BJP British Journal of Pharmacology

DOI:10.1111/bph.12688
www.brjpharmacol.org

Themed Section: Pharmacology of the Gasotransmitters

REVIEW

Novel lead structures and activation mechanisms for CO-releasing molecules (CORMs)

U Schatzschneider

Institut für Anorganische Chemie, Julius-Maximilians-Universität Würzburg, Würzburg, Germany

Correspondence
Professor Dr Ulrich Schatzschneider, Institut für Anorganische Chemie, Julius-Maximilians-Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany. E-mail: ulrich.schatzschneider@uni-wuerzburg.de

Received
3 December 2013
Revised
28 February 2014
Accepted
6 March 2014

Carbon monoxide (CO) is an endogenous small signalling molecule in the human body, produced by the action of haem oxygenase on haem. Since it is very difficult to apply safely as a gas, solid storage and delivery forms for CO are now explored. Most of these CO-releasing molecules (CORMs) are based on the inactivation of the CO by coordinating it to a transition metal centre in a prodrug approach. After a brief look at the potential cellular target structures of CO, an overview of the design principles and activation mechanisms for CO release from a metal coordination sphere is given. Endogenous and exogenous triggers discussed include ligand exchange reactions with medium, enzymatically-induced CO release and photoactivated liberation of CO. Furthermore, the attachment of CORMs to hard and soft nanomaterials to confer additional target specificity to such systems is critically assessed. A survey of analytical methods for the study of the stoichiometry and kinetics of CO release, as well as the tracking of CO in living systems by using fluorescent probes, concludes this review. CORMs are very valuable tools for studying CO bioactivity and might lead to new drug candidates; however, in the design of future generations of CORMs, particular attention has to be paid to their drug-likeness and the tuning of the peripheral 'drug sphere' for specific biomedical applications. Further progress in this field will thus critically depend on a close interaction between synthetic chemists and researchers exploring the physiological effects and therapeutic applications of CO.

LINKED ARTICLES
This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

Abbreviations
BODIPY, boron dipyrromethene difluoride; CORM, CO-releasing molecule; EPR, electron paramagnetic resonance; ET-CORM, enzyme-triggered CORM; HO, haem oxygenase; Mb, myoglobin; MbCO, carboxymyoglobin; MO, molecular orbital; MOF, metal-organic framework; PhotoCORM, photoactivatable CORM; sGC, soluble guanylate cyclase; YFP, yellow fluorescent protein

Carbon monoxide: a small signalling molecule with specific target structures

Carbon monoxide (CO) is now well established as the third small signalling molecule in higher organisms, including humans (Wu and Wang, 2005; Kim *et al.*, 2006; Piantadosi, 2008). It is endogenously generated by the action of haem oxygenase (HO) on haem (see Alexander *et al.*, 2013c), which also leads to the formation of iron(II) and biliverdin. The latter is further converted to bilirubin by biliverdin reductase. The enzymatic mechanism has been elucidated at the molecular level by a combination of spectroscopic and

theoretical approaches (Chen *et al.*, 2008; Lai *et al.*, 2010; Matsui *et al.*, 2010) as well as X-ray crystal structure analysis (Friedman *et al.*, 2003; Rahman *et al.*, 2008; 2009). The two isoforms of HO, constitutively expressed as HO-2 and inducible HO-1, are involved in a large number of important physiological processes, in particular vasodilatation and responses to stress conditions (Morse and Choi, 2002; Ryter *et al.*, 2002; 2006; Abraham and Kappas, 2008; Piantadosi, 2008; Rochette *et al.*, 2013). CO signalling is intimately intertwined with that of the other two small signal-transducing molecules, NO and H_2S (Ignarro, 1999; Mustafa *et al.*, 2009; Kajimura *et al.*, 2010; 2012; Szabo, 2010; Li *et al.*, 2011). However, there is a fundamental difference in the reactivity of CO compared to NO and H_2S (Fukuto *et al.*, 2012), since at ambient pressure and temperature and in the absence of special catalysts, CO will only bind to transition metal centres, whereas the other two species react rapidly with both metal centres and many of the organic constituents of biological systems. This relative inertness and selective reactivity of CO has special implications for both the cellular target structures of CO and the development of delivery systems for CO in potential therapeutic applications (Motterlini and Otterbein, 2010; Wegiel *et al.*, 2013). Thus, the present review will first summarize the most important properties of CO and its binding to metal centres for the non-chemist. Then, a short but critical view will be given on the currently discussed cellular target structures of CO. The main focus, however, is on artificial systems for the delivery of CO to cells and tissues and the activation mechanisms that trigger the release of CO from such carriers. The most important lead structures of CO-releasing molecules (CORMs) will be critically discussed, with particular emphasis on the latest development in this field during the past few years. Another important issue is the *in vitro* and ultimately *in vivo* detection and tracking of CO and CORMs, which will be discussed before this review concludes with an outlook on the challenges of turning CORMs into viable drug candidates.

Carbon monoxide: properties and binding to metal centres

CO is a colourless and odourless flammable gas, which, in spite of its signalling function, is highly toxic to organisms at elevated concentrations. In that context, it should be noted that it is actually not so much an interference with oxygen transport in the blood due to tight binding of CO to the haem iron centres in haemoglobin, but rather the increase in tissue CO concentrations occurring upon inhalation, which disturb normal mitochondrial function, that is responsible for CO toxicity. As elegantly demonstrated by Goldbaum and co-workers, mammals actually have a rather large 'built-in' margin of safety in the oxygen-carrying capacity of the blood, and haemoglobin levels available for O_2 transport can be reduced to about 20–30% of the normal without detrimental effects, as long as high lung CO levels do not shift the equilibrium towards increased tissue accumulation (Goldbaum *et al.*, 1975; Foresti and Motterlini, 2010). With a melting point of –205.1°C and a boiling temperature of –191.5°C, CO exists as a gas at ambient conditions, which is only sparingly soluble in water (0.35 L of CO per 1 L of H_2O or 14 mM). The short carbon-oxygen bond distance (1.1282 Å in the gas phase) and high dissociation energy (1070 $kJ \cdot mol^{-1}$) are in line with the C≡O triple bond character. This is particularly evident from the molecular orbital (MO) diagram shown in Figure 1A. The overlap of the 2*s* atomic orbitals of carbon and oxygen leads to two σ and σ* MOs, whereas the 2*p* atomic orbitals form three bonding and three anti-bonding MOs, a pair of σ/σ* character and two each of π and π* character. Four of the bonding but only one of the anti-bonding MOs are doubly occupied, resulting in this exceptionally strong bonding. However, of particular importance for the reactivity and binding of CO to metals, is the presence of relatively low-lying empty MOs of π* character. As shown in Figure 1B, the energetically highest filled orbital of CO, which is of σ* character, can overlap with symmetry-adapted empty *d* orbitals on a transition metal centre along the M-C vector to form a bonding σ-donor interaction. At the same time, there is also a π-donor interaction between the next highest occupied π orbital on the CO with properly oriented empty metal *d* orbitals. In contrast, occupied metal d orbitals can also overlap with the empty low-lying MOs on the CO, forming an additional π-acceptor interaction, the so-called backbonding, which is among the characteristic features of transition metal carbonyl complexes and is responsible for the particular stability of this type of compound (Elschenbroich, 2006). Therefore, carbonyl complexes preferentially form with transition metal centres in relatively low oxidation states, since these provide filled *d* orbitals of proper energy to facilitate the backbonding. Furthermore, the strength of the M-(CO) bond can be modulated by the presence of electron-donating or withdrawing coligands on the metal-carbonyl fragment. A decrease in the metal d electron density will result in subsequent weakening of the backbonding and thus facilitate CO release from the metal coordination sphere. This can be achieved, for example, by chemical or electrochemical oxidation of a low-valent metal centre. Alternatively, electronic excitations due to absorption of light of a proper wavelength might also reduce the metal charge density in the excited state, particularly when metal-to-ligand charge transfer transitions are involved. These binding characteristics have important implications for the triggering of CO release from stable prodrug compounds, but also on the potential cellular target structures of CO. Another special feature of CO as a ligand is that it can coordinate terminally to a metal centre but also act as a μ_2- and even μ_3-ligand, bridging two or three metal centres respectively. Furthermore, in a suitable environment, it can even change its coordination mode from terminal to bridging and *vice versa* (Figure 1C).

