Dalton Transactions

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/dalton

Examining the antimicrobial activity and toxicity to animal cells of different types of CO-releasing molecules

Lígia S. Nobre[a], Hélia Jeremias[a], Carlos C. Romão[a], and Lígia M. Saraiva[a]*

[a]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República (EAN), 2780-157 Oeiras, Portugal.

* Lígia M. Saraiva
E-mail: lst@itqb.unl.pt
Phone: +351-214469328; Fax: +351-214411277

Abstract

Transition metal carbonyl complexes used as CO-releasing molecules (CORM) for biological and therapeutic applications may exhibit interesting antimicrobial activity. However, understanding the chemical traits and mechanisms of action that rule this activity is required to establish a rationale for development of CORMs into useful antibiotics. In this work the bactericidal activity, the toxicity to eukaryotic cells, and the ability of CORMs to deliver CO to bacterial and eukaryotic cells was analysed for a set of seven CORMs that differ in the transition metal, ancillary ligands and CO release profile. Most of these CORMs exhibited bactericidal properties that decrease in the following order: CORM-2 > CORM-3 > ALF062 > ALF850 > ALF186 > ALF153 > $[Fe(SBPy_3)(CO)](BF_4)_2$. A similar yet not entirely coincident decreasing order was found for their induction of intracellular reactive oxygen species (ROS) in *E. coli.* In contrast, studies in model animal cells showed that for any given CORM, the level of intracellular ROS generated was negligible when compared with that measured inside bacteria. Importantly, these CORMs were in general not toxic to eukaryotic cells, namely murine macrophages, kidney LLC-PK1 epithelial cells, and liver cell line HepG2. CORM-2 and CORM-3 delivered CO to the intracellular space of both *E. coli* and the two types of tested eukaryotic cells, yet toxicity was only elicited in the case of *E. coli.* CO delivered by ALF186 to the intercellular space did not enter *E. coli* cells and the compound was not toxic to either bacteria or to eukaryotic cells. The Fe(II) carbonyl complex $[Fe(SBPy_3)(CO)]^{2+}$ had the reverse, undesirable toxicity profile, been unexpectedly toxic to eukaryotic cells and non-toxic to *E. coli*. ALF153, the most stable complex in the whole set was essentially devoid of toxicity or ROS induction ability to all cells. These results suggest that CORMs have a relevant therapeutic potential as antimicrobial drugs since i) they can show opposite toxicity profiles towards bacteria and eukaryotic cells; ii) their activity can be modulated through manipulation of the ancillary ligands, as shown with the three $\{Ru(CO)_3\}^{2+}$ and the two zerovalent Mo based CORMs; and iii) their toxicity to eukaryotic cells can be made acceptably low. With this new approach, this work contributes to the understanding of the roots of the bactericidal action of CORMs and help establishing strategies for their development into a new class of antibiotics.

Keywords

Carbon monoxide; CORM; drug toxicity; *E. coli*; reactive oxygen species; eukaryotic cells

Abbreviations

CO, Carbon monoxide; CORM, carbon monoxide-releasing molecule; ROS, reactive oxygen species; MS, minimal medium salts; PBS, phosphate buffered saline; FI, fluorescence intensity; DMEM, Dulbecco's modified Eagle's; FBS, heat-inactivated fetal bovine serum.

1. Introduction

Carbon monoxide (CO), an intracellular product of the haem oxygenase activity, has been administrated as a therapeutic agent in pre-clinical studies and shown to have beneficial effects in animal models of cardiovascular disease, inflammatory disorders and in organ transplantation.[1] However, in humans, the therapeutic utilization of inhaled CO presents important limitations as it may raises systemic carboxyhaemoglobin to prohibitive levels in humans. Carbon monoxide releasing molecules (CORMs) are prodrugs that deliver CO into biological systems. Since they can be designed not to affect the oxygen transport by haemoglobin they are considered a safer alternative to CO gas.[2-4] To date, a number of different molecules have been reported as CORMs, namely tertiaryaldehydes, oxalates, boroncarboxylates and silacarboxylates, but metalcarbonyl complexes proved to be the most suitable class of compounds.[2] The presence of carbonyl groups bound to transition metals such as ruthenium, iron or molybdenum appears to make CORMs unique in their ability to transfer CO into cells and amplify the mechanisms of signal transduction mediated by CO. Therefore, CORMs, as CO donors, exhibited a wide range of biological activities. In particular, selected transition metal carbonyls have repeatedly shown cytoprotective properties and other relevant curative activities in a very large range of cellular and *in vivo* animal models of diseases.[5, 6]

Our initial report on the bactericidal activity of CORM-2, CORM-3 (see Figure 1) and a few other transition-metal complexes unveiled a new type of molecules that are non-toxic to eukaryotic cells, have beneficial therapeutic effects in animal models of disease and yet are antimicrobial agents against Gram-positive and Gram-negative bacteria, grown either under aerobic or anaerobic conditions.[7-11] Several bacterial gene products have been suggested as CORM-targets, which were inferred from transcriptome data obtained for CORM-treated *Escherichia coli* cells.[12-14] In particular, it was shown that CORM-3-derived CO binds to terminal oxidases and other haem-containing proteins such as flavohaemoglobin, impairing cellular respiration and nitric oxide detoxification, respectively.[15] More recently, Wilson and co-workers proposed that non-haem proteins are also targeted by CORM-3 based on studies performed in an *E. coli* haem-deficient mutant.[16] In essence, all these studies considered that the bactericidal action was due to CO and somehow amplified relative to that of diffusing CO gas alone due to the higher intracellular concentration resulting from the delivery from a CORM. However, while exploring the mechanism of action of CORM-2 and ALF062 (see Figure 1) in order to learn how to design new CORMs with improved potency and efficacy, we realized that CORMs generate reactive oxygen species (ROS), not only in aerobic, cell free solutions but also inside *E. coli* cells. We showed for these two compounds that ROS also mediate the bactericidal effect.[8, 17] Importantly, the presence of CO was shown to be required, as addition of CO scavengers or the use of CO depleted CORMs abolished the bactericidal action.[7] However, the correlation of the intracellular ROS formation and the bactericidal activity remains to be fully understood. In any case, this important observation raised the need to uncouple the effect of intracellular CO from that of the CORM scaffold or the metal fragments that are generated

intracellularly upon CO release, as they all may contribute to the bactericidal activity. Already with this problem in mind, Ward and collaborators prepared tryptoCORM, a photoCORM bearing tryptophanate coordinated to the $[Mn(CO)_3(NCMe]^+$ fragment.[11] Their work showed that tryptoCORM efficiently kills *E. coli* only when it is irradiated with visible light in the presence of the cells, but not when pre-irradiated. Control tests revealed that tryptoCORM is not a bactericide in the dark and its photoproducts are also non-toxic to *E. coli*. In an almost simultaneous work, Nagel and co-workers prepared the photoCORM $[Mn(CO)_3(tpa\text{-}\kappa^3N]^+$ (tpa = tris(2-pyridylmethyl)amine), which is designed to trap its photoproducts (iCORM) as stable species like $[Mn(CO)_2(tpa\text{-}\kappa^4N]^+$.[18] Although this endeavour was only partially achieved it was shown that following internalization of the photoCORM in the *E. coli* cells, the compounds only show significant perturbation of growth when both irradiated and deprived of glucose. Under these conditions, the *E. coli* growth suffered only a transient reduction similar to that observed with CO gas. This result strongly suggests that, in this case, the growth perturbation is solely due to the inhibition of terminal oxidases by the CO released upon irradiation. Therefore, and in contrast with tryptoCORM, no extensive bacterial killing was observed with this photoCORM. A similar mimics of CO gas growth retardation activity was also reported for the interaction of the metal-free boranocarbonate CORM-A1 with *Pseudomonas aeruginosa*.[19]

While this manuscript was being evaluated, Simpson and collaborators reported a family of complexes of general formula $[Mn(CO)_3(bpy')X]^+$ (bpy' = substituted bipyridyl; X = anti-fungal azole ligand, (*e.g.* clotrimazol).[20] In total contrast with the two $[Mn(CO)_3]^+$ derivatives above mentioned, these molecules are very toxic in the dark, but only for Gram-positive bacteria and some parasites, reaching submicromolar MIC (minimal inhibitory concentration) values. Moreover, these molecules are much more active than their azole ligands. The toxicity of these CORMs is independent of irradiation, which is required to release CO from $[Mn(CO)_3]^+$ complexes under biological conditions. Therefore, these results are indicative of a decisive role of the ligand sphere in determining the biological activity of CORMs, which so far has been discussed in the context of animal cells, but is extensively to bacteria and other microbial cells.[2] More importantly, the relationship between bactericidal activity and toxicity to eukaryotic cells, as well as the ability to deliver CO to bacterial and eukaryotic cells still needs to be studied if the design of anti-microbial CORMs is to be made successful. To address these issues, in this work we have investigated the toxicity of several CORMs with different transition metals and CO release profiles (Figure 1), which are following briefly described.

