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Carbon-Monoxide-Releasing Molecules for the Delivery of Therapeutic CO In Vivo

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Dedicated to Professor Carlos Romão on the occasion of his 65th birthday

The development of carbon-monoxide-releasing molecules (CORMs) as pharmaceutical agents represents an attractive and safer alternative to administration of gaseous CO. Most CORMs developed to date are transition-metal carbonyl complexes. Although such CORMs have showed promising results in the treatment of a number of animal models of disease, they still lack the necessary attributes for clinical development. Described in this Minireview are the methods used for CORM selection, to date, and how new insights into the reactivity of metal-carbonyl complexes in vivo, together with advances in methods for live-cell CO detection, are driving the design and synthesis of new CORMs, CORMs that will enable controlled CO release in vivo in a spatial and temporal manner without affecting oxygen transport by hemoglobin.

1. Introduction

Carbon monoxide is a recognized therapeutic molecule involved in a multitude of defense mechanisms under physiological and pathological conditions (Figure 1).[1,2] Unlike other gaseous systems currently used in the clinic or in early-stage clinical development (e.g., nitric oxide and hydrogen sulphide) that interact with a range of intracellular targets, CO is a stable molecule which reacts predominantly with transition metals in a specific redox state. The safety and feasibility of the use of CO as an inhaled gas remains uncertain owing to the lack of specificity and toxicity at high concentrations. To sidestep this problem, Motterlini and co-workers proposed the use of CO-releasing molecules (CORMs) as pharmaceutical agents for the controlled delivery of CO.[3] Firstly, by focusing on the transition-metal carbonyl complexes $[Fe(CO)_5]$, $[Mn_2(CO)_{10}]$ ("CORM-1"), and $[\{RuCl_2(CO)_3\}_2]$ (CORM-2), it was demonstrated that CO could be carried and released under certain physiological conditions when covalently bound to a metal. Unlike iron and manganese carbonyl complexes, which require exposure to light to release CO, ruthenium carbonyl CORM-2 transferred

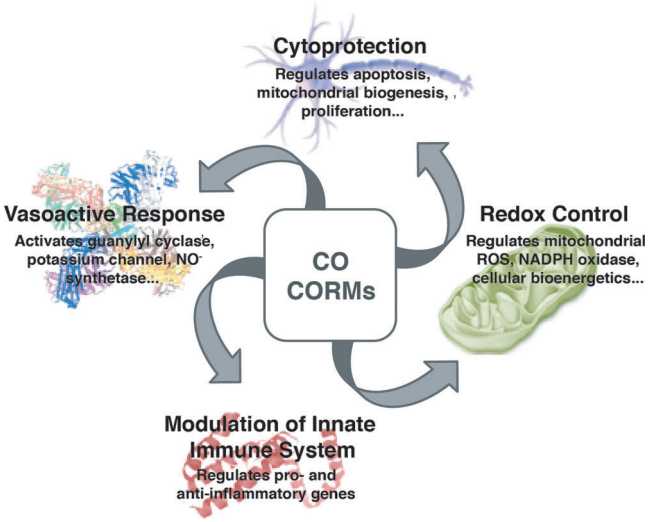

Figure 1. Biological effects of carbon monoxide delivered as a gas or as a CO-releasing molecule (CORM). Adapted from Reference [1].

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CO spontaneously, even in the dark, and exerted an identical pharmacological effect to that observed with CO gas, including vasodilatation and a decrease in arterial blood pressure.[3] This lipid-soluble system was progressed to water-soluble CORMs such as [RuCl-glycinato(CO)$_3$] (CORM3) or (Na_2-[H_3BCO_2]) (CORMA1), in which the chemical structures conferred different CO-release profiles and biological properties.[1]

In the past decade a number of different molecules have been reported as CORMs—molecules that are able to release CO under certain biological settings—and include a wide variety of organometallic complexes (M = Ru, Fe, Mn, V, Co, Ir, Cr, Mo, W) and a number of main-group compounds (α,α-dialkylaldehydes, oxalates, boroncarboxylates, and silacarboxylates).[4–7] Most of these molecules release CO by simple thermal activation or hydrolysis in biological buffers.[8] Controlled and specific CO delivery may also be achieved by using stable compounds which release CO only when activated by an internal trigger (e.g., enzymatic reaction)[9–13] or an external trigger (e.g., photoexcitation).[14–16] The latter strategy involves molecules known as photoactivated CORMs (photoCORMs), which are molecules that are capable of CO release in a specific tissue with temporal control. An ideal photoactivation profile entails long wavelengths[14] with complete stability of the CORM in the absence of light. Since the first photoCORM, [$Mn_2(CO)_{10}$], was reported by Motterlini and co-workers,[3] several research groups have exploited this strategy. For instance, Schatzschneider and co-workers have successfully developed [Mn(CO)$_3$(R-tpm)]$^+$ complexes [tpm = tris(pyrazolyl)-methane] coupled to target molecules, such as peptides[17] and SiO_2 nanoparticles,[18] without altering the photochemical CO-release properties of the metal complexes.

Despite the large collection of reported CORMs, most therapeutic studies to date have focused on the commercially available CORM-2 and water-soluble CORM-3. In a heart-attack mouse model, CORM-3 reduced cardiac muscle damage and infarct size during reperfusion,[19] and considerably prolonged the survival rate after organ transplantation. The anti-inflammatory properties of CORMs have been demonstrated in the treatment of arthritis,[20] bacterial infection,[21] or neuroinflammation.[22] In addition, their vasodilatory properties,[23] the capacity to inhibit platelet aggregation,[24] and antiapoptotic effect,[19] make CORMs interesting alternatives for the treatment of diseases including diabetes and Alzheimer's disease. Although these complexes were able to mimic the beneficial therapeutic effect of CO gas in many animal models of disease, they lack an assignable pharmacokinetic (PK) profile, which prevents their advance into clinical development. In this Minireview, we describe and discuss the methods used for CORM selection to date, and discuss how new insights into the reactivity of CORMs in vivo, in particular metal carbonyl complexes, together with advances in methods for the quantification and live-cell CO detection, are driving the design and synthesis of new and more efficient CORMs.

2. Current Limitations for the Clinical Use of CORMs

Despite the highly versatile chemistry of CORMs reported, that is, CORMs featuring several classes of metal-carbonyl compounds as well as organic molecules,[4,6,25,26] most CORMs lack the druglike properties necessary for clinical development. An ideal CORM should satisfy a number of prerequisites: effective therapeutic action and low toxicity; appropriate absorption, distribution, metabolism, and excretion (ADME) properties; solubility and stability in aerobic aqueous media; adequate PK profile as well as biocompatibility and stability in blood. In the next sections we discuss the major limitations of CORMs developed to date.

