A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia

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ABSTRACT **The search for new molecules to fight *Pseudomonas aeruginosa* is of paramount importance. Carbon monoxide (CO) is known to act as an effective inhibitor of the respiratory chain in *P. aeruginosa*, but the practical use of this gas as an antibacterial molecule is hampered by its toxicity and difficulty to manipulate. Here, we show that a water-soluble CO releaser (CORM-3) possesses bactericidal properties against laboratory and antibiotic-resistant *P. aeruginosa*. CORM-3 reduced the bacterial count by 4 logs 180 min after *in vitro* treatment. CORM-3-treated bacteria had a lower O_2 consumption than vehicle-treated bacteria, and the decrease in O_2 consumption temporally preceded the bactericidal action of CORM-3. These results support the hypothesis that the antimicrobial effect of CORM-3 is mediated by an interaction of CO liberated by the carrier with the bacterial respiratory chain. The antibacterial effect occurred at concentrations of CORM-3 that are 50-fold lower than toxic concentrations for eukaryotic cells. CORM-3 treatment compared to vehicle treatment decreased bacterial counts in the spleen and increased survival in immunocompetent and immunosuppressed mice following *P. aeruginosa* bacteremia. Our results suggest that CORMs could form the basis for developing a new therapeutic strategy against *P. aeruginosa*-induced infection.—Desmard, M., Davidge, K. S., Bouvet, O., Morin, D., Roux, D., Foresti, R., Ricard, J. D., Denamur, E., Poole, R. K., Montravers, P., Motterlini, R., Boczkowski, J. A carbon monoxide-releasing molecule (CORM-3) exerts bactericidal activity against *Pseudomonas aeruginosa* and improves survival in an animal model of bacteraemia. *FASEB J.* 23, 1023–1031 (2009)**

Key Words: pharmacology · anti-infective agents · sepsis · microbiology

Nosocomial infections represent a major public health problem. Their frequency is particularly high in intensive care units (ICUs) (1), *Pseudomonas aeruginosa* being responsible for nearly 30% of these infections (2). The high incidence of these infections is mainly attributed to the use of broad-spectrum antibiotics, which have greatly increased the antibiotic resistance of *P. aeruginosa*. Thirty percent of clinical isolates from ICU or nursing home patients are now resistant to 3 or more drugs (3). Despite this situation, no new drug against *P. aeruginosa* has been successfully introduced into the clinic in the past 2 decades.

Carbon monoxide (CO) is a stable gas that occurs in nature as a product of oxidation or combustion of organic matter. The finding that CO, an endogenous product of heme metabolism by heme oxygenase enzymes, can interact effectively with disparate targets, such as soluble guanylate cyclase, cytochrome *c* oxidase, NADPH oxidase, or potassium channels to transduce important signals within cells has opened a new area of investigation on the biological properties of this gas (4). Indeed, it has been demonstrated in recent years that CO, at defined concentrations, exerts interesting biological activities, including vasoactive, anti-inflammatory, antiapoptotic, and cytoprotective actions in various models of disease (5, 6–9). Binding of CO to

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doi: 10.1096/fj.08-122804

ferrous iron and other metal centers (reviewed in ref. 4) is thought to mediate some of its biological effects; for example, in eukaryotic cells, CO targets mitochondrial cytochrome *c* oxidase with an ensuing decrease in O_2 consumption and increased production of reactive oxygen species (ROS) (4). This effect appears to be affected by oxygen concentration (10–12). Interestingly, it was reported several years ago that CO gas is also able to bind *P. aeruginosa* cytochrome *c* oxidase (13). However, whether the persistent blockade of the pathogen's respiratory chain by CO could be used as a bactericidal strategy in a disease model of infection has never been investigated.

