The Antimicrobial Action of Carbon Monoxide Releasing Compounds

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Running Title: The Antimicrobial Action of Carbon Monoxide Releasing Compounds
Abstract

Carbon monoxide (CO) is endogenously produced in the human body mainly from the oxidation of heme catalyzed by heme oxygenase (HO) enzymes. The induction of HO and the consequent increase in CO production plays important physiological roles in vasorelaxation, neurotransmission, and in the immune system. Exogenous administration of CO gas and “Carbon Monoxide Releasing Molecules” (CO-RMs) has been shown to induce vascular effects and to alleviate hypoxia/reoxygenation injury of mammalian cells. In particular, due to its anti-inflammatory, antiapoptotic and antiproliferative properties, CO inhibits ischemic-reperfusion injury and provides potent cytoprotective effects during organ and cell transplantation. Besides these findings in the physiology and biology of mammals, nothing is known about the action of CO on bacteria. In the present work we examined the effect of CO on bacterial cell proliferation. Cell growth experiments show that CO causes rapid death of the two pathogenic bacteria tested, *Escherichia coli* and *Staphylococcus aureus*, particularly when delivered through organometallic CO releasing molecules. Quite important is the observation that the effectiveness of the CO-RMs is higher under near anaerobic environments as many pathogens are anaerobic organisms and pathogen colonization occurs in environments with low oxygen concentration. Our results constitute the first evidence that CO can be utilized as an antimicrobial agent. We anticipate our results to be the starting point for the development of novel types of therapeutic drugs designed to combat antibiotic-resistant pathogens, which are widespread and presently a major public health concern.
Introduction

Carbon monoxide (CO) is a colorless and odorless diatomic gas, chemically inert, that occurs in nature as a product of oxidation or combustion of organic matter. Owing to its lethal effect when present in high concentrations, CO was considered for many years only as an environmental toxicant that results from air pollution by automobile exhaust. The knowledge that the human body was able to produce small quantities of CO and the evidence that CO-derived from HO activity contributes to important intracellular functions has modified our perception of CO as a pernicious toxin to include its beneficial effects (15, 16, 22). In consequence, application of CO gas or CO-RMs emerged as a new therapeutic strategy in medicine (10, 13, 18). The evolution of CO from a toxicant to a molecule of mounting importance in mammals finds a parallel in another diatomic molecule, nitric oxide (NO) (17). NO is produced in the body by the nitric oxide synthase (NOS) and shares with CO many downstream signaling pathways and regulatory functions, in particular through the activation of soluble guanylyl cyclase (sGC) (7, 8, 12). In addition, there is an interplay between the two molecules, since it is proposed that CO is a modulator of NOS (10, 22) and NO up-regulates HO (19, 20), which in turn catalyses the oxidative degradation of free heme into biliverdin with the concomitant release of iron and CO. NO also constitutes one of the weapons that the mammalian immune system uses to fight pathogens (3, 4). The bactericidal function of NO relies on the deleterious effects caused in the pathogen, e.g., nitrosylation of iron centers. Although CO is a stable neutral molecule with a long half-life, it shares with NO the high affinity for iron of heme proteins, which is the basis of its toxicity. We therefore set out to explore the possible action of CO on bacterial growth rates. For this purpose, the bioactivity of CO, either in the gaseous form or via treatment with CO-RMs, was
tested on *Escherichia coli* and *Staphylococcus aureus*. These bacteria are major human pathogens which are widely spread in the community and are responsible for hospital-acquired infections, exhibiting a concerning degree of antibiotic resistance.
Materials and Methods

Reagents. The different sources of CO were: tricarbonyldichlororuthenium (II) dimer, CORM-2 (Sigma); tricarbonylchloro(glycinato)ruthenium (II), CORM-3 (6); bromo(pentacarbonyl)manganese, ALF 021 (5) and tetraethylammonium molybdenum pentacarbonyl bromide, ALF 062 (2). All compounds were freshly prepared as a 10 mM stock solution by dissolution in dimethyl sulfoxide (DMSO), pure distilled water or methanol, respectively.