2.1. Sensitivity and CO-Release Kinetics of CORMs

Most CORMs are organometallic carbonyl complexes with CO bound to a transition metal in a low oxidation state. Of these complexes, many release CO immediately after dissolution in aqueous buffer through a hydrolytic trigger. This is the case of air-stable and water-soluble CORM-3, which is unstable in aqueous solutions ($t_{1/2}$ in human plasma of just 3.6 min[27]) and has resulted in its exclusion from clinical development, despite its beneficial therapeutic activity in several animal models.[28,29] By fine-tuning of the coordination and drug spheres, CORMs with enhanced aqueous stability ($t_{1/2}$ values of up to 1 h) were synthesized and shown to improve in vivo therapeutic outcomes.[15,30–33] The kinetics of CORM delivery of CO is a key parameter which determines the degree of tissue specificity. CORMs with a fast CO release

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profile ($t_{1/2}$ = seconds, milliseconds) are particularly useful in cases in which CO release needs to occur within a short timeframe, for example in the study of ion-channel kinetics.[34] However, for in vivo therapeutic applications, temporally controlled CO release, which delivers CO to its biological target, is desirable.[15,30–33]

2.2. Cytotoxicity and Tissue Accumulation

Although a number of CORMs have resulted in promising preclinical data, with efficacy mostly relating to their cytoprotective capacity,[3,23,24] they often present a poorly understood toxicological profile. For instance, the suspected accumulation, in vivo, of the metal-containing species left after release of CO, could result in undesired side effects. Current toxicity reports, focused mainly on CORM-3, lead to contradictory conclusions. Exposure of human gingival fibroblasts or peripheral blood mononuclear cells to CORM-3 (500 μM, 24 h) did not produce any signs of toxicity,[35] whereas at the same concentration CORM-3 seems to be cytotoxic in RAW 264.7 macrophages.[36] The use of different assays to evaluate cellular toxicity (intracellular LDH release assay in the first example and cell quantification assay in the second one) may account for the observed contradictory effects, because metal-carbonyl CORMs may interfere with the assays, thus resulting in unreliable observations.[37]

To address this issue, Winburn and co-workers[37] performed a comparative study of the effects of gaseous CO, CORM-2, and its CO-absent analogue (iCORM-2) in primary rat cardiomyocytes and two different cell lines (HeK 293 and MDCK). The analysis revealed a narrow margin between the cytoprotective (< 20 μM) and cytotoxic (> 100 μM) concentration of CORM-2. Unlike CO gas, both ruthenium complexes decreased cell viability, produced abnormal cell morphology, increased apoptosis and necrosis, arrested the cell cycle, and reduced mitochondrial enzyme activity, thus indicating core-structure-mediated toxicity. A possible accumulation of iCORM-2, or any other toxic iCORM by-product, would impede their pharmaceutical development. Detailed toxicological profile evaluations of CORMs, by using robust cytotoxicity assays and suitable controls, are key for their future clinical application.

2.3. Reactivity Towards Proteins

To establish CORMs as useful pharmaceutical drugs, it is crucial to understand their interactions with biomolecules, especially those such as plasma proteins, which regulate their PK and ADME profiles. However, few studies have assessed this matter. The complex *fac*-Na[Mo(histidinato)$(CO)_3$] (ALF186) has proven to be a good model for the study of some fundamental interactions of organometallic complexes with biological media.[38] Triggered by O_2, ALF186 immediately releases all of its CO equivalents, which are then transported in the systemic circulation, bound or unbound, to hemoglobin. Meanwhile, the metal by-product forms CO-free polyoxometallates, which interact weakly with proteins, as demonstrated by X-ray diffraction with model protein hen egg white lysozyme (HEWL).

Unlike ALF186, the well-known CORM-3 and other *fac*-$[RuL_3(CO)_3]$ complexes release only residual amounts of CO in phosphate buffer solution at pH 7.4 and only produce minor quantities of carboxy hemoglobin (CO-Hb) after long incubation times in blood. A comprehensive study with inductively coupled plasma/atomic emission spectroscopy (ICP-AES), liquid-chromatography mass spectrometry (LC-MS), and infrared spectroscopy (IR) of the interactions of CORM-3 with proteins revealed that this ruthenium carbonyl reacts rapidly with plasma proteins such as horse heart myoglobin, human hemoglobin, human albumin, human transferrin, and HEWL, thus leading to ruthenium species bound to these proteins.[28] Diffracting crystals from the HEWL model protein were obtained after being soaked with CORM-3 in solution, and were subjected to X-ray diffraction analysis. The study showed that CORM-3 reacts with exposed histidine 15 of HEWL to form a stable $[Ru(CO)_2(H_2O)_3]^{2+}$ adduct after loss of a chloride ion, a glycinate, and one CO ligand (Figure 2 A). A subsequent study with $[RuCl_2$(1,3-thiazole)$(CO)_3]$, another model *fac*-$[RuL_3(CO)_3]$ CORM, showed that it also reacts with HEWL to produce ruthenium adducts, and in this case forms only monocarbonyl ruthenium species covalently bound to the histidine and to two aspartates at the protein surface (Figure 2 B).[39]

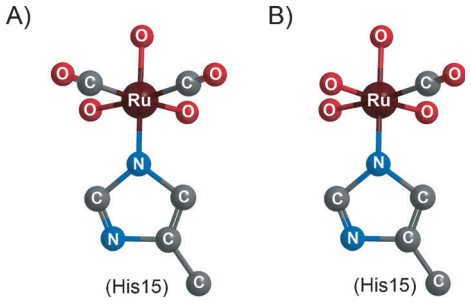

Figure 2. Schematic representation of Ru-His15 adducts of HEWL with A) CORM-3[28] and B) $[RuCl_2$(1,3-thiazole)$(CO)_3]$.[39] Ligands marked with O account for water molecules.

Collectively, these results suggest that *fac*-$[RuL_3(CO)_3]$ CORMs deliver CO in vivo through the decay of their adducts with plasma proteins. *fac*-$[RuL_3(CO)_3]$ complexes react rapidly with plasma proteins after entering the blood stream and lose one equivalent of CO in the form of CO_2, thus yielding protein–$[Ru(CO)_2]$ adducts. These adducts, which are carried throughout the body in the circulation, are responsible for CO distribution to different organs and tissues through slow loss of CO, thus preventing rapid CO-Hb elevation.[29] This mechanistic proposal is consistent with the therapeutic versatility of CORM-3[1] and suggests the use of metalloproteins as a useful strategy for therapeutic CO delivery in vivo.

2.4. CO Release Detection

The development of CORMs as lead compounds requires fine-tuning of their CO-release rates under physiological conditions. In the quantification of this process several factors need to be controlled, including the assay solution, temperature, and the presence of O_2 or light, all of which may directly influence the rate of CO release. Electrochemical devices (e.g., CO electrodes)[5] as well as methods based on manometric and gas chromatography[4] measurements have found only limited application for CO quantification owing to the low solubility of CO in water.