Cellular target structures of carbon monoxide

Keeping in mind the special stability of the M-(CO) bond due to the interplay of bonding and backbonding interactions, it is very hard to imagine a recognition and binding of CO without the involvement of a transition metal centre, at least under physiological conditions. Therefore, it is not surprising that, in particular, haem proteins have been implicated as the

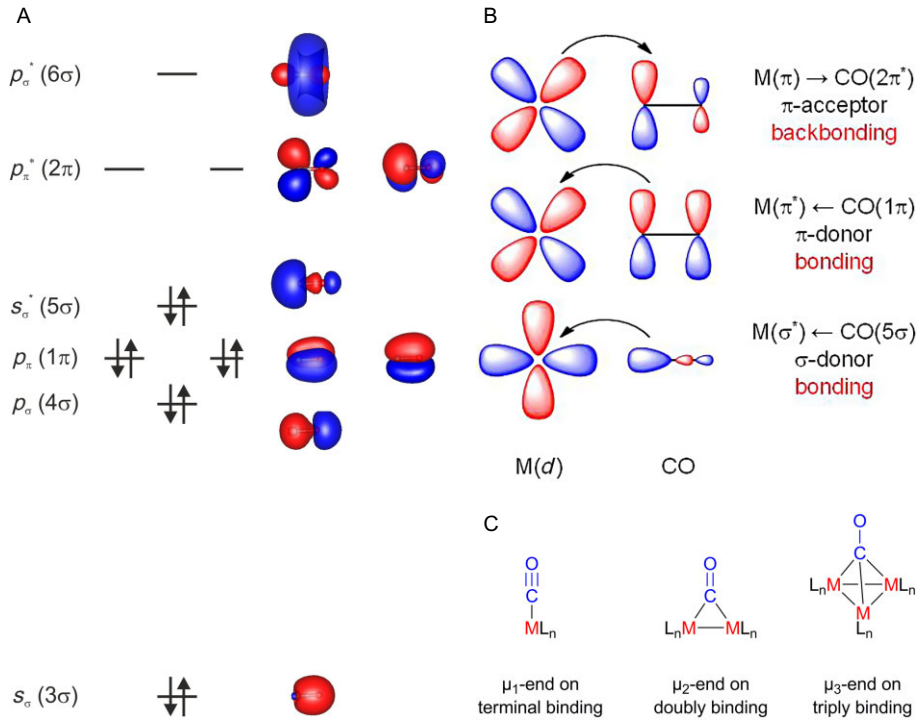

Figure 1

(A) Molecular orbital (MO) diagram of CO; (B) bonding and backbonding interactions in transition metal-CO complexes; and (C) CO coordination modes (Elschenbroich, 2006).

primary cellular targets for CO binding. Soluble guanylate cyclase (sGC) has been the focus of many earlier works in this field. It is a haem protein in which the haem co-factor is covalently anchored to the protein via an axial histidine ligation to the iron to give rise to a five-coordinate metal centre, with the position *trans* to the histidine able to bind either NO or CO, but not O_2 (Poulos, 2006; Derbyshire and Marletta, 2012). This leads to an increase in cGMP production from GTP by about two orders of magnitude, at least in the case of NO. The cGMP then triggers further down-stream signalling events, including phosphodiesterase and protein kinase activation as well as modulation of gated ion channels. The study of sGC structure and function has, however, for a long time been hampered by problems with the over-expression of the full-length protein, and thus, usually only shortened constructs are investigated. Furthermore, sGC activation by CO alone only leads to a two- to fourfold increase in cGMP production. Additional synthetic sGC activators such as YC-1 were shown to potentiate these effects (Ibrahim *et al.*, 2010), but the search for natural analogues thereof has so far remained elusive. Based on the very low sGC activation by CO alone, it is questionable whether this is indeed the main target of normal CO signalling in cells. Other haem proteins, where this activity seems to be better defined, have been identified more recently. Especially mentioned here is cystathionine-β-synthase, one of the enzymes involved in H_2S biosynthesis from sulphur-containing amino acids, and thus one of the sites of cross-talk between the different small-molecule signalling systems (Puranik *et al.*, 2006; Weeks *et al.*, 2009; Li *et al.*, 2011). Other targets include nuclear hormone receptors (Gupta and Ragsdale, 2011), and particularly in the case of very simple organisms, different gas sensor proteins (Uchida and Kitagawa, 2005; Podust *et al.*, 2008). Particularly intriguing, however, is the recent discovery that the activity of different ion channels, such as $K_{Ca}1.1$ (also know as BK_{Ca}), ENaC, $K_{2P}2.1$ (also known as TREK-1), K_v (see Alexander *et al.*, 2013b), $P2X_2$ and $P2X_4$ (Alexander *et al.*, 2013a), can be modulated by the application of CO (Wilkinson and Kemp, 2011). Taking the $K_{Ca}1.1$ channels as an example, it was demonstrated that they can be directly stimulated by CO and thus act as a gas sensor (Hou *et al.*, 2009). However, the initial hypothesis that haem bound to the channel is the CO binding site is not in line with more recent results and the location of the haem binding site and the CO interaction seem to be spatially separated. Through site-directed mutagenesis, a number of histidine and asparagine residues in the RCK1 domain were shown to be essential for the CO sensitivity, particularly His^{365}, His^{394} and Asp^{367} (Hou *et al.*, 2008). However, the molecular mechanism of the interaction of CO with this part of the ion channel is still elusive. In the absence of an X-ray crystal structure of the channel or at least of the RCK1 domain, homology arguments have been drawn upon to suggest that this sensory domain does not contain a haem co-factor or any other metal. Since membrane proteins are notoriously difficult to handle and purify, it is possible that a relatively labile metal site has been lost during the channel isolation and preparation. Clearly, more careful biophysical characterization of the native ion channels is urgently required, including metal analysis of the different preparations with inductively coupled plasma mass spectrometry or

atomic absorption spectroscopy, and electron paramagnetic resonance (EPR) as well as Mössbauer spectroscopy to try to detect a metal centre, with the latter method particularly well-suited for iron-containing biomolecules. An intriguing prospect is based on the observation that haem oxygenase 2 (HMOX2) seems to associate in the cellular membrane with $K_{Ca}1.1$ channels. Thus, it could be envisaged that both the ferrous iron as well as the CO generated by HMOX2's action on haem actually migrate within such a multi-enzyme cluster, without ever escaping to the cytosol, to form a very sensitive metal site *in situ* by iron coordination to the above-mentioned amino acid-derived ligands, which then binds the CO, but is easily lost upon handling during the purification procedure. A particularly well-studied example of such internal gas channels is found in dual-functional CO dehydrogenase/acetyl-coenzyme A synthase as part of the Wood-Ljungdahl pathway of anaerobic CO_2 and CO fixation in certain microorganisms (Ragsdale *et al.*, 2012). Furthermore, since NO has been shown to interact with iron-sulphur clusters, leading to the formation of dinitrosyl iron complexes (Tinberg *et al.*, 2010), one could speculate that CO might also target this very important class of metal centres, having functions in electron transfer and catalysis in a very wide range of proteins.

Delivery systems for carbon monoxide gas

One option to modulate the concentration of CO in an organism for biomedical applications is of course the pharmacological stimulation of haem oxygenase-1 activity (Li *et al.*, 2007; Abraham and Kappas, 2008). However, many of the current approaches are more focused on an exogenous delivery of CO. The simplest way to achieve this is inhalation of a gas mixture containing CO. Due to the very high general toxicity of CO, this has to be carried out in a carefully controlled way, to prevent adverse effects on the patient and the medical personnel. In fact, there are devices now available that will adjust the quantity of CO gas delivered, depending on the breathing rate as well as other parameters, and immediately shut off the supply under abnormal conditions (Motterlini and Otterbein, 2010). Although this mitigates the safety issues associated with this mode of delivery, a fundamental drawback of the application of CO by inhalation remains. This is related to the fact that once the CO is taken up via the respiratory system, its further distribution is determined by the partition ratio between the different body fluids and tissues. Since this is a fixed value, it is very difficult to specifically address particular disease sites in the organism using this form of application, particularly when they are not part of the vascular system.

CORMs: general design principles and activation mechanisms for CO release

Due to the above-mentioned inherent limitations in the use of CO gas, about 10 years ago, Motterlini and co-workers introduced the concept of using a chemically bound form of CO as a prodrug for physiological CO release, for which the term CO-releasing molecules (CORMs) was coined (Motterlini *et al.*, 2002). Although a number of organic compounds was initially explored for their potential as CORMs, including haloalkanes, aldehydes, oxalates and silacarboxylic acids (Mann, 2010; Romao *et al.*, 2012), it turned out that their release rate and toxicological profile were not favourable to justify further development. Consequently, due to the strong but modifiable binding of CO to transition metal centres, most of the research on CORMs has focused on metal carbonyl complexes. A notable exception, however, is a family of main group boranocarbonates introduced by Alberto's group (Motterlini *et al.*, 2004; Pitchumony *et al.*, 2008; 2010). Furthermore, very recently, two organic systems based on unsaturated cyclic 1,2-diketones and xanthene-9-carboxylic acid were introduced as the first really viable purely organic CORMs (Antony *et al.*, 2013; Peng *et al.*, 2013). Nevertheless, it is still the transition metal-based CORMs that offer the greatest flexibility in terms of molecular design and thus they will be the focus of the discussion herein. This is due to the fact that by proper selection of the metal centre, the number and spatial arrangement of CO ligands around it, and the nature of the coligands completing the preferred coordination sphere of the metal, it is possible to tune the CO-release properties in a wide range (Figure 2). Consequently, this 'inner' part of such a molecule has been termed the *CORM sphere* or *coordination sphere* by Romao *et al.* (2012). Key parameters are the number of CO molecules that can be released from the metal coordination sphere, the kinetics of the CO release process and the trigger mechanism required to initiate the liberation of the CO. Currently, there is no consensus on whether a high or a low number of CO ligands per metal complex unit would be more desirable and whether the CO release should be slow or fast. Monocarbonyl compounds of course have the simplest release kinetics, while in the case of more than one labile CO present per molecular unit, these might be liberated consecutively at a different speed, thus making kinetic analysis and identification of potential intermediates much more difficult. Furthermore, it is not clear whether a low but steady release of CO from CORMs would

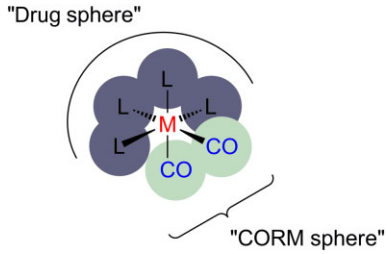

Figure 2
The ligand environment around a metal-carbonyl complex can be divided in a 'CORM sphere' and a 'drug sphere'. While the former determines the stoichiometry and kinetics of the CO release from the metal centre by proper choice of metal, metal oxidation state and coligands, the latter, as mainly defined by the periphery of the coligands L, can be modulated to enable tissue-specific targeting (Romao *et al.*, 2012).