Bacterial strains and growth conditions. *Escherichia coli* K-12 ATCC 23716 and *Staphylococcus aureus* NCTC8325 were grown in minimal medium salts MS (1.3 % (w/v) of Na$_2$HPO$_4$, 0.3 % (w/v) of KH$_2$PO$_4$, 0.05 % (w/v) of NaCl and 0.1 % (w/v) of NH$_4$Cl supplemented with 20 mM of glucose, 2 mM of MgSO$_4$, 100 µM of CaCl$_2$ and 0.25 % (w/v) of casamino acids) or in Luria-Bertani LB (1 % (w/v) of tryptone, 0.5 % (w/v) of yeast extract and 1 % (w/v) of NaCl), respectively, under different oxygen supply conditions. Aerobic experiments were undertaken in flasks filled with 1/5 of its volume, microaerobic tests in closed flasks filled with 1/2 of its volume and anaerobic conditions with rubber sealed flasks that, once filled with media and closed, were extensively fluxed with nitrogen gas.

CO gas and CO-RMs treatment. Overnight cultures of *E. coli* or *S. aureus* grown in LB or Tryptic Soy Broth (TSB), respectively, were used to inoculate fresh MS medium (*E. coli*) or LB medium (*S. aureus*) and incubated at 37°C, under the aeration conditions required, until an optical density at 600 nm (OD$_{600}$) of 0.3. At this point cells
were exposed to a flux of CO gas for 15 min or to CO-RMs. Untreated cells were bubbled with nitrogen gas or treated with DMSO, water or methanol, depending on the solvent used to dissolve CO-RM.

The inactive form of ALF 062 was prepared by mixing vigorously with 20 % of methanol, in a closed flask over 2-3 hours. The counter-ion of ALF 062, the tetraethyl ammonium bromide (BrNEt₄), and one of the products of ALF 062 decomposition, the sodium molybdate (MoO₄Na₂), were used at the same concentration of ALF 062 (50 µM).

Viability assays. The number of viable cells was evaluated by measuring the colony forming units (cfu/ml) upon plating on agar plates serial dilutions of the various cultures. The percentage of survival was calculated as the number of colonies originated by treated cultures divided by the number of colonies formed upon platting untreated cultures. Sensitivity tests were assayed by plating on agar 5 µl of serial dilutions of cultures grown for 4 hours and treated with CO-RMs, with or without the CO scavenger utilized (20 µM of hemoglobin from bovine, Sigma). The experiments were performed with a minimum of three independent cultures and the results are presented as averaged values with error bars representing one standard deviation.

The investigation of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) was carried out by the tube dilution test. Briefly, 2.5 ml of minimal medium was inoculated with an overnight culture of *E. coli* or *S. aureus* to give an OD₆₀₀ of 0.005-0.01. Different concentrations of CORM-2, between 150 µM and 2 mM, were added to the diluted suspension and incubated in 24-wells plates for at least 18 hours at 37°C and 90 rpm. The first tube in the series with no sign of visible growth
was reported as the MIC. All the cultures that exhibited lack of cell growth were then subsequently plated onto agar devoid of any drug. After incubation at 37°C for 24 hours, no growth was assumed as the MBC value.

**CO releasing kinetics.** CO-RMs were mixed with MS or LB medium in sealed vessels and incubated at room temperature under constant stirring and protected from light. Gas samples were collected after 30 min and 4 hours and analyzed using a gas chromatograph (Thermofinnigan Trace GC) equipped with a CTRI column (Alltech) and a thermal conductivity detector. CO released was quantified using a calibration curve recorded prior to reaction course.

**Inductively Coupled Plasma Mass Spectrometry (IPC-MS) analysis.** *E. coli* cells cultured in MS medium with or without 50 µM of ALF 062, were collected after 1 hour of growth and the cellular metal content was analyzed at Instituto das Pescas e do Mar. The *E. coli* intracellular concentration of Mo was assayed on a quadropole ICP-MS (Thermo Elemental, X-Series) equipped with a Peltier Impact bead spray chamber and a concentric Meinhard nebuliser. The experimental parameters were: forward power=790 W; peak jumping mode; 150 sweeps per replicate (dwell time=10 ms; dead time=30 ns). A 7-points calibration within a range of 1-100 µg L⁻¹ was used to quantify metal concentration. Coefficients of variation for metal counts (n=5) varied between 0.5 and 2%. The precision and accuracy of metal concentration measurements was determined through repeated analysis of references materials (TORT-1, TORT-2, DORM-2 and DORM-3 from National Research Council of Canada), using Indium (In) as an internal
standard, was 1-2%. Procedural blanks always accounted for less than 1% of the total molybdenum concentrations in samples.
Results and Discussion

The effect of CO on viability of bacteria was first investigated by direct delivery of CO gas. Administration of CO gas, fluxed into the growing cultures, led to a significant growth impairment of *E. coli* and *S. aureus* (Fig. 1).