To date, most CORM structures have been selected by using the so-called deoxymyoglobin carbonylation assay. This assay uses deoxymyoglobin, a CO acceptor, to quantify the amount of CO released from CORMs. The formation of Mb-CO is quantified and correlated to the amount of CO released by the CORM. However, this assay suffers from several drawbacks,[40–42] and modifications have been proposed.[43] The strong absorbance of myoglobin in the near-UV and visible regions makes the assay incompatible with photolysis in aerated media, and limits the utility of the technique for quantifying CO release by photoCORMs. In addition, it has recently been demonstrated that CO is not released from CORM-2 and CORM-3 at an appreciable rate in the presence of reduced myoglobin alone.[44] Instead, it is the reducing agent, sodium dithionite, in the assay that is responsible for triggering CO release. Consequently, evaluation of CORMs based on the Mb-CO assay is inappropriate and requires a redefinition of CORM CO-release profiles. Consequently, classification of CORM-2 and CORM-3 as fast releasers,[8] owing to the fact that CO liberation occurs within one minute after incubation with deoxymyoglobin, is no longer accepted because they are actually slow CO releasers in the absence of dithionite. It is important to revisit and study the CO-release profile of CORMs, selected based on the Mb-CO assay, to unambiguously determine their CO-release rates and how these correlate with reported biological activities.

3. Progress in CORM Development

3.1. CO-Release Rate Redefinition

Robust and accurate assays should be used to determine the rate of CO release from CORMs. An indicative preliminary value for CO release may be achieved by using a theoretical approach by means of density functional theory (DFT), as demonstrated by Vummaleti et al.[45] In this case, the predicted CO-release profiles of different manganese carbonyl complexes were in good agreement with the experimental observations. Although the utility of this approach is limited, because it does take into account speciation of CORMs in aqueous solutions or in plasma, such DFT calculations may be useful in assigning potential intermediates (iCORMs) detected by in situ IR spectroscopy.[46]

Furthermore, McLean et al. described an alternative oxy hemoglobin assay which eliminates the use of dithionite. This method is based on the assumption that the efficiency of CO release from a CORM is the result of intracellular interactions with anions.[44] Given the greater affinity of CO versus O_2 for hemoglobin, this assay also eliminates the need for deoxygenated reduced hemoglobin. The different absorption spectrum of oxy hemoglobin and carboxy hemoglobin in the Soret region ($\lambda = 422$ nm) with a peak appearing between two isosbestic wavelengths, makes it an ideal method for measuring the displacement of O_2 by CO.

More recently, two assays for efficient CO quantification in live cells were reported based on fluorescent probes.[47] Chang et al.[48] developed a turn-on fluorescent palladium probe (COP-1) in which the metal atom quenches the fluorescence of the borondipyrromethene difluoride (BODIPY) core through heavy-atom electronic effects. Interaction with CO triggers a fluorogenic carbonylation reaction (Figure 3A), thus releasing Pd^0 and a highly fluorescent compound. The second assay, established by Wang et al.,[49] is based on a genetically encoded fluorescent probe (COSer), which takes advantage of the unique CO-binding selectivity of the dimeric heme protein CooA (Figure 3B) to become an effective CO reporter. In both cases, the fluorescent probes are capable of detecting CO in live cells (and in aqueous buffer in the case of COP-1) with high selectivity for a range of biologically relevant reactive small molecules. Although COP-1 shows a higher fluorescent enhancement and sensitivity relative to COSer, the biosensor displays a much faster response and has a reversible mechanism suitable for real-time detection of CO.

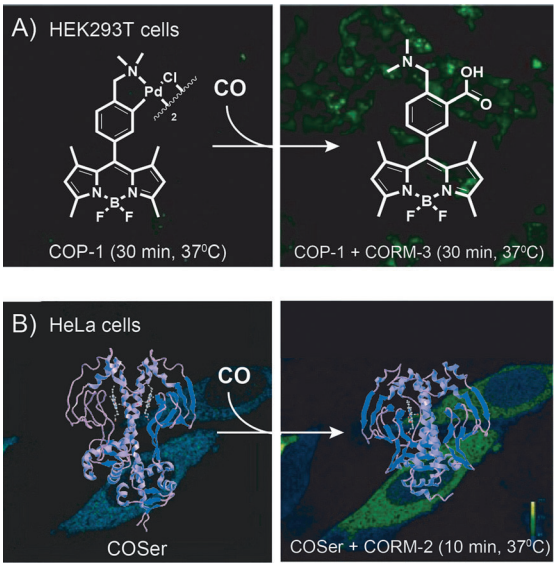

Figure 3. CO detection in live cells by using A9 COP-1[48] and B) COSer.[49]

To quantify CO release from photoCORMs, Rimmer et al.[50] reported a number of analytical procedures which leave the spectrum of the photolysis solution undisturbed and are compatible with studies in aerated media. These procedures involve sampling the gas phase that is in equilibrium with the photolyzed solution sample enclosed in a Schlenk

cuvette, and calculates a partition coefficient of 45:1 between the gas and liquid phases at equilibrium under specific conditions. The procedures differ in the methods used for CO quantification and include infrared spectroscopy, programmable gas chromatograph equipped with a carbosieve packed column, thermal conductivity detection, and flash photolysis. Finally, it is also possible to use gas chromategraphy coupled with mass spectrometry (GC-MS), although this technique relies on the low solubility of CO in water.[51] Together, these recent advances in methods for CO quantification and imaging will facilitate the design of CORMs with defined CO-release rates for biological applications.

3.2. Improved CORM Design

To help rationalize the design of CORMs with the appropriate pharmaceutical properties, Romão and colleagues proposed a conceptual model of a metal carbonyl complex,[52] made up of three different components: A) the metal center responsible for the main properties and toxicity of the molecule; B) the inner-coordination sphere (CO and ancillary ligands) for tuning the stability and reactivity towards plasma proteins, to respond to a specific trigger or to generate a specific CO-release profile; and C) the drug sphere obtained by modifying the ancillary ligands at their distal sites, thus modulating the desired pharmacological parameters (water solubility, biocompatibility, tissue targeting).

These three principles were the basis for the generation of new complexes with improved properties over CORM-3, often considered the CORM of choice.[23] ALF492, ALF795, and B_{12}-ReCORM-2 (Figure 4) are three examples of new CORMs which are equipped with favorable characteristics for in vivo applications, including enhanced solubility, biocompatibility, defined CO-release rates, and tissue specificity.