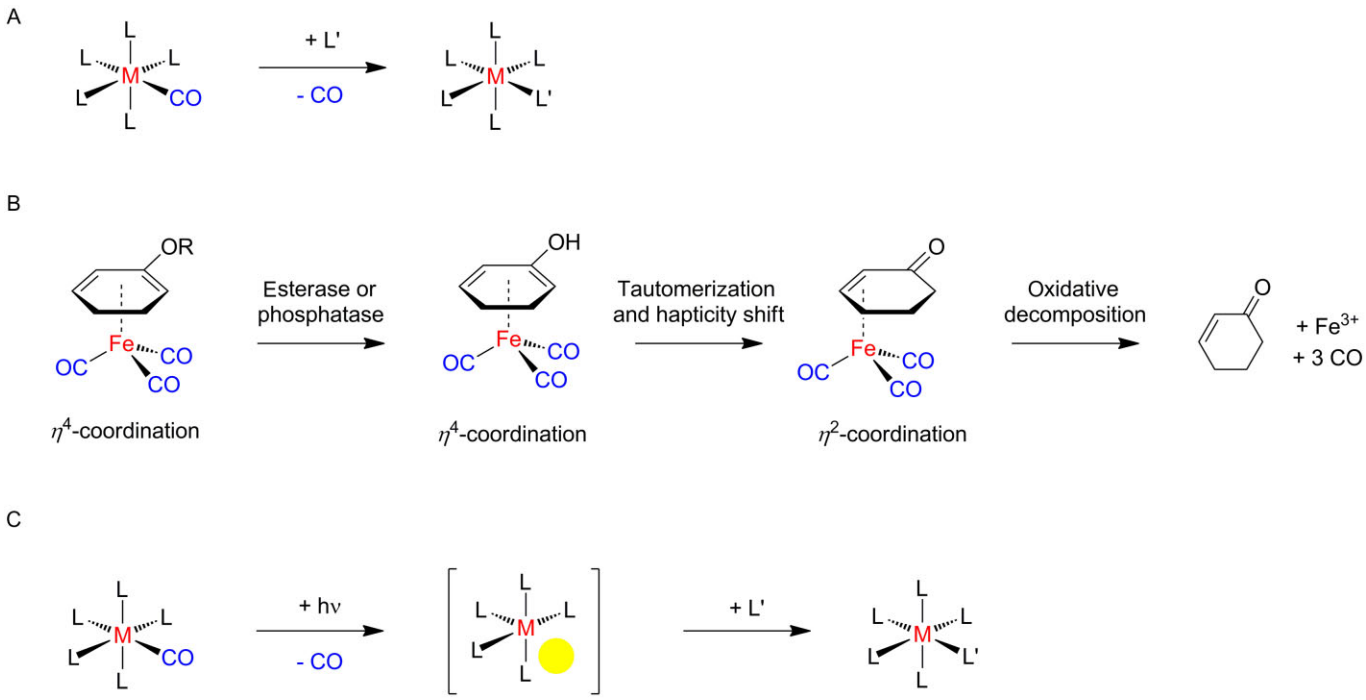

Figure 3

Common trigger mechanisms to initiate CO release from a metal coordination sphere: (A) ligand-exchange triggered, (B) enzyme-triggered and (C) photoinduced release.

be most beneficial, or rather a quick burst of CO liberated. Very likely, there is no single answer to these questions and, instead, different sets of CORMs should be designed with these properties specifically optimized for a particular medical application in mind.

In addition to the *CORM sphere*, which can be tuned to control the CO release, it is also possible to modify the (co)ligand periphery pointing away from the metal centre. This is particularly important and the most significant advantage of CORMs over simple CO gas, since it allows the modulation of their partition ratio between the different body fluids and tissues and might also enable an active or passive targeting of specific cell subpopulations. Thus, the 'outer part' of CORMs has been termed the *drug sphere* (Figure 2), since it will dominate the pharmacological profile of these compounds (Romao *et al.*, 2012). Unfortunately, much research has so far mainly focused on the CO-release properties and the proper tuning of the *drug sphere* has been more or less neglected, although this is absolutely vital if CORMs are indeed to be used beyond simple tools for fundamental biological studies on small-molecule signalling and turned into real drugs. However, the optimization of the *drug sphere* will require a clear focus on selecting and treating a particular medical condition, as nicely illustrated by liver targeting of CORMs in recent work from the Romao group (Marques *et al.*, 2012). In the context of targeting specific cells and tissues, another currently unresolved question is whether it will actually be necessary for the CORM to exert its biological action to enter the cells by passive or active uptake, or whether it will be sufficient to liberate the CO close enough to the target site, which it will then reach by diffusion through cellular membranes. Since the diffusion behaviour and maximum free path length of CO in complex biological systems is not known at present, it is difficult to estimate how close will be close enough. However, some mathematical modelling on the 'sphere of action' of H_2S has already been carried out and it would be highly desirable if such methodology is also applied to the study of CO distribution (Cuevasanta *et al.*, 2011).

Since CORMs serve as prodrugs for CO delivery, a very important question is also the trigger mechanism by which the CO liberation is actually induced, since this will be important to control site-specific delivery. In most of the metal-carbonyl complexes initially investigated for their suitability as CORMs, the release of CO from the metal coordination sphere is evoked by ligand exchange reactions with the medium (Figure 3). For example, while CORM-3, [RuCl(glycinato)$(CO)_3$], has a half-life of 98 h in distilled water at 37°C, this is reduced by three orders of magnitude to just 3.6 min in human plasma, probably due to an interaction with the thiol from glutathione (Johnson *et al.*, 2007). Since upon i.v. delivery, CORMs will immediately be exposed to high concentrations of bio(macro)molecules as potential ligands, the tissue distribution and targeting capability of such ligand-exchange triggered 'conventional' CORMs will largely depend on the fine balance between their half-life in complex medium and the time required to reach and accumulate at a specific target site in the body. One way to modulate this is by incorporation of the CORM inro a local hydrophobic environment and such an approach has indeed been explored by incorporation of CORM-3 into a triblock co-polymer composed of a CO-releasing domain flanked by hydrophilic and hydrophobic

sections, which self-assemble to a CO-releasing micelle with release kinetics slowed down compared with the free parent compound (Hasegawa *et al*., 2010).

In addition, researchers have started to explore alternative trigger mechanisms, in which a metal-carbonyl complex serves as a prodrug entirely stable towards ligand exchange even with highly abundant biological nucleophiles, and the CO release is only induced by a proper internal or external stimulus (Figure 3).

The most attractive external stimulus is probably light, since highly focused and pulsed light sources will allow for a very precise spatial and temporal control of the biological activity of such photoactivatable CO-releasing molecules (PhotoCORMs) (Schatzschneider, 2011; Rimmer *et al*., 2012). In addition to topical applications, for example, in the treatment of skin diseases, it can generally also be envisioned to target sites deeper inside solid tissue due to modern developments in waveguide technology, but the direct correlation between excitation wavelength and tissue penetration depth remains a major problem (Lane, 2003; Szacilowski *et al*., 2005; Agostinis *et al*., 2011). Red light or even infrared photoactivation is the ultimate goal, both to ensure that structures deeper inside the body can be reached and to minimize photodamage to healthy tissue in the beam pathway.

A different way of elegantly combining external stimulation with ligand-exchange triggered CO-release from metal carbonyl complexes was recently reported by Kunz *et al*. (2013). In this work, a catecholate-modified CORM-3 was anchored to maghemite (Fe_2O_3) nanoparticles, which, upon exposure to an alternating magnetic field, locally heat up; this then accelerates the ligand exchange of CO with medium, leading to a twofold increase in CO release in some of the models studied.