To evaluate the potential of CO-RMs, the compounds indicated in Figure 2 were selected. CORM-2 and CORM-3 are active in a variety of CO mediated biological processes, both *in vitro* and *in vivo* (9). In the first series of experiments, the effect of CO released from CORM-2 on cell growth was studied in *E. coli* and *S. aureus* cultured under different conditions of oxygen supply. Shortly after the exposure to CORM-2 the percentage of survival significantly diminished (Fig. 3). Experiments using water soluble CORM-3 revealed, that albeit requiring higher concentrations due to its chemical composition, the compound also strongly decreased the viability of *E. coli* and *S. aureus* cells (Fig. 4). However, while for *E. coli* the addition of CORM-3 resulted in a strong inhibition of cell growth, *S. aureus* was more resistant to CORM-3 (Fig. 4A), particularly under aerobic conditions. In general, the action of the two compounds was rapid and extended over time, as cells did not resume growth over the subsequent four hours (Fig. 3 and Fig. 4) or after eight hours (not shown).

In order to examine whether the bactericidal effect of CO-RMs was due to CO, cell growth experiments with CO-RMs were also performed in the presence of hemoglobin (Hb), a high affinity CO scavenger. In all cases the bactericidal effect towards *E. coli* and *S. aureus* was completely lost (Fig. 3B and Fig. 4B), thus demonstrating that the antimicrobial action of CO-RMs is dependent on their release of CO.
Bactericidal activity has been defined as the ratio of the Minimal Bactericidal Concentration (MBC) to the Minimal Inhibitory Concentration (MIC) of \(<4\) (14). Determination of the MBC/MIC ratio of CORM-2 of 1.5 and 1.0 for *E. coli* and *S. aureus*, respectively, revealed the bactericidal character of the drug.

The two other CO-RMs used to investigate the bactericidal effect of CO, namely manganese carbonyl ALF 021 and molybdenum carbonyl ALF 062, were also seen to be capable of strongly reducing the viability of *E. coli* and *S. aureus* (Fig. 5 and Fig. 6). Again, the addition of Hb completely eliminated the harmful action of ALFs compounds on the two bacteria (Fig. 5 and Fig. 6). Furthermore, to ensure that the activity of ALF 062 was not related to its decomposition products, we tested the effect of BrNEt₄, MoO₄Na₂ and a solution of “inactivated ALF 062”, obtained after cessation of CO released (see Methods), on bacterial growth. Neither compounds had bactericidal properties or altered growth kinetics (data not shown). Therefore, the bactericidal effects of ALF062 are due to its capacity to release CO.

It should be mentioned that neither CORM-2 nor CORM-3 release CO gas when dissolved in the media utilized, even at concentrations higher than those used in our experiments (Table 1). Furthermore, although ALF 021 and ALF 062 release CO gas upon dissolution in the medium, they do so in rather small amounts within the time scale of the experiment (Table 1). However, ICP-MS analysis of *E. coli* cells incubated with ALF 062 revealed a very large increase in the content of Mo (155 µg g⁻¹) as compared to control cells (2.5 µg g⁻¹), confirming that the Mo from ALF 062 accumulates inside the *E. coli* cells where it releases CO to the cellular targets.

Since the bactericidal effect of the CO-RMs does not require the release of CO gas to the extracellular medium, (Table 1) we must conclude that CO has to be delivered
to the cellular targets directly from the CO-RMs. Because Mo from bactericidally active (CO loaded) ALF062 is found to accumulate rapidly within cells we infer that it is transporting and delivering CO into the intracellular space where it reaches the cellular targets and causes the decrease of bacterial cell viability. If hemoglobin is present in the medium, its high affinity to CO results in a fast transfer (or abstraction) of the active CO from the CO-RMs (or from gas, obviously) to the protein hemes and its effective scavenging as COHb (see below). Under these conditions, no CO will be available for intracellular delivery and the cells remain alive.