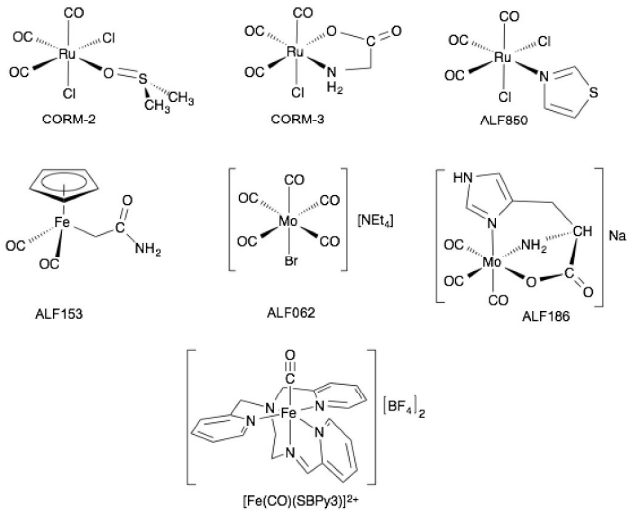
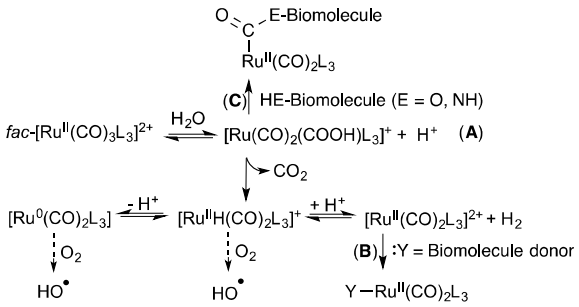


Figure 1. Schematic structures of CORMs used in this work.

CORM-2 and CORM-3 are ruthenium-based CORMs that release CO intracellularly but not to solution or its headspace.[21-24] So far, these compounds have been the more used ones in tests in animal and bacterial cells, but their mode of delivery is not completely stablished.[7, 9, 13, 25-27]

The thiazole complex ALF850 is an analogue of CORM-2 and CORM-3 with similar chemical properties, including the lack of CO release to the headspace of buffered solutions and to whole blood.[28] However, it possesses a sulphur containing ligand that can potentially interfere with the radical propagation process. Its CO release profile, aqueous chemistry and interaction with proteins have been studied but to the best of our knowledge it has not yet been used in bacterial or animal studies.[29, 30]

The chemistry of the air-stable, water sensitive $\{Ru(CO)_3\}^{2+}$ derivatives CORM-2, CORM-3, and ALF850 has been recently rationalized in some detail, confirming and extending the earlier report on CORM-3 aqueous chemistry.[31] The key reaction that initiates the aqueous chemistry of these complexes is the addition of hydroxyl ion to one of the CO ligands of the $\{Ru(CO)_3\}^{2+}$ moiety, according to equation **A** in Scheme 1.



Scheme 1. Summary of the aqueous chemistry of a generalized $\{Ru(CO)_3\}^{2+}$ based CORM.

This reaction is a pH dependent reversible process and opens the way to several possible reaction outcomes. Both the ester or amide forming pathway (**C**) and the water-gas-shift loss of CO_2 and H_2 leading to a coordinatively unsaturated fragment in pathway (**B**) provide means to anchor the $\{Ru(CO)_2\}^{2+}$ fragment to biomolecules through hydroxyl, amine or histidine groups on proteins or other donor molecules present on the surface, the cytoplasm or the nucleus of cells. The rapid formation of protein-adducts of $\{Ru(CO)_2\}^{2+}$ to histidine residues in the reaction of CORM-3 with proteins has been recently characterized.[32] Also remarkable is the formation of hydroxyl radicals, which takes place upon the reduction of oxygen by Ru^0 or Ru-H species that are formed in the medium after carbon dioxide loss.[31]

The chemical characterization of these CORMs also needs to consider that the dimeric structure of solid, commercially available CORM-2, $[Ru_2(CO)_3(\mu\text{-}Cl)_2]_2$ is not the actual structure of the compound when used in biological studies. Due to its water insolubility, dimethyl sulfoxide (DMSO) stock solutions are firstly made from which aliquots are added to the aqueous media. In DMSO solution, the chloride bridges of the dimer are broken with formation of monomeric $[RuCl_2(CO)_3(OSMe_2)]$ (Figure 1), which is believed to be the real active species in CORM-2.[33]

ALF062 is a lipophilic molecule with little solubility in water in spite of its negative charge. In contrast to the three above described CORMs, ALF062 releases free CO to the headspace of its solutions. In blood, it is proposed to release 4 equivalents of CO within 30 min, and the total load in less than 75 min.[34]

The decomposition of $[M(CO)_5X]^-$ (M = Cr, Mo, W; X = Cl, Br, I) complexes in the presence of biological molecules is mediated by X^- dissociation and formation of coordinatively unsaturated $\{M(CO)_5\}$ fragments. Such fragments are stabilized by solvent, water and other donors in solution, and can readily bind biological molecules with appropriate oxygen, nitrogen or sulfur donors.[35, 36] Ultimately, oxidation by O_2 releases all CO and forms molybdate as in the case of ALF186 described below. ALF062 has been shown therapeutic activity in an animal model of rheumatoid arthritis and is endowed with a notable antimicrobial activity.[7, 37]

ALF186 is a water-soluble zerovalent Mo complex that decomposes in aqueous medium under normoxic conditions, according to equation 1. After 30 min, the compound releases 1 CO equivalent, and after 4 h a maximum of 2.6 equivalents of CO is liberated. This decomposition becomes very fast in blood.[33]

$$[Mo(histidinate)(CO)_3]^- \xrightarrow[H_2O]{O_2} [MoO_4]^{2-} + 2.6\ CO + 0.4\ CO_2 \quad (1)$$

The stoichiometry in equation 1 may vary slightly with the composition of the medium but remains in the indicated range. Mo^0 is oxidized to molybdate that depending on the pH can oligomerise to polyoxometallates, but none of which binds proteins covalently.[33] The oxidation of ALF186 is faster than that of ALF062. In the latter, combined π-acidity of five CO ligands efficiently removes electronic charge from $\{Mo(CO)_5\}$ making it more difficult to oxidize than the $\{Mo(CO)_3\}$ fragment of ALF186. This compound has been used in several animal models of disease, presents no acute toxicity and acts *in vivo* as a CO bolus donor.[38, 39] It was reported

that ALF186 enhances intestinal clearance of bacteria and promotes the macrophage antimicrobial activity.[40]

The dicationic Fe^{2+} complex $[Fe(SBPy3)(CO)]^{2+}$ bearing a neutral pentadentate N5 ligand has the simplest CO release profile. In aqueous solution, the molecule releases a maximum of 1 equivalent of CO by a dissociative process, which follows equation 2, leaving behind an aqua-complex with the same charge and scaffold. It was reported to induce vasorelaxation of mouse aorta, and it was not yet tested in bacteria.[41]

$$[Fe(SBPy3)(CO)]^{2+} \xrightarrow{H_2O} [Fe(SBPy3)(H_2O)]^{2+} + CO \quad (2)$$

ALF153, the amphiphilic complex $[FeCp(CH_2CONH_2)(CO)_2]$, is a rather air stable compound that does not release CO in normoxic aqueous media for at least 6 h, except when in the presence of important concentrations of hydrogen peroxide.[34, 42] In this study, this compound is used as a non-CO releasing metal carbonyl.

The action of this set of diverse CORMs on bacterial and eukaryotic cells will be analysed in this work, in order to help unravelling key parameters that control CORM toxicity and CO delivery into microbial and mammalian cells.

2. Experimental

2.1. Carbon monoxide-releasing molecules

Seven compounds were used as CO donors namely, CORM-2 (Sigma), CORM-3, ALF850, ALF062, ALF186, ALF153, all from Alfama and $[(Fe(SBPy_3)(CO)](BF_4)_2$.[41] CORM-3 was prepared as previously described.[43] These CORMs were freshly prepared at concentrations in the range of 4-10 mM using as solvents dimethyl sulfoxide (DMSO) (CORM-2 and ALF850), methanol (ALF062), phosphate buffered saline (PBS) ($[(Fe(SBPy_3)(CO)](BF_4)_2$) and water (CORM-3, ALF153 and ALF186). In all assays, the correspondent solvent of each CORM was used as control.