More recently, work has also started to utilize differences in cellular microenvironments for a localized control of the CO release. While parameters like cellular pH and redox environment might also be exploited in this context, an interesting alternative approach was taken by the group of Schmalz, which is based on potential differences in cellular enzyme expression rates. These researchers prepared metal carbonyl complexes in which an organometallic ligand is 'trapped' in one of two possible tautomeric forms by ester formation or *O*-phosphorylation. Exposure of these CO prodrugs to esterases or phosphatases leads to a hapticity change of the metal-carbonyl unit, resulting in increased sensitivity to dioxygen and subsequent CO release (Romanski *et al*., 2011; 2012a,b; Botov *et al*., 2013). Although these enzyme-triggered CO-releasing molecules (ET-CORMs) are still at an exploratory stage, this approach also holds great promise for tissue-specific, internally-triggered, controlled CO bioactivity.

CORMs: important lead structures and novel developments

A significant number of different CORMs based on transition metal as well as main group elements have been reported during the past decade and a 'periodic table of CORMs' is shown in Figure 4. Since this extensive body of work has already been reviewed, for example, by Mann and co-workers (Mann, 2010; Romao *et al*., 2012; Zobi, 2013), no attempt will be made here to provide a comprehensive coverage of all CORMs published to date. Instead, the discussion will focus on some of the most important and most widely used CORMs with a particular focus on their benefits and limitations, and then particularly new developments and further prospects for the field.

Initially, iron pentacarbonyl ($Fe(CO)_5$) and dimanganese decacarbonyl ($Mn_2(CO)_{10}$) were explored. However, these were found to be only poorly bioavailable due to their non-polar nature and also require photoactivation (Motterlini *et al*., 2002). Thus, most studies on the cellular delivery and biological activity of CO today utilizes either CORM-2 ($[RuCl(\mu\text{-}Cl)(CO)_3]_2$) or CORM-3 ($[RuCl(glycinato)(CO)_3]$). Although many interesting results have been obtained employing these two CORMs (Figure 5), they are far from ideal for this purpose. In particular, CORM-3 shows a very complicated and solvent-dependent speciation in solution, which is also further influenced by the pH of the medium. A large number of different isomers and adducts can form, which are difficult to track down and might have variable CO release kinetics. This is particularly evident in the case of CORM-3, which shows a wide variation in half-life depending on the medium (Johnson *et al*., 2007). Furthermore, the $Ru(CO)_n$ fragment was shown to bind to surface-accessible amino acid side chains in proteins such as lysozyme (Santos-Silva *et al*., 2011; Santos *et al*., 2012). Since ruthenium complexes are widely explored in the context of anticancer chemotherapy (Alessio *et al*., 2004; Melchart and Sadler, 2006; Bratsos *et al*., 2007; Levina *et al*., 2009; Süss-Fink, 2010), it is very important to keep in mind that the metal–coligand fragment remaining after liberation of CO from the coordination sphere might well possess biological

H																	He
Li	Be											B	C	N	O	F	Ne
Na	Mg											Al	Si	P	S	Cl	Ar
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn

Figure 4

Periodic table of CO-releasing molecules (CORMs). Elements for which CORMs have been reported to date are highlighted in grey.

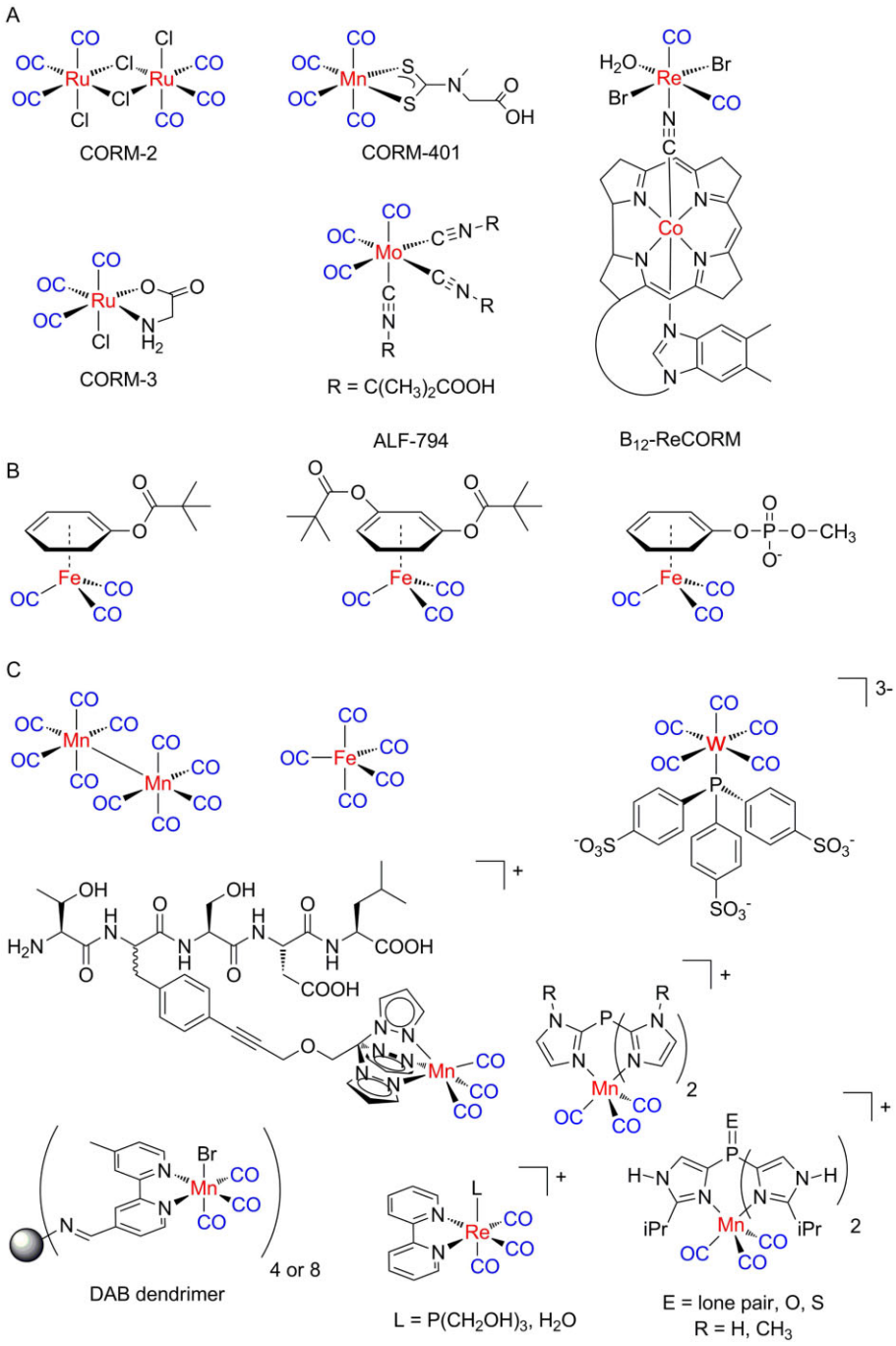

Figure 5

Selected examples of CO-releasing molecules, by activation mechanism: (A) ligand-exchange triggered CORMs, (B) enzyme-triggered CORMs (ET-CORM), and (C) photoactivated CORMs (PhotoCORMs).

activity on its own. Unfortunately, these reaction products, termed inactivated CORMs (iCORMs), are often not structurally characterized and, in many cases, not assayed for bioactivity separately. Thus, unless very careful controls are included in a study, using both CO gas as a positive and well-characterized iCORMs as a negative control, it remains doubtful whether the biological effects observed are actually indeed due to the CO only, or rather result from the metal–coligand fragment remaining, or at least a combination of both. Furthermore, since these CORMs have not been designed for a specific biomedical application, their drug sphere is probably not ideal in terms of cellular targeting, uptake and intracellular distribution.

A notably different approach has been taken by Romao *et al.*, who very carefully modified and explored a large family of molybdenum(0) tricarbonyl complexes of the general

formula $[Mo(CO)_3(L)_3]$, in which L is a neutral monodentate ligand, for the treatment of acute livery injury resulting from poisoning with acetaminophen (Marques *et al.*, 2012). It turned out that isonitriles were the ligands of choice, and the correct selection of peripheral functional groups indeed led to the desired tissue selectivity. Furthermore, for a related molybdenum(0) compound, Na[Mo(histidinate)$(CO)_3$], it was shown that this is finally metabolized to a polyoxomolybdate cluster $[PMo_{12}O_{40}]^{3-}$, as demonstrated by X-ray crystallography of this species bound to lysozyme (Seixas *et al.*, 2013). Thus, instead of somewhat arbitrarily preparing and screening further metal carbonyl complexes, there is a very clear need for compounds that are designed for a very particular biomedical application and thoroughly examined not only for their CO release behaviour, but also the follow-up products.