Albeit some minor deviations, the general pattern of our results shows that CO-RMs toxicity is enhanced when growth is performed under conditions of lower oxygen concentration. For example, ALF 021 was more effective in lowering the viability of *E. coli* cells grown anaerobically (200 µM of ALF 021) than grown aerobically (500 µM of ALF 021). The fact that the effect of CO is augmented at low oxygen concentrations may be explained by the preferential binding of CO to the ferrous form of heme proteins, which are predominant under reducing environments. More importantly, the bactericidal effect of CO-RMS under anaerobic conditions indicates that growth inhibition is not restricted to the impairment of the respiratory chain by the binding of CO to cytochrome oxidase, which is likely to contribute to the bactericidal activity of these compounds under aerobic conditions. This fact is quite important since pathogen colonization occurs in near anaerobic environments and that many pathogens are anaerobic organisms. On the other hand, the type of bacterial cell wall seems also not to interfere with the action of CO-RMs, as judged by the similar decrease in cell viability observed for the Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) species upon treatment with the same CO-RM. Hence, CO-RMs have the potential for being used as bactericides in a wide
range of microorganisms independently of the type of bacterial cell wall and oxygen growth requirements.

The difference between the degree of action of dissolved molecular CO gas and of CO-RMs is striking. When administered as gas, CO had to be present in rather high concentrations (ca. 1mM) to become effective as a bactericide. The ability of CO-RMs to accumulate inside bacterial cells before they release CO makes these compounds highly effective “CO donors” to bacterial targets, thereby strongly enhancing the bactericidal efficacy of CO. In fact, CO-RMs used in this study were able to transfer CO to Hb to form COHb, as judged by the shift of the Hb Soret band from 413 to 418 nm (not shown) and by the results depicted in Fig. 3B, Fig. 4B, Fig. 5 and Fig. 6. Hence, CO-RMs are capable of delivering CO to heme-containing molecules as had been shown before for the rapid carbonylation of myoglobin by CORM-3 (11). Likewise, carbonylation of Hb by CORM-2 and CORM-3 occurs within the mixing time while for ALF 021 and ALF 062 it takes place in less than 15 min. It is well known that the biological effect of CO in mammalian cells is mainly due to its interaction with iron-containing proteins, such as the above mentioned cytochrome oxidase. In addition to heme proteins/sensors, CO may bind to almost all transition metal-containing proteins, giving rise to structural modifications and alteration of their biological function. Hence, in bacteria there are a wide number of likely intracellular targets that can account for the toxic effect of CO revealed in this study.

In spite of the increasing expectations for the use of CO in medicine (10, 13, 18), until now, the role of CO as a bactericidal compound had remained unexplored. Nevertheless, in the early seventies it was reported that addition of CO to an aerobic culture of *E. coli* caused a decrease in DNA replication (21). However, as the authors did
not observe any effect of CO on cells growing anaerobically on glucose, they concluded that inhibition of DNA synthesis in cells grown under aerobic conditions was not due to a direct effect on the replication apparatus but resulted from indirect effects such as ATP or dNTP depletion (21). In more recent years, in spite of several public concerns, CO has been used by the food industry to generate the bright red color of the dark muscle tissue of meat and fish, resulting from the great affinity of CO for the Fe (II) binding site of myoglobin. Interestingly, a very recent study of the influence of different packing systems on meat preservation indicated that packages to which CO gas had been added exhibited lower bacterial growth. These results suggest that CO could be one of the packaging gases responsible for the inhibition of the growth of microorganisms (1). We now show that CO and, in particular, CO-RMs have the ability to kill bacteria under aerobic and anaerobic conditions. We submit that CO-RMs constitute a novel class of antibacterial molecules which may become drug candidates if and when safe and controllable methods of CO delivery to bacterial targets that avoid in vivo scavenging of CO by the red blood cells are developed (10). In particular, non-systemic bactericides could be an easier application for CO-RMs. Although this is a first visualization of a still a very distant goal, bactericides based upon completely new concepts are urgently required as the emergence and spread of drug resistant bacterial pathogens reveal a concerning decrease of the effectiveness of our currently available antibiotics.
Acknowledgements

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References


Figure Legends

Figure 1 – Effect of CO gas on *E. coli* and *S. aureus* viability. **A.** *E. coli* and *S. aureus* cells were grown under microaerobic conditions in MS or LB medium, respectively, and exposed to a flux of CO gas, for 15 min. **B.** Sensitivity tests were assayed by plating the indicated serial dilutions of the cultures collected after 4 hours of exposure to CO gas (+) or to nitrogen gas (-).