A recent fundamental development in the field of CO research has been the discovery of carbon monoxide-releasing molecules (CORMs), a group of transition metal carbonyls that serve as carriers for the delivery of controlled amounts of CO in biological systems (14). These molecules provide a useful pharmacological tool to exploit the bioactive properties of CO and at the same time minimize its toxicity (14). Among the different classes of CORMs, tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) has been characterized and successfully tested in models of inflammation, vascular dysfunction, and ischemic injury (15, 16). On the basis of the emerging pleiotropic effects of CORMs, the present study was designed to investigate whether CORM-3 affects the growth of *P. aeruginosa in vitro* and to identify the molecular mechanisms involved in its potential antibacterial actions. The relevance of CORM-3 as a prototypic CO-based pharmaceutical agent was then assessed using an *in vivo* model of *P. aeruginosa* infection in both immunocompetent and immunosuppressed mice.

MATERIALS AND METHODS

Reagents

N-acetylcysteine (NAC), cysteine, and reduced and oxidized glutathione were obtained from Sigma-Aldrich (St. Louis, MO, USA). CORM-3 [Ru(CO)(3)Cl(glycinate)] was synthesized as described previously (17).

Bacterial strains and growth conditions

P. aeruginosa PAO1 ATCC 15692 and resistant *P. aeruginosa* isolated from ICU hospitalized patients were grown aerobically for 24 h at 37°C in Luria-Bertani (LB) medium, then washed twice in NaCl 0.9% before the experiments. We used three resistant strains of *P. aeruginosa*. Strain 1 overproduced cephalosporinase; strain 2 was resistant to quinolones due to a DNA gyrase mutation; and strain 3 displayed overexpression of cephalosporinase, impermeability to carbapenems, and resistance to quinolones involving a DNA gyrase mutation.

Bacterial growth and viability assays

Experiments were performed aerobically at 37°C in minimal M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.25 g/L $MgSO_4 \cdot 7H_2O$, and 2 g/L glucose). We used an initial bacterial concentration of 4×10^6 CFU/ml. *P. aeruginosa* growth was determined by measuring the optical density at 600 nm every 10 min for 18 h. The number of viable cells was evaluated by measuring the colony-forming units per milliliter, plating serial dilutions of the various cultures onto LB agar plates. Growth and viability experiments started as soon as bacteria were treated by CORM-3. In the experiments conducted with CO gas, a solution of M9 medium was initially inoculated with 2×10^6 CFU/ml *P. aeruginosa*. CO gas (BOC Gases, Guildford, UK) was then bubbled into the solution for 5 min in a sealed tube, and bacteria were subsequently grown at 37°C. Optical density at 600 nm was measured at 6 and 24 h.

Eukaryotic cell viability

Murine RAW264.7 monocyte macrophages were purchased from the European Collection of Cell Cultures (Salisbury, UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere, and experiments were conducted on cells at ~80–90% confluence. Macrophages were exposed for 24 h to different concentrations of CORM-3. Cell viability was determined at the end of the incubation using an Alamar Blue assay kit and carried out according to the manufacturer's instructions (Serotec, Oxford, UK).

Oxygen consumption assays

Oxygen consumption was measured at 37°C using a Clark-type electrode. Bacteria (5×10^8 CFU/ml) were incubated in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, and 5 mM KH_2PO_4, pH 7.4, at 37°C. Respiration was initiated by addition of glucose (2 g/L). To make a parallel between oxygen consumption and bacterial viability, we performed viability experiments in the respiration buffer with an initial bacterial concentration of 5×10^8 CFU/ml.

Reduced glutathione measurements

The method used was a colorimetric technique based on the transformation of 5′,5-bis-dithionitrobenzoic (DTNB) into 2-nitro, 5-thiobenzoic acid (TNB), a colored compound absorbing light at wavelength of 412 nm, by reduced glutathione. Bacteria were treated with CORM-3 (10 μM) or vehicle for 30 min, centrifuged and washed, then lysed in 10% trichloroacetic acid. A neutral pH was obtained by the addition of NaOH and DTNB (600 μM) was added. Measurements were performed spectrophotometrically at an optical density of 412 nm.