Since the compounds discussed previously all start to undergo CO release as soon as they are dissolved in the medium, it is largely their half-lives in the circulation after i.v. injection that determine the target structures that can be addressed in the body. Recent work is therefore aimed at CORM prodrugs that are stable in serum and only get triggered in the targeted tissue by a very specific stimulus. A very interesting example of this class of compounds is the enzyme-triggered CORMs (ET-CORMs) from Schmalz's group (Romanski *et al.*, 2011; 2012a,b; Botov *et al.*, 2013). This concept is based on the trapping of an α,β-unsaturated ketone, such as cyclohexenone, in the enolate form by ester formation or *O*-phosphorylation. The resulting diene, with the acyloxy or phosphoryloxy group in either the 1 or 2 position, then acts as a η^4-ligand to the $Fe(CO)_3$ fragment (Figure 5), which is introduced by reaction with diiron nonacarbonyl. Cleavage of the C–O or P–O bond by suitable esterases or phosphatases regenerates the dienol, which undergoes a hapticity change from η^4 to η^2. These intermediates are much more prone to oxidative decomposition, giving rise to ferric iron and the α,β-unsaturated ketone, as well as the release of all three equivalents of carbon monoxide. Differences in tissue expression rates of esterases or phosphatases between organs as well as healthy and diseased sites might allow for a locally restricted CO release and thus bioactivity. Although the potency of the iron(III) as well as the α,β-unsaturated ketone needs to be further explored in separate controls, this system also gives rise to well-defined follow-up products, which can be independently prepared and assessed. An alternative, external trigger is based on the use of light to induce CO release from a transition metal carrier system (Schatzschneider, 2010; 2011; Rimmer *et al.*, 2012). In addition to the problems with independent evaluation of the iCORM products, which is inherent to all CORMs (*vide supra*), these PhotoCORMs face an additional difficulty due to the inverse correlation between the tissue penetration depth of light and the incident wavelength. Thus, the further the PhotoCORM absorption can be shifted to the red or even infrared part of the electromagnetic spectrum, the more likely the deeper structures can be addressed inside the tissue (Lane, 2003; Szacilowski *et al.*, 2005). In addition to tuning the PhotoCORM absorption itself, alternative strategies might be based on its conjugation to established photosensitizers, the exploration of two-photon excitation and the use of up-converting nanoparticles. A significant number of such compounds have been explored in the last few years, in particular based on iron and manganese compounds (Niesel *et al.*, 2008; Gonzalez *et al.*, 2011; 2012a,b; Ward *et al.*, 2012). A particularly interesting PhotoCORM has been reported by Pierri and Ford. The rhenium(I) compound $[Re(bpy)(CO)_3(PR_3)]^+$, with $R = CH_2OH$, upon photolysis at 405 nm, undergoes specific liberation of only one of the three carbonyl ligands, the one *trans* to the phosphane group (Pierri *et al.*, 2012). The most interesting feature of this system is due to the fact that both the starting material as well as the resulting aqua compound are luminescent and can be tracked inside cells by confocal fluorescence microscopy with excitation at 405 nm and detection at 465–495 nm versus 660 nm, respectively, for the PhotoCORM and the follow-up product (Figure 5). Also, very recently some of the first non-metal-based CORMs with photoactivation have been reported (Antony *et al.*, 2013; Peng *et al.*, 2013).

An alternative external stimulus to trigger CO-release locally from a prodrug system is the application of an alternating magnetic field to CORM-loaded magnetic nanoparticles, which leads to local heating and thus acceleration of the ligand exchange reaction of CO with medium, as demonstrated by Kunz *et al.* (2013).

Finally, a number of carrier systems have been used to confer some target specificity to CORMs. This includes conjugation to biomolecules and macromolecules as well as soft and hard nanomaterials. For example, Zobi and co-workers have attached rhenium-based CO-releasing groups to cobalamin as a potential naturally occurring carrier system (Zobi *et al.*, 2012), whereas the Schatzschneider group as well as others use peptide conjugates for CORM conjugation (Pfeiffer *et al.*, 2009; 2013; Matson *et al.*, 2012). Other hard and soft nanomaterials used for the attachment of CORMs include silica nanoparticles as well as carbon nanomaterials (Dördelmann *et al.*, 2011; 2012), dendrimers (Govender *et al.*, 2013), and micelles (Hasegawa *et al.*, 2010). Alternative approaches do not depend on the attachment of molecular metal-carbonyl units to nanoscale carrier systems, but are instead based on the direct loading of porous nanomaterials like metal-organic frameworks (MOFs) with CO gas, which is liberated again upon controlled degradation of the material (Ma *et al.*, 2013). Of course, good biocompatibility of both the organic and the inorganic parts of the MOFs has to be ensured for potential therapeutic applications.

In summary, a wide range of different trigger mechanisms to induce the CO release from the metal coordination sphere and a significant number of core structures now exist, and these have been explored for use as CORMs. Future generations of CORMs to be added to this arsenal certainly have to be designed with very clear therapeutic applications in mind, in order to properly tune the release kinetics and stoichiometry to the desired range, and with a specific emphasis on the drug-like properties of any new compound. Thus, much more emphasis has to be placed on the choice and variation of the 'drug sphere' in the future and the biomedical community has to provide information on the required amount of CO at a target site to elicit a physiological response (high or low) and the time scale over which a certain concentration level needs to be kept (fast or slow CO release).

Detecting and tracking CO and CORMs

The quantification of the number of CO ligands released from a CORM as well as the release kinetics and the nature of potential intermediates, if the CO ligands are released in a stepwise instead of a concerted process, is a very important parameter in the characterization of CORMs.

The simplest method for this purpose is the so-called 'myoglobin assay', in which the conversion of dexoymyoglobin (Mb) to carboxymyoglobin (MbCO) is followed spectrophotometrically, by the decrease in intensity of the band of Mb at 557 nm and the increase of the MbCO absorption at 540 and 577 nm. The amount of CO released per molecular unit of carbonyl compound is then determined from the plateau value at the end of the measurement, which is reached once no more spectral changes can be observed at prolonged reaction time, and the kinetics can be obtained from a proper fitting of the increase in MbCO absorption with time. For this type of experiment, one always has to ensure that the molar amount of Mb exceeds the maximum number of moles of CO that a CORM may potentially release, in order to provide at least one free Mb for each CO liberated and thus prevent premature signal saturation. Although the myoglobin assay will probably remain the procedure of choice for initial screening for CORM activity since it is very easy to carry out, there are some severe limitations with this method. First of all, especially for highly coloured metal-carbonyl compounds, which is usually the case with Photo-CORMs, there might be an overlap in the absorption bands of the myoglobin, the CORM, and potentially also the follow-up iCORM (inactivated CORM) products (Figure 6), which will require complicated spectral deconvolution techniques (Atkin *et al.*, 2011). Furthermore, the assay has to be carried out under an atmosphere of dinitrogen or argon protective gas in order to keep the iron centre in the ferrous (+II) state and to prevent formation of oxymyoglobin from air. However, some CORMs, particularly the ET-CORMs from the Schmalz group, require an oxidative process to complete the CO release from the metal coordination sphere (Romanski *et al.*, 2011). The reduction of the myoglobin is usually carried out by the addition of a large excess of aqueous sodium dithionite, which causes additional problems due to sulphite species generated from the reductant, which might react with either the CORM or the myoglobin (McLean *et al.*, 2012). Since commercially available dithionite is often not of the purity required for such experiments, it needs to be carefully checked and, if necessary, recrystallized before use in the myoglobin assay (McKenna *et al.*, 1991). Another currently unresolved question is whether the release of the CO from the CORM and its binding to the myoglobin are two totally uncoupled processes, with free CO intermediately present in solution, or whether there might be a more intimate association between the CORM and the myoglobin, with a more or less direct transfer of CO between the two metal centres, at least in some special cases.

Figure 6

Application of a trigger to a CO-releasing molecule (CORM) prodrug leads to the release of CO from the metal coordination sphere. In addition to the CO thus generated, inevitably a metal–coligand fragment, termed inactivated CORM (iCORM), is also formed, which might have biological activity on its own in addition to that of the CO. Therefore, iCORMs should always be independently prepared, fully characterized and included in bioassays as a control.

Therefore, a number of alternative methods have been explored to study the release kinetics of CO and to quantify the amount of CO liberated per mole of CORM. They are usually based on a separation of the CORM solution from the sensor unit (Mann, 2010; Rimmer *et al.*, 2012) and also circumvent the use of reducing or anoxic conditions. The most accurate but also most expensive technique is GC, which is to be considered the gold standard in the field (Vreman *et al.*, 2005; 2011; Bernardi *et al.*, 2008).

