed as mean ± standard error of the mean (n = 3).

Comparing the hydroxylation of CODD and NODD, we found that CO has a much stronger inhibitory effect on NODD hydroxylation than CODD hydroxylation (Figure 1). At 100 μM CORM-2 concentration, ~80% reduction of NODD hydroxylation was observed compared to only ~30% reduction of CODD hydroxylation under similar conditions. In order to quantify this effect, inhibition constants (IC_{50}) were determined. The IC_{50} values of CORM-2 when NODD and CODD were used as substrates were found to be 34.2 ± 1.3 μM and 142.5 ± 1.8 μM, respectively (Table 1 and Figure S10). This is an interesting observation, as our results showed that different substrates may modulate the inhibition potency of CO. CODD is a stronger binder than NODD, which is reflected by the binding constants (K_D values) (~14 μM and ~85 μM for CODD and NODD, respectively)[45] and, to a certain extent, the Michaelis constants (K_M values) for these two compounds (5.2 ± 1.3 μM and 107.1 ± 29.7 μM, respectively; Table 1 and Figure S11). In order to further study the effect of CO modulation of PHD2 activity towards different substrates, additional PHD2 substrates were explored. Previous studies showed that the N-terminal side of the PHD2 peptidyl substrates is important for interactions with the β2β3/loop region of PHD2, and the C-terminal side of the substrates is important for interactions with the α4 helix of PHD2.[73–75] It was also shown that it is possible to tune the hydroxylation of PHD2 peptidyl substrates by using CODD/NODD hybrids.[44] Using this information, we designed two CODD/NODD hybrid peptides by swapping the N- and C-terminal sides of the prolyl residue of CODD and NODD. The two peptides with the sequences DALTLLAAPYIPMDDDFQL and DLDLEMLAPYIPTIISLDF are denoted Hybrids #01 and #02, respectively. In Hybrid #01, the two leucine residues in –5 and –2 positions relative to the proline in the conserved LxxLAP motif were replaced by threonine and alanine, respectively. Huang et al. showed that PHDs could tolerate substrates with substitution in these positions.[76] Indeed, using the MADLI-TOF MS assay, it was found that both Hybrids #01 and #02 were substrates of PHD2. The K_M values of Hybrids #01 and #02 were found to be 36.6 ± 5.3 μM and 130.6 ± 31.6 μM respectively (Table 1 and Figure S11). We then measured the IC_{50} values of CORM-2 to PHD2 with Hybrids #01 and #02 as substrates. We found that the IC_{50} value of CORM-2 roughly followed the same trend (Table 1 and Figure S10), i.e. the lower the K_M value of the substrate, the higher the IC_{50} value. In comparison, the IC_{50} value of CORM-2 in the absence of peptidyl substrate (i.e. uncoupled turnover) was found to be 38.9 ± 1.1 μM (Table 1 and Figure S10).

In the consensus mechanism of 2OG-dependent oxygenases (Figure S12), the binding of the primary substrate generates an open coordination site on the Fe(II) centre at the active site by displacing a water ligand.[38,77] Our results may therefore reflect how different peptidyl substrates may modulate the access and/or binding of gaseous molecules (e.g. O_2 and CO) to the metal ion at the active site of PHD2. CO prefers to bind to Fe(II) with a linear geometry. It is possible that different substrates may distort the binding geometry of CO to different extents. The ability of peptidyl substrates to modulate the binding of gaseous molecules to 2OG-dependent oxygenases has been observed before.[78] For example, Taabazuing and co-workers reported that, by using density functional theory (DFT) calculations, the binding of peptidyl substrates may dictate and distort the binding geometry of nitric oxide (NO) at the metal centre of factor inhibiting HIF (FIH), another 2OG-dependent oxygenase that catalyses the hydroxylation of HIF.[78] Indeed, we found that the addition of excess peptidyl substrate (CODD or NODD) may rescue the activity of PHD2 against the inhibition by CO (Figure S13), further supporting the hypothesis that peptidyl substrates may affect the binding of gaseous ligands to PHD2. However, further modelling and/or structural-based experiments are needed to fully understand this effect (e.g. whether the prolyl residue of CODD/NODD directly clashes with the binding of CO in the linear geometry).