Detection of CO release

The release of CO from CORM-3 was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO), as previously reported (14, 17, 18). A small aliquot of concentrated CORM-3 solution was added to 1 ml deoxy-Mb solution in phosphate buffer (final concentrations: 40 μM CORM-3, 53 μM deoxy-Mb), and changes in the Mb spectra were recorded over time. The amount of MbCO formed was quantified by measuring the absorbance at 540 nm.

Spectral analysis of cytochromes

P. aeruginosa cells were harvested by centrifugation for 20 min at 5500 rpm, washed in buffer (0.1 M potassium phosphate, pH 7), and resuspended in 40 ml 0.1 M potassium phosphate buffer. The protein concentration was 2 mg/ml, and samples were diluted 1:2 before use. Difference spectra (CO plus reduced *minus* reduced) of whole cells were recorded at room temperature in an SDB3 dual-wavelength scanning spectrophotometer, custom built at the University of Pennsylvania School of Medicine Biomedical Instrumentation Group and Current Designs (University of Pennsylvania, Philadelphia, PA, USA. Cells were reduced with dithionite, then 100 μM CORM-3 or CO gas, was added and used to record spectra in cells of 10 mm path length. Spectra were smoothed and plotted in SigmaPlot (SPSS Inc., Chicago, IL, USA).

In vivo infection models

The experiments conducted in the present study were approved by the local Institutional Animal Care and Use Committee, and the experimental protocol was in agreement with French legal recommendations related to animal studies. Mice were housed in standard wire-topped cages and in temperature-controlled units. Food and water were supplied *ad libitum*. Pathogen-free males of the BALB/c and C57/BL6 lineage (7 to 8 wk old, 20 to 25 g) were purchased from the Centre d'Elevage R. Janvier (Le Genest, St. Isle, France). Bacteremia was obtained by intraperitoneal injection of 2×10^7 bacteria (PAO1) into each mouse. In leukopenic mouse experiments, leukopenia was induced by intraperitoneal injection of cyclophosphamide (150 mg/kg body weight) on 3 successive days before bacterial challenge. Intraperitoneal injections of CORM-3 (7.5 mg/kg, equivalent to 25 μmol/kg body weight) or vehicle were given 5 min after bacterial challenge. The dose of CORM-3 selected for our experiments is in agreement with previous *in vivo* studies showing a therapeutic effect of this compound in models of inflammation, protection against renal ischemia-reperfusion injury, and hypertension (16, 19, 20). The survival rate was evaluated every 12 h for 72 h. To measure bacterial levels in the spleen, animals were sacrificed 1 or 3 h after bacterial inoculation, the spleens were removed and homogenized, and bacteria enumerated by plate counting after serial dilutions.

Statistical analysis

Data are expressed as means ± SE. For comparisons between 2 groups, we used 2-tailed nonparametric Mann-Whitney *U* analysis. For comparisons among more than 2 groups and multiple comparisons, we used the nonparametric Kruskal-Wallis test with the Dunn's post test. Comparisons of mortality were made by analyzing Kaplan-Meier survival curves, and then log-rank test was used to assess for differences in survival. The number of samples per group (n) is specified in Results or in figure legends. Statistical significance was accepted at $P < 0.05$.

RESULTS

CORM-3 inhibits *P. aeruginosa* growth *in vitro*

As a first step in studying the antimicrobial action of CORM-3, we examined how this compound affects the growth of *P. aeruginosa* strain PAO1 *in vitro*. Experiments on this strain were performed in glucose M9 minimal medium at 37°C. CORM-3 (0.1–10 μM) inhibited bacterial growth in a concentration-dependent manner and a significant difference ($P<0.01$) in optical density (OD 600) measured at 18 h was observed at concentrations as low as 0.5 μM (**Fig. 1*A***). Bacterial growth inhibition was the consequence of a fast bactericidal effect mediated by CORM-3 with a reduction of bacterial count by 4 logs 180 min after addition of 10 μM CORM-3 to bacteria. The decrease in bacterial count exerted by CORM-3 was comparable to that induced by the antibiotic amikacine (50 mg/L=0.8 μM) and faster than the decrease induced by ticarcilline (50 mg/kg) (Fig. 1*B*). The PAO1 strain used in these experiments was sensitive to ticarcilline and ami-