An even more challenging field is the *in vitro*/*in vivo* detection of either CO endogenously generated by HO activity or exogenously delivered from CORMs. Only very recently, two fluorescent sensor systems have been described, which are initial proof of principle for this concept (Yuan *et al.*, 2013). Thus, He and co-workers developed a genetically encoded fluorescent probe, which is based on the CO binding affinity of the CooA protein, which has been fused to yellow fluorescent protein (YFP) (see Figure 7A). Upon CO binding to the CooA domain, the whole construct, called 'COSer' (CO Sensor), undergoes a conformational change, which leads to the YFP emission lighting up (Wang *et al.*, 2012). Significant signal response was found down to 1–2 μM CO and little interference was observed from imidazole, cyanide, NO and dioxygen. However, a significant drawback of this system is the need to transfect cells to be studied with a COSer-containing expression vector, which will very likely severely limit its broader application. In contrast, Chang *et al* followed an approach based on a synthetic small molecule, which is composed of a BODIPY (boron dipyrromethene difluoride) unit and an attached cyclometalated dimeric palladium(II) moiety, which has been termed 'COP-1' (CO Probe 1) (see Figure 7B). While this compound is non-luminescent, addition of CO leads to CO insertion in the carbon-palladium bond with subsequent carboxylation of the pendant substituent and the probe then lights up (Michel *et al.*, 2012). Challenge experiments with a number of small molecules were carried out and a detection limit of about 1 μM was estimated. Although this small-molecule probe is much more suitable for general use, both in solution and in cell cultures, important questions remain. Firstly, what is the fate of the palladium metal liberated during the CO binding process by the probe, and secondly, what is the membrane permeability of COP-1 and whether a uniform distribution inside cells can generally be reached. In addition, the concentration ratio of COP-1 to CORM as well as the proper order and timing of the addition of both the fluorescent probe and the CORM to cell cultures seems to play a very important role in obtaining meaningful results. Thus, although these two systems finally

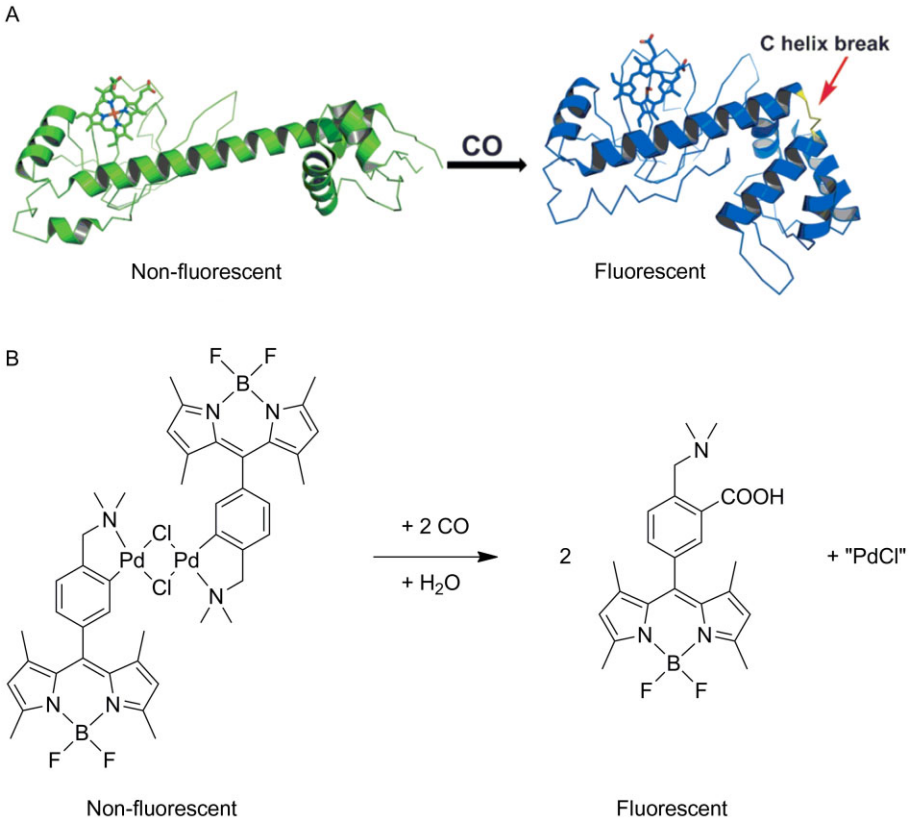

Figure 7
(A) 'CO Sensor' (COSer) and (B) 'CO Probe 1' (COP-1) as fluorescent switch-on probes for CO.

provide some initial tools to study *in vitro*/*in vivo* CO generation and delivery, there is clearly an urgent need for further improvement in fluorescent CO detection in complex biological environments.

Summary, conclusion and outlook: challenges to turn CORMs into drugs

CO is now well established as a small signalling molecule in higher organisms. While its endogenous production by the action of HO enzymes on haem and the regulation of this process is quite well understood now at the molecular level, this is not the case with the direct down-stream effects of the CO generated. Although the chemical properties of CO strongly suggest that binding to a transition metal centre will be required for its recognition, surprisingly little is known about its primary targets in the cell. Although implicated for a long time, sGC is rather questionable since its activation by CO is very low compared to NO in the absence of additional synthetic effectors, for which no natural analogue has been found so far. However, a fascinating prospect is the activation of ion channels by CO, although further light needs to be shed on the molecular details of this process. It is quite possible that a rather labile metal site has so far been lost during isolation and purification of these delicate to handle membrane proteins and better application of current biophysical techniques such as Mössbauer and EPR spectroscopy as well as metal analysis will certainly be required to solve this riddle. To modulate these signalling pathways by exogenous application of CO sources, for either fundamental biological studies or potential therapeutic applications, metal carbonyl complexes as CORMs have emerged as very valuable tools and a wide variety of such compounds with different trigger mechanisms (ligand exchange, enzymatic activation, photoactivation) is now available with modifiable release stoichiometry and kinetics. Recent efforts are also under way to modify the outer periphery of such compounds, the so-called 'drug sphere', to modulate the target specificity of CORMs, either by careful choice of peripheral functional groups or attachment to carrier systems such as bio(macro)molecules or hard and soft nanomaterials. Further progress in the field will depend on reliable methods to study the CO release from these carrier systems and in particular on the tracking of CORMs as well as CO in complex biological environments, both *in vitro* and *in vivo*. Although some recent progress has been made in the latter field with the introduction of the first fluorescent probes for CO, further development is urgently needed. The major challenge is now to design systems suitable for real clinical applications and take them beyond animal models of disease.

Acknowledgements

Our own work in this field was greatly facilitated by COST action BM 1005 'Gasotransmitters: from basic science to therapeutic applications (ENOG: European Network on Gasotransmitters)' through a number of travel grants and a short-term scientific mission (STSM) of C. Nagel from Würzburg to Sheffield (laboratory of Prof. Dr. R.K. Poole).

Conflict of interest

The author has no conflict of interest to declare.

References

Abraham NG, Kappas A (2008). Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 60: 79–127.

Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO *et al*. (2011). Photodynamic therapy of cancer: an update. CA Cancer J Clin 61: 250–281.

Alessio E, Mestroni G, Bergamo A, Sava G (2004). Ruthenium antimetastatic agents. Curr Top Med Chem 4: 1525–1535.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013a). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-gated ion channels. Br J Pharmacol 170: 1582–1606.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Ion channels. Br J Pharmacol 170: 1607–1651.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Antony LAP, Slanina T, Sebej P, Solomek T, Klan P (2013). Fluorescein analogue xanthene-9-carboxylic acid: a transition-metal-free CO releasing molecule activated by green light. Org Lett 15: 4552–4555.

Atkin AJ, Lynam JM, Moulton BE, Sawle P, Motterlini R, Boyle NM *et al*. (2011). Modification of the deoxy-myoglobin/carbonmonoxy-myoglobin UV-vis assay for reliable determination of CO-release rates from organometallic carbonyl complexes. Dalton Trans 40: 5755–5761.

Bernardi C, Chiesa LM, Soncin S, Passero E, Biondi PA (2008). Determination of carbon monoxide in tuna by gas chromatography with micro-thermal conductivity detector. J Chromat Sci. 46: 392–394.

Botov S, Stamellou E, Romanski S, Guttentag M, Alberto R, Neudörfl J *et al*. (2013). Synthesis and performance of acyloxy-diene-Fe$(CO)_3$ complexes with variable chain lengths as enzyme-triggered carbon monoxide-releasing molecules. Organometallics 32: 3587–3594.

Bratsos I, Jedner S, Gianferrara T, Alessio E (2007). Ruthenium anticancer compounds: challenges and expectations. Chimia (Aarau) 61: 692–697.

Chen H, Moreau Y, Derat E, Shaik S (2008). Quantum mechanical/molecular mechanical study of mechanisms of heme degradation by the enzyme heme oxygenase: the strategic function of the water cluster. J Am Chem Soc 130: 1953–1965.

Cuevasanta E, Denicola A, Alvarez B, Möller MN (2011). Solubility and permeability of hydrogen sulfide in lipid membranes. PLoS ONE 7: e34562.

Derbyshire ER, Marletta MA (2012). Structure and regulation of soluble guanylate cyclase. Ann Rev Biochem. 81: 533–559.

Dördelmann G, Pfeiffer H, Birkner A, Schatzschneider U (2011). Silicium dioxide nanoparticles as carriers for photoactivatable CO releasing molecules (PhotoCORMs). Inorg Chem 50: 4362–4367.