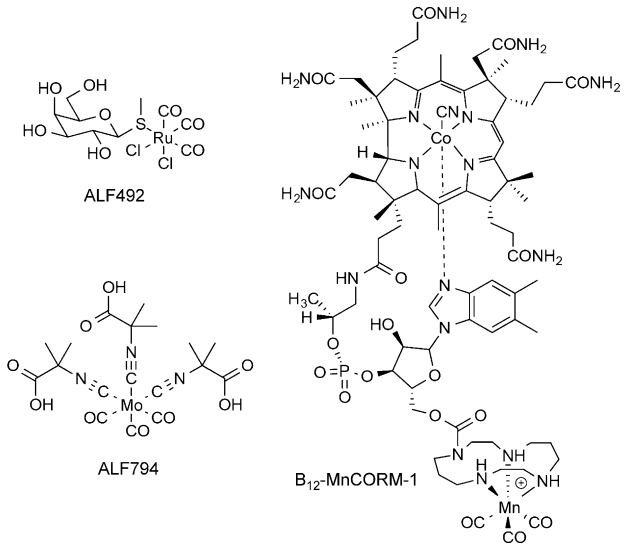

Figure 4. The chemical structure of three new CORMs which have produced promising results as therapeutic agents.[46,53,54]

ALF492: This ruthenium-based CORM, [$RuCl_2$-thiogalactopyranoside$(CO)_3$],[53] shows a significant improvement in terms of druglike properties and CO-release profile relative to other those of CORMs. ALF492 has a galactose-derived ligand bound to the metal and it confers enhanced water solubility and biocompatibility, and results in preferential distribution to the liver in vivo by targeting asialoglycoprotein receptors. ALF492 provides full protection against experimental cerebral malaria (ECM) and acute lung injury without affecting oxygen transport by hemoglobin in mice. The observed protective effect is CO dependent and induces the expression of heme oxygenase-1, which contributes to the observed protection. Additionally, when used in combination with the antimalarial drug, artesunate, ALF492 offers protection against ECM after the onset of severe disease. This marked protective effect highlights the potential of CORMs in the treatment of severe forms of malaria.

ALF794: This molybdenum-based CORM, [$Mo(CNCMe_2CO_2H)_3(CO)_3$], has low toxicity, favorable druglike properties, and delivers CO in a specific manner to the liver in the treatment of acetaminophen-induced acute liver failure in mice.[54] The incorporation of methyl groups on the isocyanoacetate ligand significantly increased CO delivery to the liver relative to that of the parent complex, [$Mo(CNCH_2CO_2H)_3(CO)_3$] (ALF795). Five minutes after intravenous administration, ALF794 displays concentration values in the liver/blood and liver/kidney of 5.27 and 12.58, respectively, relative to 0.33 and 0.5 obtained with ALF795. Although ALF795 appears to be rapidly cleared by the kidneys, ALF794 is not, and allows efficient liver targeting to take place to produce a favorable curative therapeutic outcome in animal models of acute liver disease.

B_{12}-MnCORM-1: Zobi and co-workers have produced CORMs based on biocompatible scaffolds such as cyanocobalamin (B_{12}). The rhenium(II) conjugate, B_{12}-ReCORM-2, with the metal moiety coordinated to the axial cyano group of the vitamin, produces cytoprotective effects on an ischemia-reperfusion injury model, but shows poor cellular uptake.[32] Optimization of the synthetic strategy through the incorporation of the metal moiety, in this case manganese, in the ribose group of the scaffold yielded the conjugate B_{12}-MnCORM-1.[46] After its active internalization in live 3T3 fibroblasts, this system released CO through visible-light photoinduction and prevented fibroblasts from dying under conditions of hypoxia and metabolic depletion. A number of CORMs of potential pharmacological value may be synthesized by means of this strategy and delivered intracellularly.

3.3. Controlled CO Release and Tissue Targeting

Efficient CORM targeting depends on a suitable half-life profile for CO release to allow the molecule to reach the intended disease site. Hydrolytic or ligand exchange mechanisms have been the most explored CO release triggers, but a number of other processes which promote CO release from metal-carbonyl complexes have also been pursued. In particular, mechanisms that allow a degree of control over CO-release kinetics have received considerable attention. Among

these, boranocarbonate CORM-A1 exhibits pH- and temperature-dependent CO release.[25] It has a half-life of about 21 minutes at 37 °C and pH 7.4, and can be accelerated by decreasing the pH and increasing the temperature. Enzyme-triggered systems (ET-CORMs), such as acyloxybutadiene iron tricarbonyl complexes,[9] are emerging as an attractive alternative to the traditional chemically triggered methods. These compounds, which are stable in buffer under normal conditions, are activated by the enzymatic cleavage of an ester bond, thus leading to a keto–enol tautomeric rearrangement which weakens the Fe–CO bond and results in CO release. In addition, Kunz et al.[55] reported the use of magnetic Fe_2O_3 nanoparticles decorated with [RuCl-(π-DOPA)$(CO)_3$] moieties which, upon exposure to a magnetic field, lead to controlled CO release. This alternative strategy opens new possibilities for the development of tissue-specific CORMs.

A widespread strategy that enables a high degree of control over the kinetics of CO release is based on light as a trigger.[14,15] Most photoCORMs are metal-carbonyl complexes which are stable in the dark in aqueous solution for a period of time long enough for them to accumulate in the targeted tissue. Upon photoexcitation, these systems release one (or more) CO equivalents which then diffuse into the biological target. The development of photoCORMs currently focuses on the photolytic release of CO by light within a phototherapeutic window ($\lambda > 600$ nm). Three main strategies have been proposed:[15] A) to shift the absorption maximum of the photoCORMs to the red end of the spectrum through suitable metal and co-ligand combinations (e.g., chelators with an extended aromatic π system); B) to attach a photosensitizer for the CO release process to a metal-carbonyl moiety, such as an organic dye or metal complex; and C) to use two-photon absorption to achieve photolytic liberation of CO from a photoCORM prodrug. These strategies have been successfully used to generate a new series of photoCORMs which can be activated to release CO by visible light. Among the new transition-metal photoCORMs,[14,15] Gonzalez et al.[56] synthesized manganese carbonyl complexes equipped with azaheteroaromatic ligands which contain extended conjugation and electron-rich donors on their frames. These systems readily release CO upon exposure to light ($\lambda = 400$–550 nm), and the release and quantum yield values, at $\lambda = 509$ nm ($\lambda 509$), of the photoCORMs increase steadily with increased conjugation in the ligand frame and inclusion of a SMe group. Addition of bromide as an ancillary ligand also improves the CO-donating capacity. Govender et al. prepared the first CO-releasing metallodendrimers, which the authors describe as the next-generation photoCORMs.[57] These consist on polypyridyl first- and second-generation dendritic scaffolds functionalized with four and eight [$Mn(CO)_3$] moieties, respectively. These scaffolds are stable in the dark in aqueous buffer for up to 16 hours, but show photoinduced CO release after excitation at $\lambda = 410$ nm. The half-life and quantum yield of CO release were similar for the first- and second-generation metallodendrimers, thus indicating that each [$MnBr(bpy)(CO)_3$] end-group behaves independently from the others, but that the total amount of CO released per molecular unit increases with dendrimer generation, to reach a value of 15 CO per molecule