2.2. Bacterial strains, growth conditions and viability assays

Escherichia coli K12 MG1655, was grown aerobically in minimal medium salts (MS),[7] at 37ºC and 150 rpm, to an optical density at 600 nm (OD_{600nm}) of 0.3. At this point, *E. coli* cells were left untreated or exposed to CORM (250 μM). Cell viability was determined by the number of colony forming units (CFU) formed on agar plates per mL. The percentage of survival represents the ratio of the number of colonies obtained after CORM exposure and when treated with the solvent only.

2.3. Minimal Inhibitory Concentration and Minimal Bactericidal Concentration experiments

Determination of the Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC) values were carried out by the tube dilution test. Briefly, 2 mL of MS was inoculated with an overnight culture of *E. coli* to give an OD_{600nm} of 0.01. CORMs, in a concentration range of 50 μM to 2 mM, were added to the bacterial cell suspensions contained in 24-well plates. Plates were incubated for 24 h, at 37°C and 90 rpm. The concentration of CORM in the first well in the series with no sign of visible growth was reported as the MIC. Cells were subsequently plated onto agar devoid of any drug. After incubation at 37°C, for 24 h, the lowest concentration of CORM used in a culture that showed no growth was considered the MBC.

2.4. Determination of endogenous ROS content in *E. coli* cells

For all compounds, *E. coli* cells were treated, for 1 h, with CORM (250 μM) or the correspondent solvent. Cells were harvested, washed twice and resuspended in PBS buffer. Probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma) was added to cell suspensions, at a final concentration of 10 μM, and the fluorescence intensity (FI) was acquired up to 2 h using a Varian Eclipse 96-well spectrofluorimeter (wavelength of excitation at 485 nm and of emission at 538 nm). To determine the ROS content, the FI of cultures treated only with solvent were subtracted from those of cells treated with CORM. The FI was normalized in relation to the OD_{600nm} of each culture.

2.5. Eukaryotic cell lines and cytotoxicity assays

The eukaryotic cell lines used in this study were: murine macrophage RAW 264.7 (ECACC 91062702), porcine kidney epithelial cells LLC-PK1 (ECACC 86121112, obtained from the American Type Culture Collection), and human hepatoma HepG2 cells (ECACC 85011430, Sigma). Cell lines were routinely maintained in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 0.7% (v/v) penicillin-streptomycin (Pen-Strep) mixture, all from Gibco-Invitrogen, and incubated in a 5% CO_2 atmosphere, at 37ºC, for 48 h. Cells used in the assays described below, were seeded on 24-well plates at $5x10^5$ cells/mL and incubated, at 37ºC, in a 5% CO_2atmosphere for 24 h.

The toxicity of CORMs to eukaryotic cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this colorimetric assay, the cell viability is determined by metabolisation of the yellow tetrazole (MTT) into purple formazan crystals. Cells, previously incubated in 24-well plates for 24 h, were washed three times with PBS, the medium replaced by DMEM supplemented with 10% FBS and treated with CORMs, for 24 h. At this point, the medium was replaced by fresh DMEM supplemented with 10% FBS and 1 mg/mL MTT, and cells incubated for1 h. The formazan crystals formed were solubilized with DMSO (1 mL) and the absorbance of the final solution was determined at 550 nm.

2.6. Endogenous ROS content in mammalian cells

The production of ROS was evaluated in two cell lines, namely the murine macrophages RAW264.7 and kidney epithelial LLC-PK1. Cells grown in 24-well plates for 24 h were treated with CORM at a 250 μM final concentration or with the correspondent solvent. The cells were incubated for 1 h, then washed with PBS and left untreated or exposed to the DCFH-DA probe (5 μM). After 30 min, cells were washed and 200 μL aliquots were distributed on 96-well plates. FI measurements were done up to 2 h for cells not exposed (FI0) and exposed to the probe (FI), and the FI/FI0 ratio was calculated. The percentage of fluorescence of CORM-treated cells refers to the FI/FI0 ratio of CORM- and solvent-treated cells.

2.7. CO detection in eukaryote and bacterial cells using COP-1

To assess CO inside the eukaryotic cells, murine macrophages RAW 264.7 and kidney epithelial cells LLC-PK1 were inoculated ($5x10^5$ cells/mL) in 6-well plates, containing a sterile coverslip, and incubated for 24 h. After removal of the medium, cells were washed three times with PBS buffer and exposed, for 15 min, to CORM-3 (100 μM) and next incubated, for 30 min, with 0.1 μM COP-1, which was prepared in DMSO as previously described.[22]

To evaluate the presence of CO into *E. coli,* bacterial cells grown in MS to an OD_{600} 0.3 were left untreated or exposed, for 15 min, to CORM-2, CORM-3, ALF186 (all at 250 μM) and $[RuCl_2(DMSO)_4]$ (concentration of 500 μM), the latter compound was used to mimic Ru^{2+} toxicity, and is herein designed iCORM. After treatment with COP-1 (1 μM), for 15 min, cells were collected by centrifugation, washed with PBS, and resuspended in 1/10 of its initial

volume in PBS. Cell suspensions were loaded onto a slide previously covered with a thin agarose layer (1.7%).

Fluorescence microscopy imaging experiments were performed in Leica DM6000B microscope equipped with a phase contrast Uplan F1 10x objective (100x to bacteria) and a CCD Ixon camera (Andor Technologies). Images were acquired and analysed using the Metamorph software suite version 5.8 (Universal Imaging). COP-1 was excited using a 488 nm Ar laser, and the emission was collected using a META detector between 500 and 650 nm.

3. Results

The seven selected CORMs (Figure 1) differ on the metal, coordination sphere types and, consequently, on their chemical and biocompatibility profiles (Table S1). These compounds were analysed in terms of antimicrobial action, amount of ROS released into bacterial cells, toxicity to eukaryotic cells, and ability to deliver CO to bacterial and eukaryotic cells. *Escherichia coli* was used as the microbial model and CORMs were tested in three types of eukaryotic cells that are widely used in *in vitro* assays, namely, the murine macrophage RAW 264.7, the porcine kidney LLC-PK1 epithelial cells and the human hepatocellular liver carcinoma cell line HepG2. The last two cell types have been widely used for nephrotoxicity and hepatotoxicity studies of drug candidates.[44, 45]

3.1. CORMs reduced the *E. coli* viability

CORMs were added to *E. coli* cells growing aerobically in minimal medium[7] and the viability was evaluated by CFU counting. After treatment, all compounds impaired the bacterial growth but to different extents, with CORM-2, CORM-3 and ALF062 being the more effective ones (Figure 2). After 4 h, *E. coli* exposed to CORM-2, CORM-3 and ALF062 showed a drastic viability decrease of at least 4 logs, which corresponds to a drop in survival higher than 99% (Figure 2B). For ALF850, ALF186 and ALF153, a much lower reduction of the cell viability was observed after 1 h (10-25 % survival), with cells recovering viability after 4 h to values similar to those of untreated cells. The antimicrobial capacity of $[(Fe(SBPy_3)(CO)](BF_4)_2$ was below that of all other CORMs after 1 h, but its toxicity increased along time.

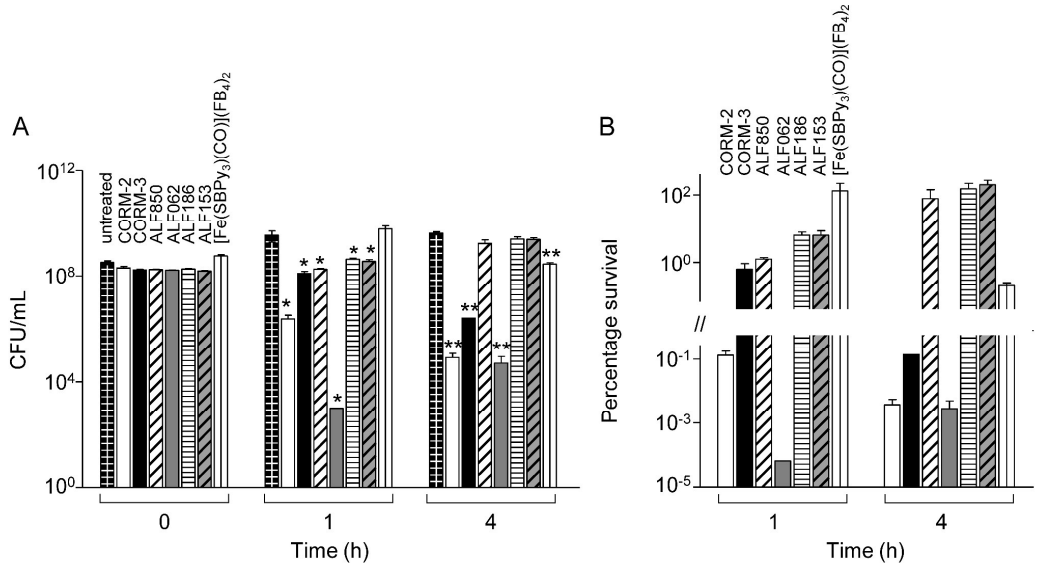


Figure 2. Effect of CORM on *E. coli* viability.