Dördelmann G, Meinhardt T, Sowik T, Krüger A, Schatzschneider U (2012). CuAAC click functionalization of azide-modified nanodiamond with a photoactivatable CO-releasing molecule (PhotoCORM) based on $[Mn(CO)_3(tpm)]^+$. Chem Commun 48: 11528–11530.

Elschenbroich C (2006) Organometallics, 3rd edn. Wiley-VCH: Weinheim.

Foresti R, Motterlini R (2010). Interaction of carbon monoxide with transition metals: evolutionary insights into drug target discovery. Curr Drug Targets 11: 1595–1604.

Friedman J, Lad L, Deshmukh R, Li H, Wilks A, Poulos TL (2003). Crystal structure of the NO- and CO-bound heme oxygenase from *Neisseriae meningitidis*. Implications for O_2 activation. J Biol Chem 278: 34654–34659.

Fukuto JM, Carrington SJ, Tantillo DJ, Harrison JG, Ignarro LJ, Freeman BA *et al*. (2012). Small molecule signaling agents: the integrated chemistry and biochemistry of nitrogen oxides, oxides of carbon, dioxygen, hydrogen sulfide, and their derived species. Chem Res Toxicol 25: 769–793.

Goldbaum LR, Ramirez RG, Absalon KB (1975). What is the mechanism of carbon monoxide toxicity? Aviat Space Environ Med 46: 1289–1291.

Gonzalez MA, Fry NL, Burt R, Davda R, Hobbs A, Mascharak PK (2011). Designed Iron carbonyls as carbon monoxide (CO) releasing molecules: rapid CO release and delivery to myoglobin in aqueous buffer, and vasorelaxation of mouse aorta. Inorg Chem 50: 3127–3134.

Gonzalez MA, Carrington SJ, Fry NL, Martinez JL, Mascharak PK (2012a). Syntheses, structures, and properties of new manganese carbonyls as photoactive CO-releasing molecules: design strategies that lead to CO photolability in the visible region. Inorg Chem 51: 11930–11940.

Gonzalez MA, Yim MA, Cheng S, Moyes A, Hobbs AJ, Mascharak PK (2012b). Manganese carbonyls bearing tripodal polypyridine ligands as photoactive carbon monoxide-releasing molecules. Inorg Chem 51: 601–608.

Govender P, Pai S, Schatzschneider U, Smith G (2013). Next generation PhotoCORMs: polynuclear tricarbonylmanganese(I)-functionalized polypyridyl metallodendrimers. Inorg Chem 52: 5470–5478.

Gupta N, Ragsdale SW (2011). Thiol-disulfide redox dependence of heme binding and heme ligand switching in nuclear hormone receptor Rev-erbβ. J Biol Chem 286: 4392–4403.

Hasegawa U, van der Vlies AJ, Simeoni E, Wandrey C, Hubbell JA (2010). Carbon monoxide-releasing micelles for immunotherapy. J Am Chem Soc 132: 18273–18280.

Hou S, Xu R, Heinemann SH, Hoshi T (2008). The RCK1 high-affinity Ca^{2+} sensor confers carbon monoxide sensitivity to Slo1 BK channels. Proc Natl Acad Sci 105: 4039–4043.

Hou S, Heinemann SH, Hoshi T (2009). Modulation of BKCa channel gating by endogenous signaling molecules. Physiology 24: 26–35.

Ibrahim M, Derbyshire ER, Marletta MA, Spiro TG (2010). Probing soluble guanylate cyclase activation by CO and YC-1 using resonance Raman spectroscopy. Biochemistry 49: 3815–3823.

Ignarro LJ (1999). Nitric oxide: a unique endogenous signaling molecule in vascular biology. Angew Chem Int Ed 38: 1882–1892.

Johnson TR, Mann BE, Teasdale IP, Adams H, Foresti R, Green CJ *et al*. (2007). Metal carbonyls as pharmaceuticals? $[Ru(CO)_3Cl(glycinate)]$, a CO-releasing molecule with an extensive aqueous solution chemistry. Dalton Trans: 1500–1508.

Kajimura M, Fukuda R, Bateman RM, Yamamoto T, Suematsu M (2010). Interactions of multiple gas-transducting systems: hallmarks and uncertainties of CO, NO, and H_2S gas biology. Antioxid Redox Signal 13: 157–192.

Kajimura M, Nakanishi T, Takenouchi T, Morikawa T, Hishiki T, Yukutake Y *et al*. (2012). Gas biology: tiny molecules controlling metabolic systems. Resp Physiol Neurobiol 184: 139–148.

Kim HP, Ryter SW, Choi AMK (2006). CO as a cellular signaling molecule. Ann Rev Pharmacol Toxicol 46: 411–449.

Kunz PC, Meyer H, Barthel J, Sollazzo S, Schmidt AM, Janiak C (2013). Metal carbonyls supported on iron oxide nanoparticles to trigger the CO-gasotransmitter release by magnetic heating. Chem Commun 49: 4896–4898.

Lai W, Chen H, Matsui T, Omori K, Unno M, Ikeda-Saito M *et al*. (2010). Enzymatic ring-opening mechanism of verdoheme by the heme oxygenase: a combined X-ray crystallography and QM/MM study. J Am Chem Soc 132: 12960–12970.

Lane N (2003). New light on medicine. Sci Am January: 38–45.

Levina A, Mitra A, Lay PA (2009). Recent developments in ruthenium anticancer drugs. Metallomics 1: 458–470.

Li C, Hossieny P, Wu BJ, Qawasmeh A, Beck K, Stocker R (2007). Pharmacological induction of heme oxygenase-1. Antioxid Redox Signal 9: 2227–2239.

Li L, Rose P, Moore PK (2011). Hydrogen sulfide and cell signaling. Ann Rev Pharmacol Toxicol 51: 169–187.

Ma M, Noei H, Mienert B, Niesel J, Bill E, Muhler M *et al*. (2013). Iron metal–organic frameworks MIL-88B and NH2-MIL-88B for the loading and delivery of the gasotransmitter carbon monoxide. Chem Eur J 19: 6785–6790.

Mann BE (2010) Carbon monoxide: an essential signalling molecule. In: Metzler-Nolte N, Jaouen G (eds). Top. Organomet. Chem., Vol. 32. Springer: Berlin, pp. 247–285.

Marques AR, Kromer L, Gallo DJ, Penacho N, Rodrigues SS, Seixas JD *et al*. (2012). Generation of carbon monoxide releasing molecules (CO-RMs) as drug candidates for the treatment of acute liver injury: targeting of CO-RMs to the liver. Organometallics 31: 5810–5822.

Matson JB, Webber MJ, Tamboli VK, Weber B, Stupp SI (2012). A peptide-based material for therapeutic carbon monoxide delivery. Soft Matter 8: 6689–6692.

Matsui T, Unno M, Ikeda-Saito M (2010). Heme oxygenase reveals its strategy for catalyzing three successive oxygenation reactions. Acc Chem Res 43: 240–247.

McKenna CE, Gutheil WG, Song W (1991). A method for preparing analytically pure sodium dithionite. Dithionite quality and observed nitrogenase-specific activities. Biochim Biophys Acta 1075: 109–117.

McLean S, Mann BE, Poole RK (2012). Sulfite species enhance carbon monoxide release from CO-releasing molecules: implications for the doxymyoglobin assay of activity. Anal Biochem 427: 36–40.

Melchart M, Sadler PJ (2006). Ruthenium arene anticancer complexes. In: Jaouen G (ed.). Bioorganometallic. Wiley-VCH.: Weinheim, pp. 39–64.

Michel BW, Lippert AR, Chang CJ (2012). A reaction-based fluorescent probe for selective imaging of carbon monoxide in living cells using a palladium-mediated carbonylation. J Am Chem Soc 134: 15668–15671.

Morse D, Choi AMK (2002). Heme oxygenase-1: the 'emerging molecule' has arrived. Am J Respir Cell Mol Biol 27: 8–16.

Motterlini R, Otterbein LE (2010). The therapeutic potential of carbon monoxide. Nature Rev Drug Discovery 9: 728–743.

Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ (2002). Carbon-monoxide-releasing molecules – characterization of biochemical and vascular activity. Circ Res 90: e17–e24.

Motterlini R, Sawle P, Bains S, Hammad J, Alberto R, Foresti R *et al*. (2004). CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. FASEB J 18: 284–286.

Mustafa AK, Gadalla MM, Snyder SH (2009). Signaling by gasotransmitters. Sci Signal. 2: 1–8, re2.

Niesel J, Pinto A, Peindy N, Dongo HW, Merz K, Ott I *et al*. (2008). Photoinduced CO release, cellular uptake, and cytotoxicity of a tris(pyrazolyl)methane manganese tricarbonyl complex. Chem Commun: 1798–1800.

Peng P, Wang C, Shi Z, Johns VK, Ma Y, Oyer J *et al*. (2013). Visible-light activatable organic CO-releasing molecules (PhotoCORMs) that simultaneously generate fluorophores. Org Biomol Chem 11: 6671–6674.