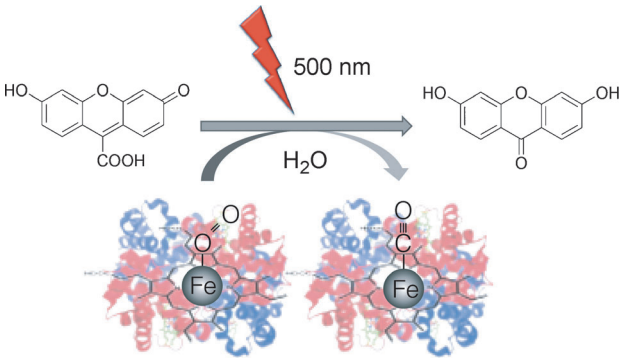

Figure 5. The first water-soluble, transition-metal-free CORM which can be activated by visible light.[58]

on the second-generation metallodendrimer. Recently, Antony et al. introduced the first water-soluble, transition-metal-free CORM which can be activated by visible light (Figure 5).[58] Based on 6-hydroxy-3-oxo-3*H*-xanthene-9-carboxylic acid, this CORM is able to release CO in both water and methanol upon irradiation at $\lambda = 500$ nm. Its favorable spectroscopic properties, aqueous solubility, and transformation into a non-interfering photoproduct highlight the potential of these photoCORMs for applications in biology and medicine.

The selective delivery of CORMs to target cells is facilitated by conjugation of a metal-carbonyl scaffold to a targeting ligand, such as peptides, antibodies, or nanoparticles. For instance, the attachment of the lead photoCORM, $[Mn(tpm)(CO)_3]^+$, to a carrier peptide was performed by using a copper-catalyzed 1,3-dipolar azide–alkyne cycloaddition reaction (CuAAC) in a post-labelling strategy.[17,59] More recently, grafting of this same moiety to silicon dioxide and carbon nanomaterials produced systems designed to deliver CO to solid tumours.[18,60] Bischof et al.[61] reported a ruthenium(II) dicarbonyl complex conjugated to a peptide nucleic acid (PNA) monomer, [$RuCl_2$(Cpp-L-PNA)$(CO)_2$], which enhanced cellular uptake and specific CO delivery. In all cases, the photoinduced CO-release properties were similar to that of the parent compound. In addition, in an efficient bioorthogonal synthetic route, a catalyst-free oxime ligation reaction led to the targeting system [$Mo(bpy^{CH3,CH=Aoa\text{-}TGF\beta1\text{-}OH})(CO)_4$] which was prepared by Pfeiffer et al.[62] In this case, the molybdenum carbonyl complex with an aldehyde group at the periphery of the 2,2′-bipyridine (bpy) ligand was coupled to a bioactive peptide (transforming growth factor β-targeting; TGFβ) functionalized at the N-terminus with aminoxy acetic acid. CO release was shown to be significantly accelerated by an LED array at $\lambda = 468$ nm, thus ensuring deeper tissue penetration than previously reported photoCORMs.

Regardless of the trigger mechanism for CO release, the use of CORMs inevitably leads to the formation of a metal/CO-ligand fragment, with possible biological adverse effects. In the case of photoCORMs, the remaining metal–ligand fragments (released upon light irradiation) or the secondary products (readily formed by subsequent reactions with the medium or with O_2) should be fully characterized and

identified. Such characterization was recently reported by Berends et al. for manganese(I) complexes,[40] and the possible acute and long-term toxicities should be thoroughly investigated to make photoCORMs useful pharmaceutical agents.

3.4. Avoiding By-Products: Macromolecular Carriers

Macromolecular systems have also been exploited as CORM carriers in an attempt to improve pharmacokinetic properties (Figure 6). This improvement may be achieved by

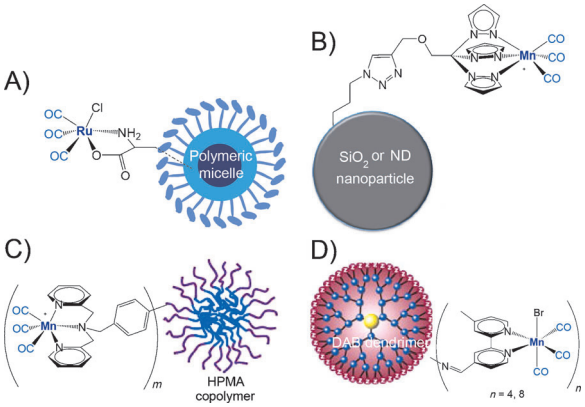

Figure 6. Examples of macromolecular CORMs: A) a micelle,[63] B) a SiO_2 or nanodiamond system,[18,60] C) a HPMA copolymer,[64] and D) a diaminobutane dendrimer.[57]

1) avoiding rapid kidney elimination, 2) targeting organs that capture nanoparticles such as the liver and fast growing tumours, and/or 3) using targeting ligands. The combination of CORMs and nanosized carriers was first used in a micellar system and in functionalized SiO_2 nanoparticles. Hasegawa et al.[63] sought to develop micellar forms of metal-carbonyl complexes which would display slow diffusion in tissues and thus improve the ability to target distal tissue drainage sites. They developed a new CO-delivery system by using a polymeric micelle with a [RuCl(amino acidate)$(CO)_3$] structure as the CO-releasing segment. This system has a high CO-loading capacity, delayed CO release, and capacity to release CO in response to thiol-containing compounds such as cysteine and glutathione. They efficiently attenuated the lipopolysaccharide-induced NF-κB activation of human monocytes and significantly reduced the cytotoxicity of the [RuCl(amino acidate)$(CO)_3$] moiety. Dördelmann et al. functionalized SiO_2 nanoparticles[18] and nanodiamonds[60] with modified [Mn(tpm) $(CO)_3]^+$ moieties through CuAAC reaction. These nanoparticles displayed similar photoinducible CO-release properties relative to the free complex, and may provide a useful platform as delivery agents for CORMs in solid tumours. In addition, the previously mentioned [RuCl-(π-DOPA)$(CO)_3$] moieties, covalently bound to the surface of magnetic Fe_2O_3 nanoparticles,[55] have been explored in the construction of tissue-specific CORMs which release CO upon exposure to a magnetic field.