Viability (A) and percentage survival (B) of *E. coli* cells grown aerobically in MS medium left untreated (squared) and treated with CORMs (250 μM) were evaluated at the indicated times: CORM-2 (white), CORM-3 (black), ALF850 (striped black), ALF062 (dark grey), ALF186

(horizontal strips), ALF153 (striped grey) and [(Fe(SBPy$_3$)(CO)](BF$_4$)$_2$ (vertical striped). The results are averaged values of at least two biological samples with error bars representing standard errors. Percentage survival was determined in relation to the initial number of cells (T=0 h). **P < 0.01, *P < 0.05.

The bactericidal activity of CORMs was also evaluated through determination of the MIC and MBC values using CORM concentrations ranging between 0 - 2 mM. Table 1 shows that CORM-2, CORM-3, ALF062 and ALF850 exhibited bactericidal character with a MBC/MIC ratio close to 1. On the contrary, ALF153 and [(Fe(SBPy$_3$)(CO)](BF$_4$)$_2$ were not effective against *E. coli*. Also, ALF186 presented a rather high MIC value, which indicates a reduced antimicrobial activity.

Table 1. Bactericidal activity of CORMs against *E. coli*

CORM	MIC (μM)	MBC (μM)	MBC/MIC
CORM-2	350	500	1.4
CORM-3	400	500	1.2
ALF850	650	750	1.1
ALF062	450	600	1.3
ALF186	2000	2000	1.0
ALF153	Na	na	nd
[Fe(SBPy$_3$)(CO)](BF$_4$)$_2$	Na	na	nd

na-not available (no MIC value was observed up to 2 mM)
nd-not determined

3.2. CORMs induce the intracellular formation of ROS in *E. coli*

The amount of ROS formed inside CORM-treated *E. coli* was determined in cells grown to the initial log phase and exposed to 250 μM CORMs, for 1 h. Among the seven CORMs, CORM-2, CORM-3, ALF850 and ALF062 were found to increase the intracellular ROS content. CORM-3, ALF850 and ALF062 generated similar amounts of intracellular ROS in *E. coli*, which were lowered than that originated by CORM-2 (Figure 3). In contrast, ALF186, ALF153 and [(Fe(SBPy$_3$)(CO)](BF$_4$)$_2$ did not cause a relevant raise in the intracellular ROS content (Figure 3).

These results suggest a possible correlation between the bactericidal character of this type of CORMs and their ROS inducing ability, as the CORMs that kill *E. coli* more efficiently (CORM-2, CORM-3, ALF062 and, ALF850) produce also higher levels of intracellular ROS, whereas those that are less bactericidal (ALF186, ALF153 and [(Fe(SBPy$_3$)(CO)](BF$_4$)$_2$) generate lower

intracellular ROS content. Nevertheless, such correlation cannot be generalized as, for example, ALF850 induces an amount of ROS equivalent to that produced by CORM-3 yet has a lower antimicrobial activity.

Therefore, we concluded that although CORM-derived ROS contribute to toxicity, the antimicrobial action of the studied CORMs is not solely sustained by the intracellular ROS formation.

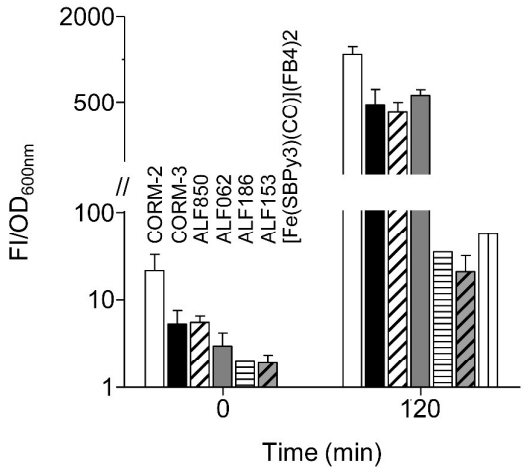


Figure 3. Intracellular ROS content in CORM-treated *E. coli* cells.

E. coli was exposed to 250 μM CORM, for 1h, and ROS were measured by fluorescence intensity (FI) of cell suspensions treated with 10 μM DCFH-DA. FI represents the subtraction of cultures treated from cultures not exposed to CORMs, and data are presented after normalization in relation to the correspondent OD_{600nm}.

CORM-2 (white), CORM-3 (black), ALF850 (striped black), ALF062 (dark grey), ALF186 (horizontal strips), ALF153 (striped grey) and $[(Fe(SBPy_3)(CO)](BF_4)_2$ (vertical strips). Each bar represents the average of four assays with the correspondent standard error.

3.3. CORMs are non-toxic to eukaryotic cells

CORM toxicity was evaluated in eukaryotic cells, namely murine macrophages RAW 264.7, porcine kidney LLC-PK1 and human liver HepG2. The most toxic CORM to eukaryotic cells was $[(Fe(SBPy3)(CO)](BF_4)_2$, which caused a severe reduction of the macrophage viability and decreased the HepG2 survival between 20%-40%. Nevertheless, it appears to be harmless to LLC-PK1 cells (Figure 4A-C). Due to the compound toxicity to two of the three cell lines, we chose to exclude it from further studies.

In general, CORMs did not decrease the viability of LLC-PK1 cells even at the highest utilized concentrations (Figure 4A). The only exception was ALF062 that above 400 μM decreased cell viability by ~20%. Most CORMs were also innocuous to RAW 264.7 macrophages up to 250 μM; again the of-trend CORM was ALF062 that reduced very strongly the cell viability (~90%). LLC-PK1 cells submitted to CORM-2 and ALF850 concentrations higher than 400 μM suffered

a viability reduction above 50%. On the contrary, all the other CORMs remained non-toxic for concentrations up to 500 μM (Figure 4B).

Comparatively, HepG2 cells were the most sensitive cells to metal carbonyls as only CORM-3 and ALF153 showed to be non-poisonous to this cell line. ALF062 were detrimental above 100 μM and CORM-2, ALF850 and ALF186 diminished the cell viability for concentrations equal and above 250 μM (Figure 4C).

The toxicity of the solvents used to dissolve CORMs was also determined (Figure 4A'-C'). DMSO did not affect the viability of any of the tested cell types in concentrations up to 5%, while methanol damaged RAW 264.7 and HepG2 cells when used in concentrations above 2.5%. Hence, part of the ALF062 toxicity is linked to its solvent, as methanol itself reduces the viability of macrophages (Figure 4B and B').