Pfeiffer H, Rojas A, Niesel J, Schatzschneider U (2009). Sonogashira and 'Click' reactions for the *N*-terminal and side chain functionalization of peptides with $[Mn(CO)_3(tpm)]^+$-based CO releasing molecules (tpm = tris(pyrazolyl)methane). Dalton Trans: 4292–4298.

Pfeiffer H, Sowik T, Schatzschneider U (2013). Bioorthogonal oxime ligation of a $Mo(CO)_4$ PhotoCORM to a bacteria-targeting phage peptide. J Organomet Chem 734: 17–24.

Piantadosi CA (2008). Carbon monoxide, reactive oxygen signaling, and oxidative stress. Free Rad Biol Med 45: 562–569.

Pierri AE, Pallaoro A, Wu G, Ford PC (2012). A luminescent and biocompatible PhotoCORM. J Am Chem Soc 134: 18197–18200.

Pitchumony TS, Spingler B, Motterlini R, Alberto R (2008). Derivatives of sodium boranocarbonate as novel CO-releasing molecules (CO-RMs). Chimia (Aarau) 62: 277–279.

Pitchumony TS, Spingler B, Motterlini R, Alberto R (2010). Syntheses, structural characterization and CO releasing properties of boranocarbonate $[H_3BCO_2H]^-$ derivatives. Org Biomol Chem 8: 4849–4954.

Podust LM, Ioanoviciu A, Ortiz de Montellano PR (2008). 2.3 Å X-ray structure of the heme-bound GAF domain of sensory histidine kinase DosT of Mycobacterium tuberculosis. Biochemistry 47: 12523–12531.

Poulos TL (2006). Soluble guanylate cyclase. Curr Opin Struct Biol 16: 736–743.

Puranik M, Weeks CL, Lahaye D, Kabil Ö, Taoka S, Nielsen SB *et al*. (2006). Dynamics of carbon monoxide binding to cystathionine β-synthease. J Biol Chem 281: 13433–13438.

Ragsdale SW, Yi L, Bender G, Gupta N, Kung Y, Yan L *et al.* (2012). Redox, haem and CO in enzymatic catalysis and regulation. Biochem Soc Trans 40: 501–507.

Rahman MN, Vlahakis JZ, Szarek WA, Nakatsu K, Jin Z (2008). X-ray crystal structure of human heme oxygenase-1 in complex with 1-(Adamantan-1-yl)-2-(1*H*-imidazol-1-yl)ethanone: a common binding mode for imidazole-based heme oxygenase-1 inhibitors. J Med Chem 51: 5943–5952.

Rahman MN, Vlahakis JZ, Vukomanovic D, Szarek WA, Nakatsu K, Jia Z (2009). X-ray crystal structure of human heme oxygenase-1 with (2*R*,4*S*)-2-[2-(4-Chlorophenyl)ethyl]-2-[(1*H*-imidazol-1-yl)methyl]-4[((5-trifluoromethylpyridin-2-yl)thio)methyl]-1,3-dioxolane: a novel, inducible binding mode. J Med Chem 52: 4946–4950.

Rimmer RD, Pierri AE, Ford PC (2012). Photochemically activated carbon monoxide release for biological targets. Toward developing air-stable photoCORMs labilized by visible light. Coord Chem Rev 256: 1509–1519.

Rochette L, Cottin Y, Zeller M, Vergely C (2013). Carbon monoxide: mechanisms of action and potential clinical implications. Pharmacol Therap 137: 133–152.

Romanski S, Kraus B, Schatzschneider U, Neudörfl J, Amslinger S, Schmalz H-G (2011). Acyloxybutadiene iron tricarbonyl complexes as enzyme-triggered CO-releasing molecules (ET-CORMs). Angew Chem Int Ed 50: 2392–2396.

Romanski S, Kraus B, Guttentag M, Schlundt W, Rücker H, Adler A *et al.* (2012a). Acyloxybutadiene tricarbonyl iron complexes as enzyme-triggered CO-releasing molecules (ET-CORMs): a structure-activity relationship study. Dalton Trans 41: 13862–13875.

Romanski S, Rücker H, Stamellou E, Guttentag M, Neudörfl J, Alberto R *et al.* (2012b). Iron dienylphosphate tricarbonyl complexes as water-soluble enzyme-triggered CO-releasing molecules (ET-CORMs). Organometallics 31: 5800–5809.

Romao CC, Blättler WA, Seixas JD, Bernardes GJL (2012). Developing drug molecules for therapy with carbon monoxide. Chem Soc Rev 41: 3571–3583.

Ryter SW, Otterbein LE, Morse D, Choi AMK (2002). Heme oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol Cell Biochem 234–235: 249–263.

Ryter SW, Alam J, Choi AMK (2006). Heme oxygenase-1/carbon monoxide: from Basic science to therapeutic applications. Physiol Rev 86: 583–650.

Santos MFA, Seixas JD, Coelho AC, Mukhopadhyay A, Reis PM, Romao MJ *et al.* (2012). New insights into the chemistry of *fac*-[Ru(CO)$_3$]2 + fragments in biologically relevant conditions: the CO releasing activity of [Ru(CO)$_3$Cl$_2$(1,3-thiazole)], and the X-ray crystal structure of its adduct with lysozyme. J Inorg Biochem 117: 285–291.

Santos-Silva T, Mukhopadhyay A, Seixas JD, Bernardes GJL, Romao CC, Romao MJ (2011). CORM-3 reactivity towards proteins: the crystal structure of a Ru(II) dicarbonyl-lysozyme complex. J Am Chem Soc 133: 1192–1195.

Schatzschneider U (2010). Photoactivated biological activity of transition metal complexes. Eur J Inorg Chem: 1451–1467.

Schatzschneider U (2011). PhotoCORMs: light-triggered release of carbon monoxide from the coordination sphere of transition metal complexes for biological applications. Inorg Chim Acta 374: 19–23.

Seixas JD, Mukhopadhyay A, Santos-Silva T, Otterbein LE, Gallo DJ, Rodrigues SS *et al.* (2013). Characterization of a versatile organometallic pro-drug (CORM) for experimental CO based therapeutics. Dalton Trans 42: 5985–5998.

Süss-Fink G (2010). Arene ruthenium complexes as anticancer agents. Dalton Trans 39: 1673–1688.

Szabo C (2010). Gasotransmitters: new frontiers for translational science. Science Translat Med 2: 59ps54.

Szacilowski K, Macyk W, Drzewiecka-Matuszek A, Brindell M, Stochel G (2005). Bioinorganic photochemistry: frontiers and mechanisms. Chem Rev 105: 2647–2694.

Tinberg CE, Tonzetich ZJ, Wang H, Do LH, Yoda Y, Cramer SP *et al.* (2010). Characterization of iron dinitrosyl species formed in the reaction of nitric oxide with a biological Rieske center. J Am Chem Soc 132: 18168–18176.

Uchida T, Kitagawa T (2005). Mechanism for transduction of the ligand-binding signal in heme-based gas sensory proteins revealed by resonance Raman spectroscopy. Acc Chem Res 38: 662–670.

Vreman HJ, Wong RJ, Kadotani T, Stevenson DK (2005). Determination of carbon monoxide (CO) in rodent tissue: effect of heme administration and environmental CO exposure. Anal Biochem 341: 280–289.

Vreman HJ, Wong AP, Stevenson DK (2011). Quantitating carbon monoxide production from heme by vascular plant preparations *in vitro*. Plant Physiol Biochem 49: 61–68.

Wang J, Karpus J, Zhao BS, Luo Z, Chen PR, He C (2012). A selective fluorescent probe for carbon monoxide imaging in living cells. Angew Chem Int Ed 51: 9652–9656.

Ward JS, Lynam JM, Moir JWB, Sanin DE, Mountford AP, Fairlamb IJS (2012). A therapeutically viable photo-activated manganese-based CO-releasing molecule (photo-CO-RM). Dalton Trans 41: 10514–10517.

Weeks CL, Singh S, Madzelan P, Banerjee R, Sprio TG (2009). Heme regulation of human cystathionine β-synthase activity: insights from fluorescence and Raman spectroscopy. J Am Chem Soc 131: 12809–12816.

Wegiel B, Hanto DW, Otterbein LE (2013). The social network of carbon monoxide in medicine. Trends Mol Med 19: 3–11.

Wilkinson WJ, Kemp PJ (2011). Carbon monoxide: an emerging regulator of ion channels. J Physiol 589: 3055–3062.

Wu L, Wang R (2005). Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol Rev 57: 585–630.

Yuan L, Lin W, Tan L, Zheng K, Huang W (2013). Lighting up carbon monoxide: fluorescent probes for monitoring CO in living cells. Angew Chem Int Ed 52: 1628–1630.

Zobi F (2013). Carbon monoxide and CO-releasing molecules in medicinal chemistry. Future Med Chem 5: 175–188.

Zobi F, Blacque O, Jacobs RA, Schaub MC, Bogdanova AY (2012). 17 e$^-$ rhenium dicarbonyl CO-releasing molecules on a cobalamin scaffold for biological application. Dalton Trans 41: 370–378.