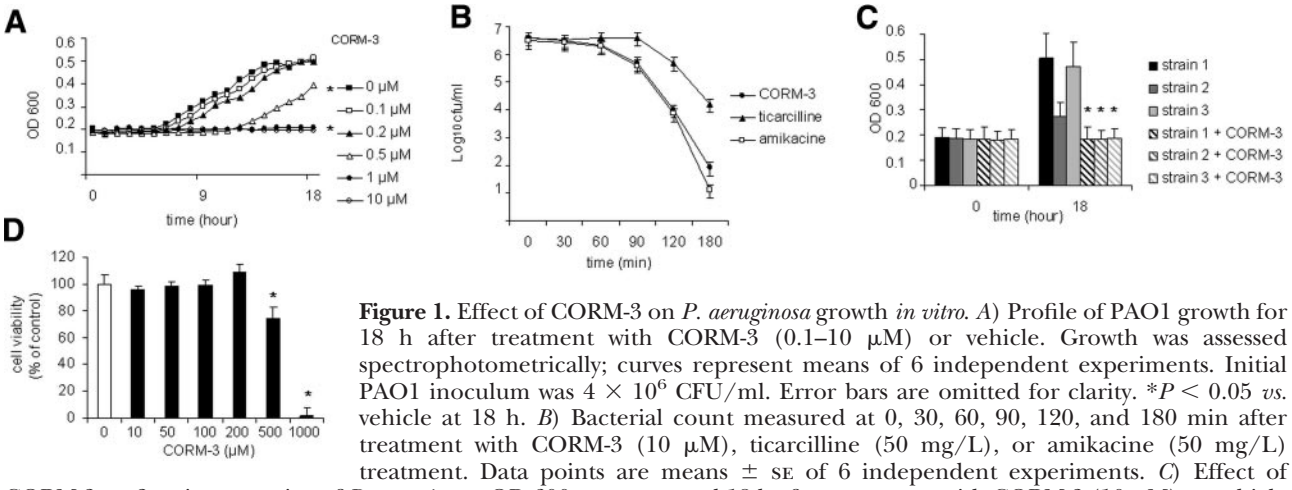

Figure 1. Effect of CORM-3 on *P. aeruginosa* growth *in vitro*. *A*) Profile of PAO1 growth for 18 h after treatment with CORM-3 (0.1–10 μM) or vehicle. Growth was assessed spectrophotometrically; curves represent means of 6 independent experiments. Initial PAO1 inoculum was 4×10^6 CFU/ml. Error bars are omitted for clarity. $*P < 0.05$ *vs.* vehicle at 18 h. *B*) Bacterial count measured at 0, 30, 60, 90, 120, and 180 min after treatment with CORM-3 (10 μM), ticarcilline (50 mg/L), or amikacine (50 mg/L) treatment. Data points are means ± SE of 6 independent experiments. *C*) Effect of CORM-3 on 3 resistant strains of *P. aeruginosa*. OD 600 nm measured 18 h after treatment with CORM-3 (10 μM) or vehicle was assessed spectrophotometrically. Bars represent means of 6 independent experiments. Initial inoculum was 4×10^6 CFU/ml. Growth of each strain treated with CORM-3 was compared with growth of same strain treated with vehicle. $*P < 0.05$. *D*) Cytotoxicity profile of CORM-3 in RAW 264.7 macrophages. Cells were incubated for 24 h with CORM-3 (0–1000 μM). Cell viability was determined spectrophotometrically using an Alamar Blue assay kit and expressed as a percentage of control. $*P < 0.05$ *vs.* control (medium alone). All comparisons by nonparametric Kruskal-Wallis test with Dunn's posttest).

kacine with minimum inhibitory concentrations of 3 and 2 mg/ml, respectively (unpublished data).