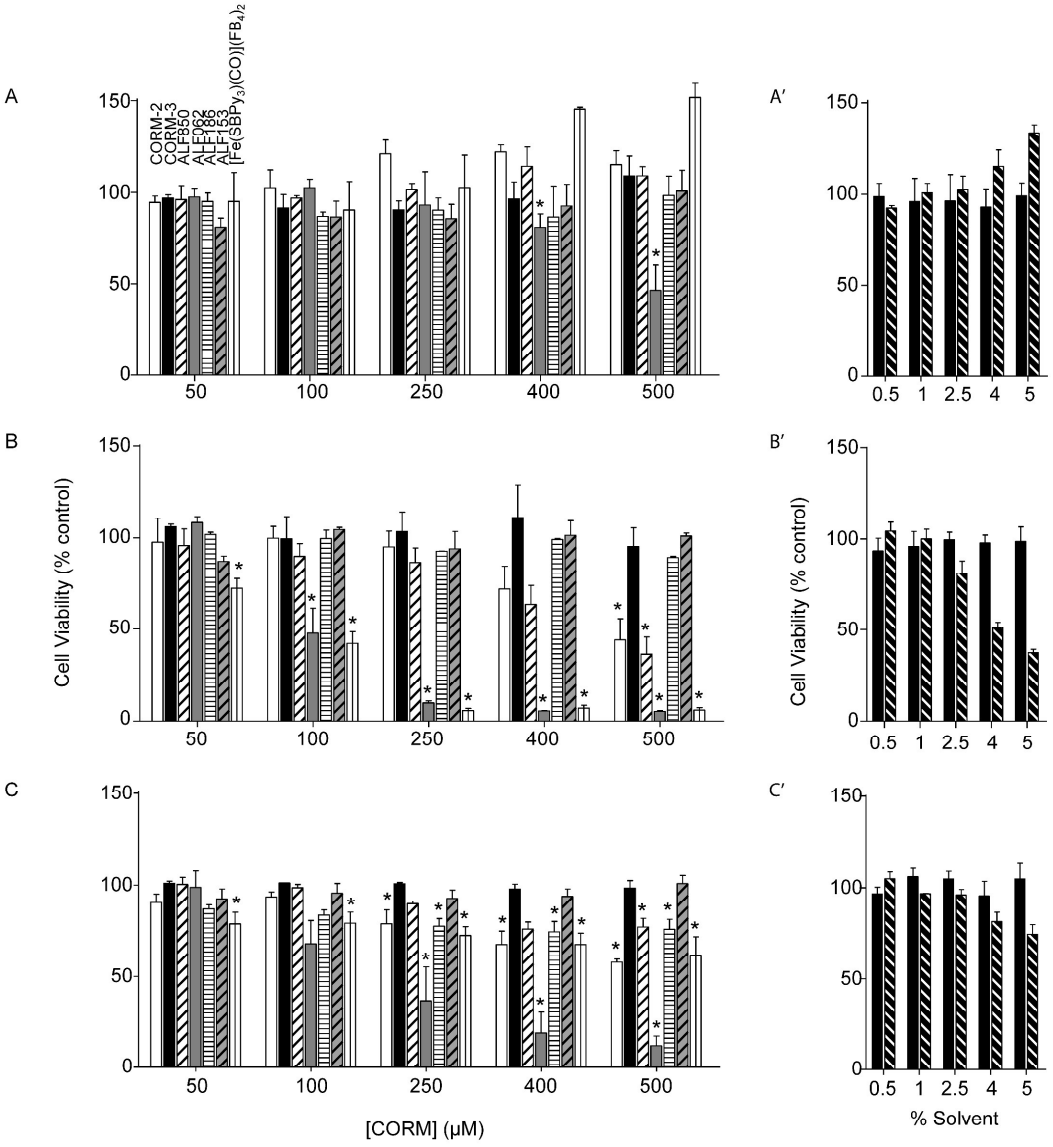


Figure 4. CORMs are not cytotoxicity to eukaryotic cells.

Cytotoxicity of CORMs and solvents was evaluated by the MTT assay in porcine kidney epithelial cells LLC-PK1 (A, A'), murine macrophage RAW 264.7 (B, B'), and human hepatoma HepG2 cells (C, C') using CORMs (50-500 μM): CORM-2 (white), CORM-3 (black), ALF850 (striped black), ALF062 (dark grey), ALF186 (horizontal strips), ALF153 (striped grey) and $[(Fe(SBPy_3)(CO)](BF_4)_2$ (vertical strips). Panels A' to C' depict the toxicity of the solvents DMSO (black) and methanol (striped black). Cell viability was determined in relation to untreated cells. Each bar represents the average of three independent sample measurements with the corresponding standard errors*P < 0.01.

3.4. CORMs do not generate significant intracellular ROS content in eukaryotic cells

The generation of ROS within eukaryotic cells was also analysed. Due to the similar toxicity of CORMs to macrophages and HepG2 cells, as described above, the CORM-associated ROS formation was tested only in murine macrophage RAW 264.7 and kidney epithelial LLC-PK1 cells. Figure 5 shows that ALF850 and CORM-2 generated a significant level of ROS in macrophage and kidney cells, respectively, while all the other compounds failed to increase ROS to levels above those measured in untreated cells. Interesting, ALF062, which decreases the viability of macrophages when administrated at 100 μM, did not augment the ROS content of these cells. Furthermore, ALF850 that significantly increased the macrophages' ROS content was non-toxic to these cells at concentrations up to 250 μM (Figure 4 and 5).

These results indicate that: i) the low level of ROS generated inside the eukaryotic cells does not correlate with lack of cytotoxicity, as some of the CORMs tested are toxic to cells in which they are unable to induce a ROS burst; and ii) the induction of ROS by CORMs does not necessarily correlate with cytotoxicity to eukaryotic cells. Hence, one needs to consider that different mechanisms of action of CORMs, other than ROS, are in place when addressing the toxicity of CORMs to bacterial and eukaryotic cells.

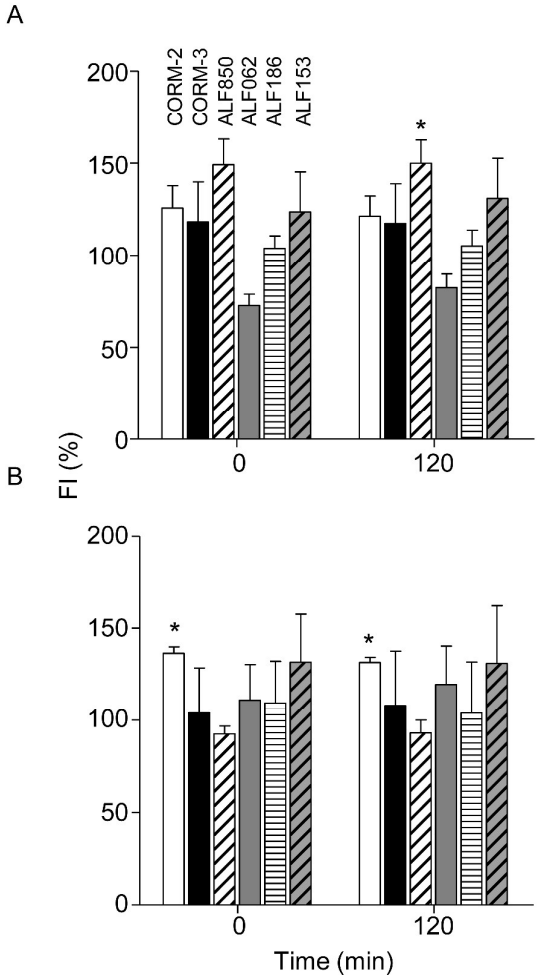


Figure 5. Intracellular content of ROS in eukaryotic CORM-treated cells.

Murine macrophage RAW 264.7 (A) and porcine kidney epithelial cells LLC-PK1 (B) were exposed to 250 μM CORM, for 1 h: CORM-2 (white), CORM-3 (black), ALF850 (striped black), ALF062 (dark grey), ALF186 (horizontal striped) and ALF153 (striped grey). For each compound/solvent, the fluorescence intensity of cells exposed to the probe (FI) was normalised to the FI of untreated cells (FI0) and the percentage of fluorescence was calculated in relation to solvent treated cells. *$P < 0.05$.

3.5. CORMs accumulate CO intracellularly in bacteria and eukaryotic cells

The two more effective antimicrobial agents, namely CORM-2 and CORM-3, and the less effective antimicrobial agent used in this study, ALF186, were chosen to evaluate the capacity of CORMs to release CO inside the bacterial cells. The intracellular accumulation of CO was detected with the COP-1 probe that has been reported to react with CO to form a fluorescent carbonylation product.[22, 46]

The results show that no CO was present inside *E. coli* cells after exposure to ALF186 followed by incubation with COP-1. However, a significant increase of the fluorescence intensity was

noted in *E. coli* cells treated with CORM-3, when compared with *E. coli* cells exposed only to COP-1 (Figure 6 and S1). Likewise, an intense fluorescence signal was observed in cells treated with CORM-2 whereas no fluorescence was detected in cells exposed to the CO-depleted molecule, the iCORM form (Figure S1).

The capacity of CORM-3 to release CO to eukaryotic cells such as macrophages and LLC-PK1 was also examined. Incubation of RAW 264.7 and LLC-PK1 cells with CORM-3 led to the intracellular accumulation of CO as judged by the development of a fluorescence response signal, which was not seen in untreated cells (Figure 7).

Hence, ALF186, which rapidly delivers CO to the cell growth medium,[33] does not accumulate CO into cells and, therefore, is harmless to both bacterial and eukaryotic cells. Ru-based CORM-3 that releases CO intracellular both into *E. coli* and animal cells (RAW 264.7 and LLC-PK1) is only toxic to bacterial cells.

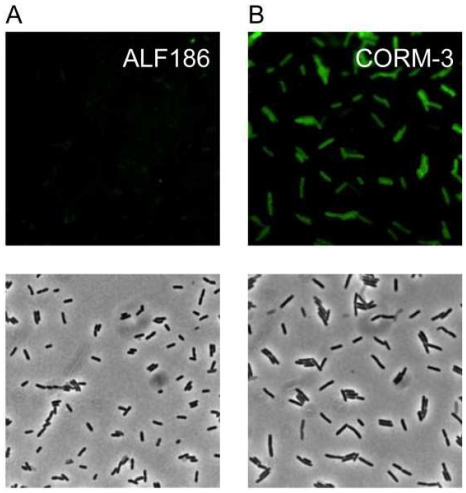


Figure 6. Fluorescence microscopy images of *E. coli* cells exposed to CORM-3 and ALF186.