Other systems, based on polymers and dendrimers, were also suggested as alternative macromolecular CORM carriers. By conjugation of an organometallic *fac*-[$Mn(CO)_3$] fragment to methacrylate or methacrylamide polymers, Brückmann et al. generated copolymer conjugates which displayed photoCORM behavior with macromolecular weights and size distributions suitable for passive drug targeting.[64] Dendrimers, such as the tetranuclear and octanuclear [$Mn(CO)_3$]-functionalized metallodendrimers described by Govender et al.,[57] represent an attractive alternative of macromolecular carrier owing to their monodisperse nature and ease of preparation. These macromolecular systems passively and selectively accumulated in tumour tissue owing to an enhanced permeability and retention (EPR) effect.[64] Additionally, Matson et al. reported a peptide-based material that spontaneously releases CO.[65] This material consists of a nanofiber gel containing a peptide amphiphile with a covalently bound ruthenium tricarbonyl moiety which spontaneously releases CO with prolonged release kinetics relative to soluble CO donors. Oxidatively stressed cardiomyocytes showed improved viability when treated with this peptide-based material, thus highlighting its potential as a biodegradable gel for localized therapeutic CO delivery.

3.5. Beyond Simple CO Carriers

Even though most CORMs exert a biological action owing to the release of CO, several studies reveal that these effects cannot be fully explained by the released CO alone.[66,67] Some of these properties, which cannot be mimicked by CO gas, include the potent antibacterial activity of CORM-3,[21,68] inhibition of cellular respiration, and promotion of cation transport across spheroblast membranes.[66,69]

A partial explanation for the antibacterial activity exhibited by CORMs could be the rapid accumulation of metal-carbonyl complexes inside bacterial cells leading to high CO concentrations at the target site(s).[68] A number of CORMs have shown potential as bactericides against a wide range of microorganisms, including gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria.[21] In addition, CORM-3 also increased the survival of mice infected with *Pseudomonas aeruginosa*.[36,39] Tavares et al. demonstrated that the bacteria-killing capacity of CORMs is linked with the cellular oxidative stress produced by the metal complexes formed after CO release.[70] This conclusion is supported by increased levels of reactive oxygen species (ROS) and DNA damage, and by decreased activity when ROS-scavenging systems are used. Finally, a Trojan horse mechanism to explain CORM's antibacterial activity has also been proposed. In brief, after internalization of the CORMs, intracellular thiol-containing molecules promote CO release,[44] and binds to the terminal oxidases of the aerobic respiratory chain and induces inhibition of respiration (Figure 7).[68,71] The inhibitory effects of CORMs are prevented by thiol-containing compounds such as *N*-acetyl cysteine,[36,67] behavior which is not attributable to their antioxidant properties (prevention of CO targeting the oxidases) but

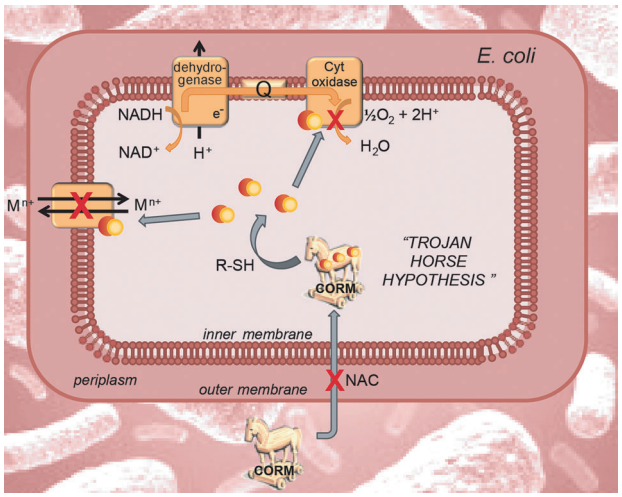

Figure 7. Proposed Trojan horse mechanism for the bactericide action of CORMs.[69,71]

rather to the inhibition of uptake of CORM into bacterial cells.[71]

CORMs may also provide an innovative approach for the treatment of other infectious diseases. For instance, [RuCl-(thiogalactopyranoside)$(CO)_3$] (ALF492)[53] was demonstrated to be an effective adjunctive/adjuvant treatment in severe forms of malaria infection. Furthermore, the decrease in the effectiveness of some currently available drugs, especially antibiotics, manifest an urgent need for therapies based upon new concepts, which avoid the development of drug resistance. The use of CORMs, with dissimilar modes of action to that of currently used medicines, represents a promising avenue for the development of pharmaceutical agents for clinical use.

4. Conclusions

CORMs, prodrugs able to deliver CO in a spatial- and time-controlled manner, have been proposed as safer alternatives to the administration of gaseous CO for CO-based therapies. Most CORMs developed to date are transition-metal-carbonyl complexes and produce beneficial therapeutic outcomes in many animal models of disease without affecting oxygen transport by hemoglobin, a major drawback of CO gas administration.

Despite progress, many challenges remain to fully realize the promise of CORMs as pharmaceutical agents. For instance, the key assay used for selecting most CORM structures reported, an assay which is based on the quantification of CO released from CORMs in the presence of reduced myoglobin by measuring the formation of Mb-CO, has been shown to be unsuitable for CO-release quantification. In fact, CO is not liberated from CORM-2 and CORM-3 at an appreciable rate in the presence of reduced myoglobin alone, but it is the reducing agent sodium dithionite used in the assay that promotes release of CO from these complexes. In addition, it has been shown that the products of decomposition of CORMs can generate additional effects which contribute to the observed biological outcome. For example, the known antibacterial activity of CORM-3 is mainly a result of its ability to cause oxidative stress rather than from CO-mediated activity. Therefore, significant attention has been devoted in the past couple of years to developing robust analytical methods for the quantification of CO as well as building carbonyl complexes with defined CO-release profiles and tissue specificity, for which the decomposition products are biologically inert.

To avoid toxic side effects, a metal-carbonyl CORM must distribute preferentially to the site of disease and, once triggered at that site, release its CO load whilst avoiding unspecific CO release. In addition, the biocompatibility of the scaffold must be modulated by the nature and properties of the metal and its coordination and drug spheres. Combining organometallic chemistry in water and medicinal chemistry principles with advances that enable precise quantification and imaging of CO release, even within live cells, will generate therapeutically active CORMs for safe, advanced experimental studies in vivo.