CORM-3 is a ruthenium-containing carbonyl complex that liberates CO when exposed to cells or tissues (17). As other transition metals, ruthenium might exert some biological properties, which could contribute to the bactericidal effect mediated by CORM-3 (21). To assess any contribution of ruthenium in the antibacterial effect elicited by CORM-3, we examined the growth of PAO1 in the presence of ruthenium chloride. However, bacterial growth was not altered by ruthenium chloride used at concentrations between 1 and 10 μM (**Fig. 2*A***), suggesting a predominant role of CO in the action of CORM-3. This hypothesis was further tested by examining the growth of PAO1 over time in a solution saturated with CO gas (≈860 μM). Interestingly, after 6 h, bacterial growth was reduced to a similar extent by both CO gas and CORM-3; however, inhibition of growth at 24 h was strongly evident in bacteria treated with CORM-3 but less so with CO gas (Fig. 2*B*). These results indicate a stronger prevention of bacterial growth by CORM-3 than CO gas.

In clinical practice, *P. aeruginosa* is able to develop numerous resistance mechanisms to antibiotics; moreover, the phenotype of these strains may be different from our laboratory strain. Therefore, we extended our *in vitro* experiments by investigating the effect of CORM-3 on growth of 3 resistant *P. aeruginosa* strains isolated from infected patients. As shown in Fig. 1*C*, the growth of these 3 strains was completely inhibited by 10 μM CORM-3, whereas no changes in bacterial growth were observed after treatment with vehicle.

Having demonstrated that CORM-3 exerts a powerful bactericidal activity, we evaluated its toxicity profile in eukaryotic cells. Murine RAW264.7 macrophages were exposed for 24 h to different concentrations of CORM-3 (from 10 to 1000 μM). We found that cell viability determined at the end of the incubation period was fully preserved after exposure of macrophages to concentrations between 10 and 200 μM CORM-3, while a significant loss in cell viability was evident only at concentrations of CORM-3 equal or greater than 500 μM (Fig. 1*D*). These results indicate that the cytotoxicity of CORM-3 in macrophages occurred at concentrations 50-fold higher than its bactericidal activity. Thus, it appears that mammalian cells are not susceptible to the toxic effects of CORM-3 at the concentrations that are detrimental for the pathogenic bacteria.

Antimicrobial effect of CORM-3 is mediated by inhibition of pathogen's respiratory chain

Since iron uptake is essential for bacterial growth (22) and this process involves heme-containing proteins sensitive to inhibition by CO (23), we first examined whether iron supplementation altered the effect of CORM-3. The results of these experiments showed that iron or heme supplementation does not affect the antimicrobial activity of CORM-3 on PAO1 (unpublished data).

Second, because CO is known to bind efficiently to cytochromes both in mitochondria and the bacterial respiratory chain, ultimately inhibiting their function (11, 23), we reasoned that the bactericidal effect of CORM-3 could be mediated by binding of CO to the PAO1 respiratory chain. Thus, we analyzed the absorption spectra obtained from cytochromes of PAO1 following exposure to CORM-3. This experiment showed that CORM-3 added to intact PAO1 targets at least two spectroscopically distinct CO-binding cytochromes, presumably terminal oxidases. The resemblance of the spectra measured after treatment with CORM-3 and CO gas confirmed that the effects of CORM-3 on the respiratory chain were mediated by CO release (**Fig. 3*A***). This interaction could lead to an inhibition of bacterial respiration as it occurs with the mitochondrial respiratory chain. To test this hypothesis, we evaluated the effect of CORM-3 on PAO1 oxygen consumption. This experiment required a high bacterial load (5×10^8 CFU/ml), in order to reach a level of O_2 consumption that could be detected by the electrode. Treatment with CORM-3 (0–100 μM) induced a dose-dependent decrease in O_2 consumption, and this decrease temporally preceded the bactericidal action of CORM-3 (Fig. 3*B*). These results support the hypothesis