E. coli cells were treated with 250 μM ALF186 (A) or CORM-3 (B), and incubated with COP-1. Fluorescence images acquired with a FITC filter are presented in the upper panels, and the correspondent bright field images (100x objective) are shown in the lower panels.

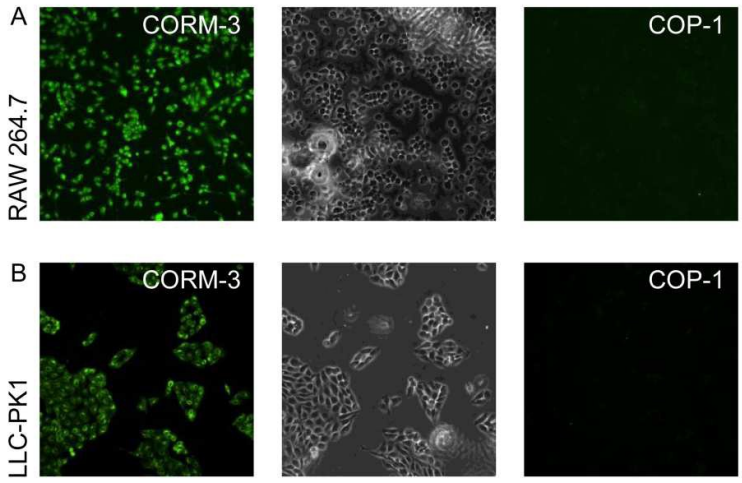


Figure 7. Fluorescence microscopy images of CORM-3 treated eukaryotic cells.
Murine macrophage RAW 264.7 (A) and porcine kidney epithelial cells LLC-PK1 (B) treated with 100 μM CORM-3 and 1 μM COP-1 (15 min). Cells exposed to COP-1 are also shown (right panel). Fluorescence images were acquired with a FITC filter (left and right panel) and bright field images were obtained with a 10x objective (middle panel).

4. Discussion

The antimicrobial action of CO was first shown for *E. coli* and *S. aureus* in studies that used CO gas and CORMs. Although the latter presented a more pronounced bactericidal action than CO gas,[7] the molecular basis of their antimicrobial action remains largely obscure, a fact that hampers the search for pharmacologically improved and more efficient bactericidal CORMs. At the outset of this work, three questions were raised, namely: i) how does the bactericidal activity depend on CO release from a CORM; ii) are bactericidal CORMs toxic to eukaryotic cells; and iii) how does CO release into cells correlate with intracellular ROS formation. In order to help clarifying such issues we compared the bactericidal action, the cytotoxicity to eukaryotic cells, the intracellular ROS production and CO-releasing ability of the panel of CORMs presented in Figure 1. As outlined in the introduction, these CORMs were selected due to their different chemistry and CO release profiles and include several compounds that have been widely tested as antimicrobials, as CO delivery drugs or both.

The data presented in Figure 2 and Table 1 confirm that *E. coli* cells are not equally killed by all types of CORMs or metal-carbonyl complexes studied herein, as some have a significant antimicrobial activity whereas others are totally innocuous. Considering the CO release profiles, the seven compounds can be divided in two large groups: those that provide CO gas to the biological medium (ALF062, ALF186, $[(Fe(SBPy_3)(CO)][BF_4]_2$) and those that do not release CO gas to the medium (CORM-2, CORM-3, ALF850, ALF153). In the former group, solely ALF062 is a bactericide while in the latter group only ALF153 is not. Indeed, both ALF186 and ALF062 liberate over 2 equivalents of CO to the medium within 2 h of incubation under aerobic conditions,[37] yet their antimicrobial activity is different. Figure 6 shows that ALF186 while efficiently delivering CO to the medium was unable to accumulate CO into cells in order to trigger fluorescence by carbonylation of COP-1. On the other hand, CORM-3 and CORM-2, which do not deliver CO gas to the medium, but are able to store CO inside bacteria, have improved ability to kill *E. coli*. Therefore, it can be concluded that the bactericidal activity does not correlate with the ability of CORMs to release free CO gas to the medium. In other words, the release of CO gas to the medium is not a sufficient condition to turn a metal carbonyl CORM into a bactericide. These results strongly suggest that in order to achieve bactericidal activity, CORMs must enter the bacterial cells and release CO inside them.

The mechanisms by which internalization and CO liberation within bacterial cells take place are not fully understood and will certainly depend on the actual chemistry of the CORM. For example, in the case of Ru based CORMs it is known that intracellular compounds such as sulphites might trigger the release of CO,[21] but this chemistry is not extensive to Mo^0 complexes like ALF062. Obviously, there are other ways in which CORMs may decompose intracellularly.

In a previous study we reported that CORM-2 and ALF062 elicit bacterial death mediated by ROS.[17] The chosen set of CORMs also allowed us to investigate whether the bactericidal activity is proportional or otherwise correlates to the amount of ROS generated by the CORMs into the *E. coli* cells. We observed that, although the induction of intracellular ROS is a general

effect of the analysed CORMs, the amounts generated vary amongst compounds. Nevertheless, the qualitative graphic comparison of the increasing order of bactericidal activity (according to MIC values, Table 1) and ROS production in *E. coli* (Figure 3) shows that they run parallel to each other (Figure 8).

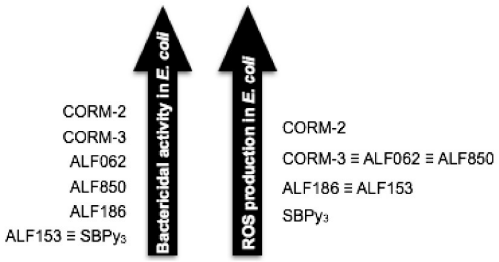


Figure 8. Comparison between CORM bactericidal activity and generation of intracellular ROS in *E. coli*.

In spite of this correlation, ROS seem not to be the sole cause of bactericidal cell death, as previously suggested by us.[17] For instance, ALF850, ALF062 and CORM-3 produced similar values of ROS but the latter is clearly a more potent bactericide. ALF153 has no antimicrobial effect yet it generates almost the same level of ROS as ALF186, which still has some bactericidal activity. In agreement with these conclusions, the bactericidal activity of another type of metal carbonyls, like e.g. photoCORMs were also shown to be bactericidal and yet might not leading to the production of ROS.[11, 18]

The mechanism of intracellular ROS generation remains an open issue. As we and others have previously shown, ALF186, CORM-3, CORM-2 and related molecules originate hydroxyl radicals when dissolved in aqueous aerobic medium, in the absence of cells.[34] This is a common property of metal carbonyl CORMs inasmuch as their low oxidation state metal centres are usually oxidized by O_2, thus generating superoxide and other ROS species. If oxygen reduction seems evident for zerovalent metal centres as those in ALF186 and ALF062, this is less clear with respect to CORM-2 and CORM-3, which are air stable solids. However, once in aerobic solutions, their water-gas shift driven chemistry (Scheme 1) leads to reducing Ru^0 or Ru-H species that can produce ROS.[33] For the complexes in the panel, this chemical mechanism of ROS generation will be active as long as $\{Mo(CO)_x\}^0$ and $\{Ru(CO)_3\}^{2+}$ derived species are in the presence of oxygen, either inside, at the walls, or outside the cells. In fact, we have previously reported that ROS generation triggered by CORM-2 and ALF062 in *E. coli* is strictly CO dependent in the sense that it requires $\{Mo(CO)_x\}^0$and $\{Ru(CO)_3\}^{2+}$ to mediate the ROS formation.[17] When formed at the walls or inside bacterial cells, it is quite likely that such CORM based ROS are at the origin of the oxidative stress processes that lead to cell death.[17] Concerning, ALF186, its low bactericidal activity seems to result from a too fast oxidation with very rapid loss of CO and ROS formation in the intercellular space, but not inside the cells. Hence, the trend indicates that the intracellular ROS burst is a major contributor to bactericidal

death by these CORMs. As for the CORMs that release CO to the intracellular space, the accumulation of CO inside bacterial cells is expected to target essential cellular proteins, whose impaired function results in cell death. Likewise, the bactericidal activity of tryptoCORM required that the CO release was triggered intracellularly.[11]

The behaviour of the same panel of CORMs tested on three different eukaryotic cell lines provided a significantly different pattern of results that varies with the type of cells. The only CORM that affects LLC-PK1 cells viability is ALF062, although its toxicity is only visible above 250 μM. HepG2 and RAW 264.7 cells proved to be more susceptible: the dose dependent toxicity of ALF062 increases between 50-500 μM for HepG2 cells and has a steeper rise for macrophages RAW 264.7 (Figure 4). The three $\{Ru(CO)_3\}^{2+}$ CORMs are non-toxic to macrophages RAW 264.7 and HepG2 cells up to 250 μM. Above this concentration both CORM-2 and ALF850 are toxic whereas CORM-3 remains harmless up to 500 μM. ALF153 is non-toxic to the three cell lines in the 50-500 μM range, while ALF186 shows very little toxicity for HepG2 cells even at 500 μM. $[(Fe(SBPy_3)(CO)](BF_4)_2$ is the more erratic of all compounds analysed. It is totally non-toxic (actually has a proliferating activity) to LLC-PK1 cells, slightly detrimental to HepG2 cells at all concentrations without a clear dose-response, and is the most toxic member of the panel for macrophages RAW 264.7 with a very steep dose dependent slope. Hence, in general, CORM-3, ALF153 and ALF186 are the least toxic CORMs whereas ALF062 is the more toxic one. CORM-2 and ALF850 only reveal toxicity above 250 μM.