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[1] R. Motterlini, L. E. Otterbein, *Nat. Rev. Drug Discov.* **2010**, *9*, 728–743.
[2] T. R. Johnson, B. E. Mann, J. E. Clark, R. Foresti, C. J. Green, R. Motterlini, *Angew. Chem.* **2003**, *115*, 3850–3858; *Angew. Chem. Int. Ed.* **2003**, *42*, 3722–3729.
[3] R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann, C. J. Green, *Circ. Res.* **2002**, *90*, 17e–24e.
[4] B. E. Mann, *Top. Organomet. Chem.* **2010**, *32*, 247–285.
[5] R. Motterlini, P. Sawle, J. Hammad, S. Bains, R. Alberto, R. Foresti, C. J. Green, *FASEB J.* **2005**, *19*, 284–286.
[6] T. S. Pitchumony, B. Spingler, R. Motterlini, R. Alberto, *Org. Biomol. Chem.* **2010**, *8*, 4849–4854.
[7] F. Zobi, *Future Med. Chem.* **2013**, *5*, 175–188.
[8] M. Desmard, R. Foresti, D. Morin, M. Dagouassat, M. Dagoussat, A. Berdeaux, E. Denamur, S. H. Crook, B. E. Mann, D. Scapens, P. Montravers, J. Boczkowski, R. Motterlini, *Antioxid. Redox Signaling* **2012**, *16*, 153–163.
[9] S. Romanski, B. Kraus, U. Schatzschneider, J.-M. Neudoerfl, S. Amslinger, H.-G. Schmalz, *Angew. Chem.* **2011**, *123*, 2440–2444; *Angew. Chem. Int. Ed.* **2011**, *50*, 2392–2396.
[10] S. Romanski, B. Kraus, M. Guttentag, W. Schlundt, H. Rucker, A. Adler, J.-M. Neudorfl, R. Alberto, S. Amslinger, H.-G. Schmalz, *Dalton Trans.* **2012**, *41*, 13862–13875.
[11] S. Romanski, E. Stamellou, J. T. Jaraba, D. Storz, B. K. Krämer, M. Hafner, S. Amslinger, H. G. Schmalz, B. A. Yard, *Free Radical Biol. Med.* **2013**, *65*, 78–88.
[12] S. Romanski, H. Rücker, E. Stamellou, M. Guttentag, J.-M. Neudörfl, R. Alberto, S. Amslinger, B. Yard, H.-G. Schmalz, *Organometallics* **2012**, *31*, 5800–5809.