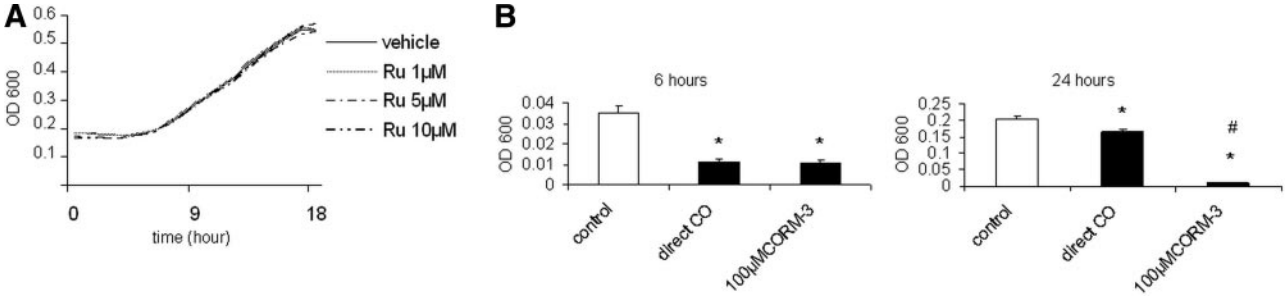

Figure 2. Effect of ruthenium chloride or CO gas on *P. aeruginosa* growth. *A*) Growth curves of PAO1 for 18 h after treatment with ruthenium chloride (Ru) (1–10 μM) or vehicle were assessed spectrophotometrically; curves represent means of 6 independent experiments. Initial PAO1 inoculum was 4×10^6 CFU/ml. Error bars are omitted for clarity. Different curves are superimposed, so it is very difficult to observe them individually. Differences are nonsignificant. *B*) OD 600 nm measured 6 and 24 h after control, CO gas (≈860 μM), or CORM-3 (100 μM) treatment. Histograms represent means ± SE of 6 independent experiments. $^*P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* CO gas; nonparametric Kruskal-Wallis test with Dunn's posttest.