In contrast to the results obtained with *E. coli*, CORMs did not raise significantly the amount of ROS within eukaryotic cells (Figure 5). Although CORM-3 delivers CO to LLC-PK1 and RAW 264.7 cells, it does not raise the intracellular ROS content. Moreover, there seems to be no interconnection between the ROS production and toxicity: ALF062 that is the most toxic CORM to eukaryotic cells does not induce any relevant amount of ROS in these cells (Figure 4). Note that, although the concentration values tested are very high when compared with those used in therapeutic tests in animal cells and animal models of disease (<100 μM), the CORM concentrations required to kill bacteria showed no significant cytotoxicity to eukaryotic cells.

We have also demonstrated that the $\{Ru(CO)_3\}^{2+}$ derived CORMs are able to enter cells where they liberate CO. Given their chemical lability in aqueous media, summarized in Scheme 1 and discussed elsewhere,[33] we believe that the internalization process is mediated by coordinatively unsaturated fragments that first bind and then cross cell walls. The nature and stability of such fragments can be controlled by the ancillary ligands in 18-electron complexes of general formula $[RuX_2(CO)_3L]$, $[RuX(CO)_3L_2]^+$, $[Ru(CO)_3L_3]^{2+}$ (X = monoanionic ligand; L = neutral, $2e^-$ ligand), according to previously work shown by some of us.[33] The structures of CORM-2, ALF850 and CORM-3 already confirm this kind of control since the small variations among their structures are sufficient to yield different activities.

For Ru-derived CORMs, the chemistry of Scheme 1 starts with the attack of hydroxyl ion to the $\{Ru(CO)_3\}^{2+}$ fragment. A similar attack is not possible when $[Mo(CO)_5Br]^-$ is dissolved in biological media as it is a negatively charged, highly electron rich molecule. Nevertheless, such charge excess can be alleviated by facile Br^- dissociation, generating a relatively air stable,

solvent stabilized $\{Mo(CO)_5\}$ fragment. This coordinatively unsaturated, lipophilic species can easily bind other biological targets/molecules accumulating at the cellular surfaces, from where it may enter cells just like $\{Ru(CO)_x\}^{2+}$ species. The very substitutionally and redox stable analogues $[W(CO)_5X]^-$ (e.g. X = CN, SCN, SCH_2Ph) freely cross membranes of eukaryotic cells as shown by electromanipulation studies.[47, 48] So, the lipophilic $\{Mo(CO)_5\}$ fragmentor or its adducts generated from ALF062 is expected to enter the cells with similar ease. Yet, the $[Mo(CO)_5X]^-$ species are much more labile than their W analogues as demonstrated previously[35] and will easily decompose inside cells. Hence, this intracellular delivery of the large load of CO may explain their high toxicity towards both bacteria and eukaryotic cells.

In any case, the active bactericide species must be some kind of metal carbonyl fragment $[M(CO)_x]$ with the ability to enter the cell. Notwithstanding, the exact nature of such species, as well as their intimate killing mechanism remains unknown and is likely to vary with the metal used. Putting together the information gathered herein, we propose that the anchoring of $\{M(CO)_x\}$ to the cell walls, followed by their transport or delivery of CO into the cells, is crucial to generate the bactericidal activity. Adequate selection of ancillary ligands to unsaturated $\{M(CO)_x\}$ fragments may eventually lead to more potent CORM bactericides and antibiotics. Importantly, this work reinforces the notion that it is possible to develop bactericidal CORMs to be used *in vivo* in mammals, since the toxicity profiles of CORMs are very different for bacteria and animal cells.

Conclusion

Most of the work done in this area has been concerning the mechanisms of bacterial killing of a reduced number of molecules and bacteria. As mentioned above, our studies on CORM-2 and ALF062 favoured the formation of ROS as the trigger for bacterial cell killing[16]. Moreover, Poole and co-workers have shown that CORM-3 has a range of haem and non-haem targets, disturbs the bacterial membrane and penetrates *E. coli*, as ascertained by intracellular Ru.[13,15] This complexity may result from the bewildering Ru^{z+} speciation expected in aqueous, let alone biological solutions of CORM-2 and CORM-3.[27] Using a novel approach in which we analysed the activity of a set of potential bactericidal CORMs along the lines of their relevant chemical traits, like nature of the metal, oxidation state, stability to CO release, ancillary ligands and structure, on *E. coli* and three eukaryotic cell lines we concluded that: the ancillary ligands in a given $\{M(CO)_x\}$ fragment modulate the bactericidal activity; along a series of CORMs the order of decreasing bactericidal activity and decreasing intracellular ROS formation is roughly parallel but not entirely coincident; for any given CORM, the induction of ROS in eukaryotic cells is negligible in comparison with bacterial cells; most CORMs are non-toxic to eukaryotic cells even at their high bactericidal concentrations; CORM-2 and CORM-3 do not deliver CO to the intercellular medium but deliver CO to the intracellular space of both animal cells and bacteria, but are only toxic to the latter; CORMs that only deliver CO to the intercellular space are devoid of bactericidal activity; and, inert coordination spheres do not favour bactericidal activity. Moreover, we show that some CORMs can kill bacteria at concentrations that are innocuous

towards several animal cells as was also seen by other authors.[11] Although, the MIC values of the best CORMs in this panel are still too high for practical use, these data contribute to envisage pathways that will increase the bactericidal potency of {$M(CO)_x$} fragments.

Acknowledgements

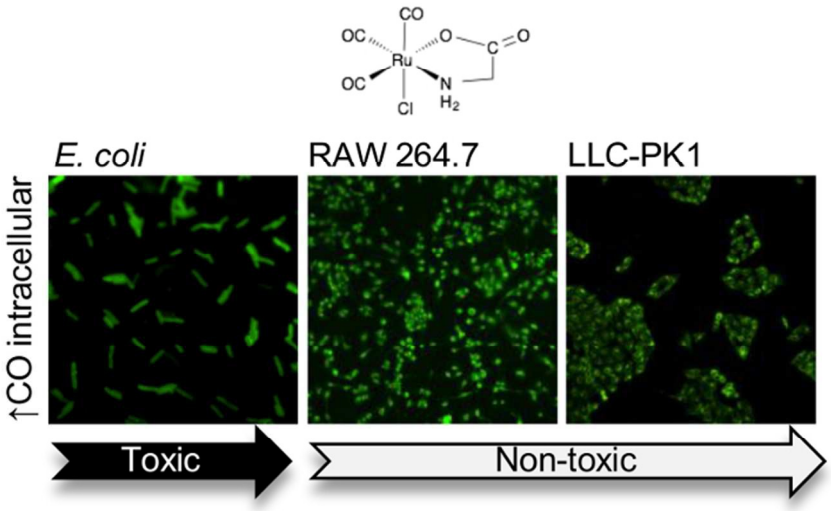
The work was funded by FCT project PTDC/BBB-BQB/0937/2012, FCT R&D Unit, UID/CBQ/04612/2013 and by the following FCT fellowship SFRH/SRFH/BPD/69325/2010 (LSN). We thank Ana F.N. Tavares and Mafalda C. Figueiredo for technical support and Alfama Inc. for providing the CORMs used.