[13] S. Botov, E. Stamellou, S. Romanski, M. Guttentag, R. Alberto, J.-M. Neudörfl, B. Yard, H.-G. Schmalz, *Organometallics* **2013**, *32*, 3587 – 3594.
[14] R. D. Rimmer, A. E. Pierri, P. C. Ford, *Coord. Chem. Rev.* **2012**, *256*, 1509 – 1519.
[15] U. Schatzschneider, *Inorg. Chim. Acta* **2011**, *374*, 19 – 23.
[16] D. Crespy, K. Landfester, U. S. Schubert, A. Schiller, *Chem. Commun.* **2010**, *46*, 6651 – 6662.
[17] H. Pfeiffer, A. Rojas, J. Niesel, U. Schatzschneider, *Dalton Trans.* **2009**, 4292 – 4298.
[18] G. Dördelmann, H. Pfeiffer, A. Birkner, U. Schatzschneider, *Inorg. Chem.* **2011**, *50*, 4362 – 4367.
[19] J. E. Clark, P. Naughton, S. Shurey, C. J. Green, T. R. Johnson, B. E. Mann, R. Foresti, R. Motterlini, *Circ. Res.* **2003**, *93*, 178.
[20] M. I. Guillén, J. Megías, V. Clérigues, F. Gomar, M. J. Alcaraz, *Rheumatology* **2008**, *47*, 1323 – 1328.
[21] L. S. Nobre, J. D. Seixas, C. C. Romao, L. M. Saraiva, *Antimicrob. Agents Chemother.* **2007**, *51*, 4303 – 4307.
[22] M. G. Bani-Hani, D. Greenstein, B. E. Mann, C. J. Green, R. Motterlini, *J. Pharmacol. Exp. Ther.* **2006**, *318*, 1315 – 1322.
[23] R. Foresti, J. Hammad, J. E. Clark, T. R. Johnson, B. E. Mann, A. Friebe, C. J. Green, R. Motterlini, *Br. J. Pharmacol.* **2004**, *142*, 453 – 460.
[24] S. Chlopicki, R. Olszanecki, E. Marcinkiewicz, M. Lomnicka, R. Motterlini, *Cardiovasc. Res.* **2006**, *71*, 393 – 401.
[25] R. Motterlini, P. Sawle, J. Hammad, S. Bains, R. Alberto, R. Foresti, C. J. Green, *FASEB J.* **2004**, *19*, 284 – 286.
[26] G. L. Bannenberg, H. L. A. Vieira, *Expert Opin. Ther. Pat.* **2009**, *19*, 663 – 682.
[27] T. R. Johnson, B. E. Mann, I. P. Teasdale, H. Adams, R. Foresti, C. J. Green, R. Motterlini, *Dalton Trans.* **2007**, 1500 – 1508.
[28] T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao, M. J. Romao, *J. Am. Chem. Soc.* **2011**, *133*, 1192 – 1195.
[29] T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romao, M. J. Romao, *Curr. Med. Chem.* **2011**, *18*, 3361 – 3366.
[30] W.-Q. Zhang, A. J. Atkin, R. J. Thatcher, A. C. Whitwood, I. J. S. Fairlamb, J. M. Lynam, *Dalton Trans.* **2009**, 4351 – 4358.
[31] W.-Q. Zhang, A. C. Whitwood, I. J. S. Fairlamb, J. M. Lynam, *Inorg. Chem.* **2010**, *49*, 8941 – 8952.
[32] F. Zobi, A. Degonda, M. C. Schaub, A. Y. Bogdanova, *Inorg. Chem.* **2010**, *49*, 7313 – 7322.
[33] R. Kretschmer, G. Gessner, H. Görls, S. H. Heinemann, M. Westerhausen, *J. Inorg. Biochem.* **2011**, *105*, 6 – 9.
[34] M. L. Dallas, J. L. Scragg, C. Peers in *Arterial Chemoreceptors, Vol. 648* (Eds.: C. Gonzalez, C. Nurse, C. Peers), Springer, Dordrecht, **2009**, pp. 89 – 95.
[35] H. Song, H. Zhao, Y. Qu, Q. Sun, F. Zhang, Z. Du, W. Liang, Y. Qi, P. Yang, *J. Periodontal Res.* **2011**, *46*, 48 – 57.
[36] M. Desmard, K. S. Davidge, O. Bouvet, D. Morin, D. Roux, R. Foresti, J. D. Ricard, E. Denamur, R. K. Poole, P. Montravers, R. Motterlini, J. Boczkowski, *FASEB J.* **2009**, *23*, 1023 – 1031.
[37] I. C. Winburn, K. Gunatunga, R. D. McKernan, R. J. Walker, I. A. Sammut, J. C. Harrison, *Basic Clin. Pharmacol. Toxicol.* **2012**, *111*, 31 – 41.
[38] J. D. Seixas, A. Mukhopadhyay, T. Santos-Silva, L. E. Otterbein, D. J. Gallo, S. S. Rodrigues, B. H. Guerreiro, A. M. L. Goncalves, N. Penacho, A. R. Marques, A. C. Coelho, P. M. Reis, M. J. Romao, C. C. Romao, *Dalton Trans.* **2013**, *42*, 5985 – 5998.
[39] M. F. A. Santos, J. D. Seixas, A. C. Coelho, A. Mukhopadhyay, P. M. Reis, M. J. Romao, C. C. Romao, T. Santos-Silva, *J. Inorg. Biochem.* **2012**, *117*, 285 – 291.
[40] H.-M. Berends, P. Kurz, *Inorg. Chim. Acta* **2012**, *380*, 141 – 147.
[41] S. H. Crook, B. E. Mann, A. J. H. M. Meijer, H. Adams, P. Sawle, D. Scapens, R. Motterlini, *Dalton Trans.* **2011**, *40*, 4230 – 4235.
[42] W. Huber, R. Linder, J. Niesel, U. Schatzschneider, B. Spingler, P. C. Kunz, *Eur. J. Inorg. Chem.* **2012**, 3140 – 3146.
[43] A. J. Atkin, J. M. Lynam, B. E. Moulton, P. Sawle, R. Motterlini, N. M. Boyle, M. T. Pryce, I. J. S. Fairlamb, *Dalton Trans.* **2011**, *40*, 5755 – 5761.
[44] S. McLean, B. E. Mann, R. K. Poole, *Anal. Biochem.* **2012**, *427*, 36 – 40.
[45] S. V. C. Vummaleti, D. Branduardi, M. Masetti, M. De Vivo, R. Motterlini, A. Cavalli, *Chem. Eur. J.* **2012**, *18*, 9267 – 9275.
[46] F. Zobi, L. Quaroni, G. Santoro, T. Zlateva, O. Blacque, B. Sarafimov, M. C. Schaub, A. Y. Bogdanova, *J. Med. Chem.* **2013**, *56*, 6719 – 6731.
[47] L. Yuan, W. Lin, L. Tan, K. Zheng, W. Huang, *Angew. Chem.* **2013**, *125*, 1670 – 1672; *Angew. Chem. Int. Ed.* **2013**, *52*, 1628 – 1630.
[48] B. W. Michel, A. R. Lippert, C. J. Chang, *J. Am. Chem. Soc.* **2012**, *134*, 15668 – 15671.
[49] J. Wang, J. Karpus, B. S. Zhao, Z. Luo, P. R. Chen, C. He, *Angew. Chem.* **2012**, *124*, 9790 – 9794; *Angew. Chem. Int. Ed.* **2012**, *51*, 9652 – 9656.
[50] R. D. Rimmer, H. Richter, P. C. Ford, *Inorg. Chem.* **2010**, *49*, 1180 – 1185.
[51] G. Bartolucci, E. Droghetti, C. Focardi, M. Bambagiotti-Alberti, M. Nocentini, G. Smulevich, *J. Mass Spectrom.* **2010**, *45*, 1041 – 1045.
[52] C. C. Romão, W. A. Blättler, J. D. Seixas, G. J. L. Bernardes, *Chem. Soc. Rev.* **2012**, *41*, 3571 – 3583.
[53] A. C. Pena, N. Penacho, L. Mancio-Silva, R. Neres, J. D. Seixas, A. C. Fernandes, C. C. Romao, M. M. Mota, G. J. L. Bernardes, A. Pamplona, *Antimicrob. Agents Chemother.* **2012**, *56*, 1281 – 1290.
[54] A. R. Marques, L. Kromer, D. J. Gallo, N. Penacho, S. S. Rodrigues, J. D. Seixas, G. J. L. Bernardes, P. M. Reis, S. L. Otterbein, R. A. Ruggieri, A. S. G. Goncalves, A. M. L. Goncalves, M. N. D. Matos, I. Bento, L. E. Otterbein, W. A. Blättler, C. C. Romao, *Organometallics* **2012**, *31*, 5810 – 5822.
[55] P. C. Kunz, H. Meyer, J. Barthel, S. Sollazzo, A. M. Schmidt, C. Janiak, *Chem. Commun.* **2013**, *49*, 4896 – 4898.
[56] M. A. Gonzalez, S. J. Carrington, N. L. Fry, J. L. Martinez, P. K. Mascharak, *Inorg. Chem.* **2012**, *51*, 11930 – 11940.
[57] P. Govender, S. Pai, U. Schatzschneider, G. S. Smith, *Inorg. Chem.* **2013**, *52*, 5470 – 5478.
[58] L. A. P. Antony, T. Slanina, P. Sebej, T. Solomek, P. Klan, *Org. Lett.* **2013**, *15*, 4552 – 4555.
[59] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108 – 7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974 – 6998.
[60] G. Dördelmann, T. Meinhardt, T. Sowik, A. Krueger, U. Schatzschneider, *Chem. Commun.* **2012**, *48*, 11528 – 11530.
[61] C. Bischof, T. Joshi, A. Dimri, L. Spiccia, U. Schatzschneider, *Inorg. Chem.* **2013**, *52*, 9297 – 9308.
[62] H. Pfeiffer, T. Sowik, U. Schatzschneider, *J. Organomet. Chem.* **2013**, *734*, 17 – 24.
[63] U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey, J. A. Hubbell, *J. Am. Chem. Soc.* **2010**, *132*, 18273 – 18280.
[64] N. E. Brückmann, M. Wahl, G. J. Reiss, M. Kohns, W. Waetjen, P. C. Kunz, *Eur. J. Inorg. Chem.* **2011**, 4571 – 4577.
[65] J. B. Matson, M. J. Webber, V. K. Tamboli, B. Weber, S. I. Stupp, *Soft Matter* **2012**, *8*, 6689 – 6692.
[66] J. L. Wilson, H. E. Jesse, R. K. Poole, K. S. Davidge, *Curr. Pharm. Biotechnol.* **2012**, *13*, 760 – 768.
[67] S. McLean, R. Begg, H. E. Jesse, B. E. Mann, G. Sanguinetti, R. K. Poole, *Antioxid. Redox Signaling* **2013**, *19*, 1999 – 2012.
[68] K. S. Davidge, G. Sanguinetti, C. H. Yee, A. G. Cox, C. W. McLeod, C. E. Monk, B. E. Mann, R. Motterlini, R. K. Poole, *J. Biol. Chem.* **2009**, *284*, 4516 – 4524.

[69] J. L. Wilson, H. E. Jesse, B. Hughes, V. Lund, K. Naylor, K. S. Davidge, G. M. Cook, B. E. Mann, R. K. Poole, *Antioxid. Redox Signaling* **2013**, *19*, 497 – 509.
[70] A. F. N. Tavares, M. Teixeira, C. C. Romao, J. D. Seixas, L. S. Nobre, L. M. Saraiva, *J. Biol. Chem.* **2011**, *286*, 26708 – 26717.
[71] H. E. Jesse, T. L. Nye, S. McLean, J. Green, B. E. Mann, R. K. Poole, *Biochim. Biophys. Acta Proteins Proteomics* **2013**, *1834*, 1693 – 1703.