Overall, our work shows for the first time that CO is an inhibitor of HIF PHDs. There is evidence showing the involvement of CO in cellular oxygen sensing and signalling, including the stabilisation of HIF in normoxia.[23,24] Although previous work proposed that CO may induce stabilisation of HIF through a PHD-independent pathway (by increasing the interactions between HIFα and heat shock protein 90),[79] our results show that CO is an inhibitor of PHD2 and suggest that the PHD-dependent pathway could, at least in part, play a role in the stabilisation of HIF in normoxia. The regulation of PHDs and FIH by endogenously produced gasotransmitters such as CO and NO is an underexplored yet important area. It is already known that PHDs and FIH could be inhibited by NO.[80–82] There is also evidence that CO and NO may work together for their

physiological functions.[83] The cellular concentration of CO was estimated to be ~2 nM,[84] which is similar to the values reported for NO (~100 pM to ~5 nM).[85] The reported IC_{50} value of NO to PHD2 was 10 μM,[86] which is in the same range as the IC_{50} values obtained for CORM-2 in this study. Although these values are higher than the cellular concentrations of CO and NO, it has also been hypothesised that local CO concentrations, for example in the immediate vicinity where CO is being produced, may be significantly higher than the background concentration so that signalling can be triggered.[84] Therefore, our results may open up a new area of study, in particular addressing how CO and NO may interfere with the activity of PHDs (and other 2OG-dependent oxygenases) as well as their physiological relevance. Finally, there is increasing interest to exploiting the therapeutic potential of CO in medicine.[87,88] The observation that CO may inhibit PHDs, which themselves are molecular targets for the treatment of a number of diseases, may open up further avenues for the development of CO therapies.

Table 1. Table summarising the K_M and K_D values of the various peptidyl substrates of PHD2, and the IC_{50} values of CORM-2 when these peptides were used as substrates.

Peptidyl substrate	K_M / μM	K_D / μM	IC_{50} / μM
CODD: DLDLEMLAPYIPMDDDFQL	5.2 ± 1.3	~14 [a]	142.5 ± 1.8
NODD: DALTLLAPAAGDTIISLDF	107.1 ± 29.7	~85 [a]	34.2 ± 1.3
Hybrid #01: DALTLLAAPYIPMDDDFQL	36.6 ± 5.3	N/A	46.8 ± 1.1
Hybrid #02: DLDLEMLAPYIPTIISLDF	130.6 ± 31.6	N/A	24.4 ± 1.2
No substrate (uncoupled turnover)	N/A	N/A	38.9 ± 1.1

[a] K_D values for CODD and NODD were obtained from reference [45].

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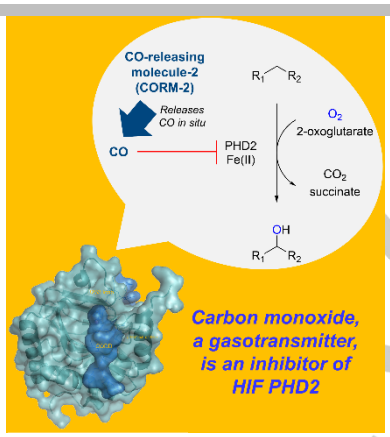
Carbon monoxide (CO) is an endogenously produced gasotransmitter that is involved in a range of physiological roles including the stabilisation of HIF, a transcription factor that regulates oxygen homeostasis. Herein, by using CO-releasing molecule-2 as a CO donor, we showed that CO is an inhibitor of HIF PHD2, one of the most important oxygen sensing enzymes in humans that regulates HIF stability at the protein level.



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