References

1. R. Motterlini and L. E. Otterbein, *Nat Rev Drug Discov*, 2010, **9**, 728-743.
2. C. C. Romao, W. A. Blattler, J. D. Seixas and G. J. Bernardes, *Chem Soc Rev*, 2012, **41**, 3571-3583.
3. F. Zobi, *Future Med Chem*, 2013, **5**, 175-188.
4. S. H. Heinemann, T. Hoshi, M. Westerhausen and A. Schiller, *Chem Commun (Camb)*, 2014, **50**, 3644-3660.
5. S. Garcia-Gallego and G. J. Bernardes, *Angew Chem Int Ed Engl*, 2014, **53**, 9712-9721.
6. R. Motterlini, B. Haas and R. Foresti, *Med Gas Res*, 2012, **2**, 28.
7. L. S. Nobre, J. D. Seixas, C. C. Romao and L. M. Saraiva, *Antimicrob Agents Chemother*, 2007, **51**, 4303-4307.
8. A. F. Tavares, M. R. Parente, M. C. Justino, M. Oleastro, L. S. Nobre and L. M. Saraiva, *PLoS One*, 2012, **8**, e83157.
9. 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 and J. Boczkowski, *Faseb J*, 2009, **23**, 1023-1031.
10. W. C. Shen, X. Wang, W. T. Qin, X. F. Qiu and B. W. Sun, *Acta Pharmacol Sin*, 2014, **35**, 1566-1576.
11. J. S. Ward, J. M. Lynam, J. Moir and I. J. Fairlamb, *Chemistry*, 2014, **20**, 15061-15068.
12. L. S. Nobre, F. Al-Shahrour, J. Dopazo and L. M. Saraiva, *Microbiology*, 2009, **155**, 813-824.
13. K. S. Davidge, G. Sanguinetti, C. H. Yee, A. G. Cox, C. W. McLeod, C. E. Monk, B. E. Mann, R. Motterlini and R. K. Poole, *J Biol Chem*, 2009, **284**, 4516-4524.
14. J. L. Wilson, H. E. Jesse, B. Hughes, V. Lund, K. Naylor, K. S. Davidge, G. M. Cook, B. E. Mann and R. K. Poole, *Antioxid Redox Signal*, 2013, **19**, 497-509.
15. M. Tinajero-Trejo, K. J. Denby, S. E. Sedelnikova, S. A. Hassoubah, B. E. Mann and R. K. Poole, *J Biol Chem*, 2014, **289**, 29471-29482.
16. J. Wilson, L. Wareham, S. McLean, R. Begg, S. Greaves, B. Mann, G. Sanguinetti and R. Poole, *Antioxid Redox Signal*, 2015, **0**, 1-15.
17. A. F. Tavares, M. Teixeira, C. C. Romao, J. D. Seixas, L. S. Nobre and L. M. Saraiva, *J Biol Chem*, 2011, **286**, 26708-26717.
18. C. Nagel, S. McLean, R. K. Poole, H. Braunschweig, T. Kramer and U. Schatzschneider, *Dalton Trans*, 2014, **43**, 9986-9997.
19. M. Desmard, R. Foresti, D. Morin, M. Dagouassat, A. Berdeaux, E. Denamur, S. H. Crook, B. E. Mann, D. Scapens, P. Montravers, J. Boczkowski and R. Motterlini, *Antioxid Redox Signal*, 2012, **16**, 153-163.
20. P. V. Simpson, C. Nagel, H. Bruhn and U. Schatzschneider, *Organometallics*, 2015, **34**, 3809-3815.
21. S. McLean, B. E. Mann and R. K. Poole, *Anal Biochem*, 2012, **427**, 36-40.

22. B. W. Michel, A. R. Lippert and C. J. Chang, *J Am Chem Soc*, 2012, **134**, 15668-15671.
23. T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. Bernardes, C. C. Romao and M. J. Romao, *J Am Chem Soc*, 2011, **133**, 1192-1195.
24. T. R. Johnson, B. E. Mann, I. P. Teasdale, H. Adams, R. Foresti, C. J. Green and R. Motterlini, *Dalton Trans*, 2007, 1500-1508.
25. R. Motterlini, J. E. Clark, R. Foresti, P. Sarathchandra, B. E. Mann and C. J. Green, *Circ Res*, 2002, **90**, E17-24.
26. B. E. Mann, *Organometallics*, 2012, **31**, 5728–5735.
27. H. Smith, B. E. Mann, R. Motterlini and R. K. Poole, *IUBMB Life*, 2011, **63**, 363-371.
28. R. Cini, S. Defazio, G. Tamasi, M. Casolaro, L. Messori, A. Casini, M. Morpurgo and M. Hursthouse, *Inorganic Chemistry*, 2007, **46**, 79-92.
29. D. Valensin, P. Anzini, E. Gaggelli, N. Gaggelli, G. Tamasi, R. Cini, C. Gabbiani, E. Michelucci, L. Messori, H. Kozlowski and G. Valensin, *Inorg Chem*, 2010, **49**, 4720-4722.
30. M. F. Santos, J. D. Seixas, A. C. Coelho, A. Mukhopadhyay, P. M. Reis, M. J. Romão, R. C. C and S.-S. T, *J Inorg Biochem*, 2012, **117**, 285–291.
31. J. D. Seixas, M. F. Santos, A. Mukhopadhyay, A. C. Coelho, P. M. Reis, L. F. Veiros, A. R. Marques, N. Penacho, A. M. Goncalves, M. J. Romao, G. J. Bernardes, T. Santos-Silva and C. C. Romao, *Dalton Trans*, 2014, **44**, 5058-5075.
32. M. Chaves-Ferreira, I. S. Albuquerque, D. Matak-Vinkovic, A. C. Coelho, S. M. Carvalho, L. M. Saraiva, C. C. Romao and G. J. Bernardes, *Angew Chem Int Ed Engl*, 2014, **54**, 1172-1175.
33. J. D. Seixas, A. Mukhopadhyay, T. Santos-Silva, L. E. Otterbein, D. J. Gallo, S. S. Rodrigues, B. H. Guerreiro, A. M. Goncalves, N. Penacho, A. R. Marques, A. C. Coelho, P. M. Reis, M. J. Romao and C. C. Romao, *Dalton Trans*, 2013, **42**, 5985-5998.
34. J. D. Seixas, Ph.D., New University of Lisbon, 2011.
35. W. Q. Zhang, A. J. Atkin, R. J. Thatcher, A. C. Whitwood, I. J. Fairlamb and J. M. Lynam, *Dalton Trans*, 2009, 4351-4358.
36. H. Liu, P. Wang, Q. Zhao, Y. Chen, B. Liu, B. Zhang and Q. Zheng, *Applied Organometallic Chemistry*, 2014, **28**, 169-179.
37. G. L. Bannenberg and H. L. Vieira, *Expert Opin Ther Pat*, 2009, **19**, 663-682.
38. S. Z. Sheikh, R. A. Hegazi, T. Kobayashi, J. C. Onyiah, S. M. Russo, K. Matsuoka, A. R. Sepulveda, F. Li, L. E. Otterbein and S. E. Plevy, *J Immunol*, 2011, **186**, 5506-5513.
39. N. Schallner, C. C. Romao, J. Biermann, W. A. Lagreze, L. E. Otterbein, H. Buerkle, T. Loop and U. Goebel, *PLoS One*, 2013, **8**, e60672.
40. J. C. Onyiah, S. Z. Sheikh, N. Maharshak, E. C. Steinbach, S. M. Russo, T. Kobayashi, L. C. Mackey, J. J. Hansen, A. J. Moeser, J. F. Rawls, L. B. Borst, L. E. Otterbein and S. E. Plevy, *Gastroenterology*, 2013, **144**, 789-798.

41. M. A. Gonzalez, N. L. Fry, R. Burt, R. Davda, A. Hobbs and P. K. Mascharak, *Inorg Chem*, 2011, **50** 3127–3134.
42. V. W. Ng, M. K. Taylor and C. G. Young, *Inorg Chem*, 2012, **51**, 3202-3211.
43. J. E. Clark, P. Naughton, S. Shurey, C. J. Green, T. R. Johnson, B. E. Mann, R. Foresti and R. Motterlini, *Circ Res*, 2003, **93**, e2-8.
44. G. S. Baldew, A. P. Boymans, J. G. Mol and N. P. Vermeulen, *Biochem Pharmacol*, 1992, **44**, 382-387.
45. P. V. Nerurkar, K. Dragull and C. S. Tang, *Toxicol Sci*, 2004, **79**, 106-111.
46. S. Pai, M. Hafftlang, G. Atongo, C. Nagel, J. Niesel, S. Botov, H. G. Schmalz, B. Yard and U. Schatzschneider, *Dalton Trans*, 2014, **43**, 8664-8678.
47. K. Nielsen, W. A. Schenk, M. Kriegmeier, V. L. Sukhorukov and U. Zimmermann, *Inorg. Chem.*, 1996, **35**, 5762–5763.
48. M. Kurschner, K. Nielsen, C. Andersen, V. L. Sukhorukov, W. A. Schenk, R. Benz and U. Zimmermann, *Biophys J*, 1998, **74**, 3031-3043.



72x39mm (300 x 300 DPI)