Figure 2 – Chemical structure of CO-RMs used in this study.

Figure 3 – Effect of CORM-2 on *E. coli* and *S. aureus* cell viability. **A.** *E. coli* grown in MS under aerobic and anaerobic conditions and treated with 250 μM of CORM-2. *S. aureus* cells grown aerobically and microaerobically, in LB medium, and exposed to 250 μM of CORM-2. **B.** Sensitivity tests for CORM-2 assayed in cultures (see Methods), treated with CORM-2 (+) (250 μM) or left untreated (-), and in the absence or in the presence of hemoglobin (Hb).

Figure 4 – Effect of CORM-3 on *E. coli* and *S. aureus* cell viability. **A.** *E. coli* cells, grown in MS medium either aerobically or anaerobically, and treated with 400 μM of CORM-3. *S. aureus* cells aerobically and microaerobically grown in LB medium to which 500 μM or 400 μM of CORM-3 were added, respectively. **B.** Sensitivity tests were assayed, by plating dilutions of cultures grown as described in Methods, after exposure to CORM-3 (+), or not treated (-), in the absence or in the presence of hemoglobin (Hb). The concentrations of CORM-3 used were the same indicated in A.
Figure 5 – Sensitivity of *E. coli* to ALF 021 and ALF 062 compounds. *E. coli* cells grown under aerobic and anaerobic conditions were treated with 500 µM or 200 µM of ALF 021, respectively, and with 50 µM of ALF 062 (see methods) in the absence or in the presence of hemoglobin (Hb).

Figure 6 – Sensitivity of *S. aureus* to ALF 021 and ALF 062 compounds. *S. aureus* cells grown under aerobic and microaerobic conditions were treated with 600 µM of ALF 021 and with 50 µM of ALF 062. Sensitivity tests were assayed in cultures exposed to CO-RMs (+) or not exposed (-), and in the absence or in the presence of hemoglobin (Hb), as described in Methods.
Table 1. Carbon monoxide released by CO-RMs to the medium

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<th>MS</th>
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<td>ALF 062 (6 mM)</td>
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Fig. 1

A. Graph showing percent survival over time for E. coli and S. aureus. Error bars indicate variability.

B. Microscopic images of E. coli and S. aureus at different concentrations: 10^5, 10^6, 10^7, 10^8. (+) and (-) symbols indicate growth conditions.

Legend:
- Black bars represent E. coli.
- White bars represent S. aureus.

Time (h) vs. Percent Survival

- Time points: 0, 1, 2, 3, 4, 5
- S. aureus:
  - 10^{-5}
  - 10^{-6}
  - 10^{-7}
  - 10^{-8}
- E. coli:
  - 10^{5}
  - 10^{6}
  - 10^{7}
  - 10^{8}
Fig. 2
Fig. 3

A

E. coli

- Aerobic
- Anaerobic

Percent Survival

0 0.5 1 2 3 4

Time (h)

B

S. aureus

- Aerobic
- Microaerobic

Percent Survival

0 0.5 1 2 3 4

Time (h)

CORM-2

- Aerobic
- Anaerobic

- Microaerobic

- (−)
- (+)
- (−, Hb)
- (+, Hb)

10^5 10^6 10^7 10^8
Fig. 4

A

E. coli

[Graph showing percent survival over time for aerobic and anaerobic conditions for E. coli.]

B

S. aureus

[Graph showing percent survival over time for aerobic and microaerobic conditions for S. aureus.]

Legend:

- Aerobic
- Anaerobic
- Microaerobic

CORM-3

(-)
(-, Hb)
(+)
(+, Hb)

E. coli

S. aureus

Downloaded from http://aac.asm.org/ on November 12, 2017 by guest
**Fig. 5**

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### S. aureus

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|          | $10^7$  | $10^8$       |
|          | $10^8$  | $10^9$       |
| ALF 062  | $10^5$  | $10^5$       |
|          | $10^6$  | $10^6$       |
|          | $10^7$  | $10^7$       |
|          | $10^8$  | $10^8$       |
| (-)      | (-)     | (-, Hb)     |
| (+)      | (+)     | (+, Hb)     |