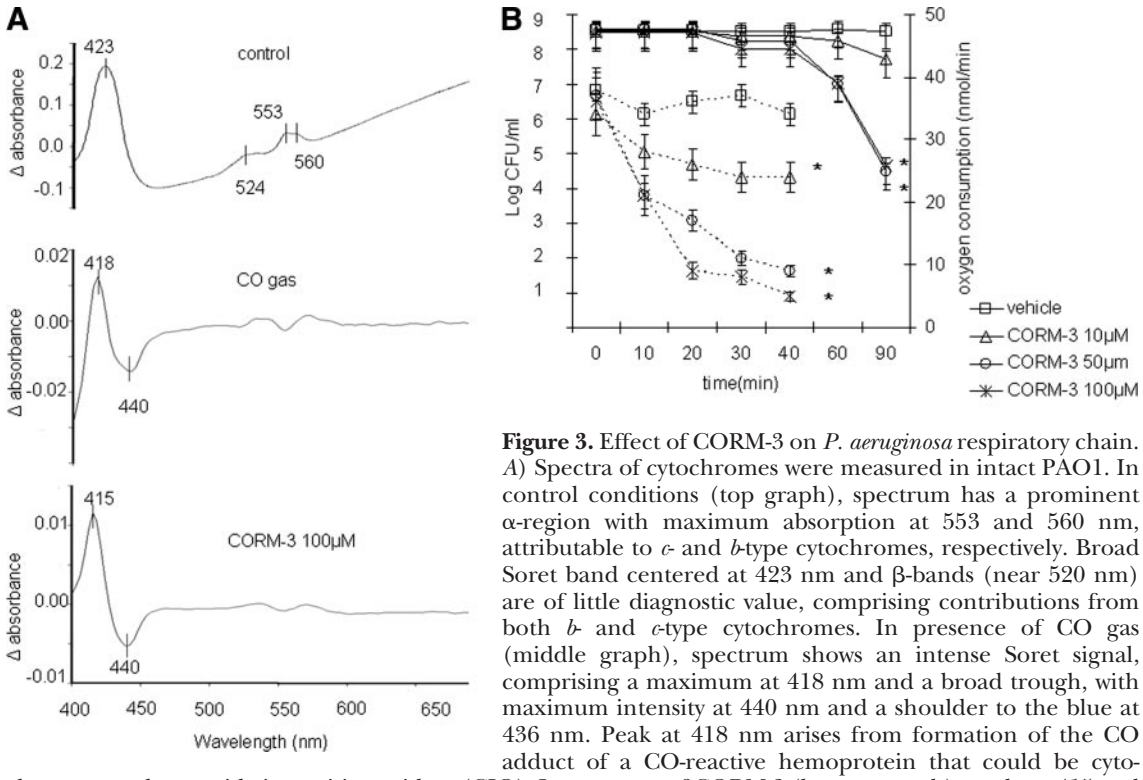

Figure 3. Effect of CORM-3 on *P. aeruginosa* respiratory chain. *A*) Spectra of cytochromes were measured in intact PAO1. In control conditions (top graph), spectrum has a prominent α-region with maximum absorption at 553 and 560 nm, attributable to *c*- and *b*-type cytochromes, respectively. Broad Soret band centered at 423 nm and β-bands (near 520 nm) are of little diagnostic value, comprising contributions from both *b*- and *c*-type cytochromes. In presence of CO gas (middle graph), spectrum shows an intense Soret signal, comprising a maximum at 418 nm and a broad trough, with maximum intensity at 440 nm and a shoulder to the blue at 436 nm. Peak at 418 nm arises from formation of the CO adduct of a CO-reactive hemoprotein that could be cytochrome *o* or the cyanide-insensitive oxidase (CIO). In presence of CORM-3 (bottom graph), peaks at 415 and 440 nm resemble features of the CO difference spectrum of cytochrome *o*. *B*) Oxygen consumption measured with a Clark-type oxygen electrode is represented by a discontinuous line (right axes). Bacterial counts in same conditions as for oxygen consumption measurements are represented by a continuous line (left axes). PAO1 strain (5×10^8 CFU/ml) was treated with CORM-3 (10, 50, 100 μM) or vehicle. Measurements were performed at 0, 10, 20, 30, and 40 min after treatment. Curves represent means of 4 experiments. $^*P < 0.05$ *vs.* vehicle; nonparametric Kruskal-Wallis test with Dunn's posttest.

that the antimicrobial effect of CORM-3 is mediated by an interaction of CO liberated by the carrier with cytochrome oxidase leading to an inhibition of the respiratory chain.

Antimicrobial effect of CORM-3 does not involve oxidative stress reactions

We and others have recently shown that CO-RMs increase the production of ROS by mitochondria in eukaryotic cells (24–26). Therefore, we tested the hypothesis that the bactericidal effect of CORM-3 could be secondary to an increase in ROS production by the bacterial respiratory chain. As a first step, we evaluated whether CORM-3 could increase bacterial H_2O_2 production. We found that CORM-3 did not increase bacterial H_2O_2 production measured by DCFH-DA fluorescence at 0, 30, and 60 min after CORM-3 treatment. In fact, H_2O_2 levels measured in PAO1 60 min after addition of CORM-3 were even lower than vehicle-treated PA01 (control group; **Fig. 4*B***). Second, we assessed whether antioxidants could reverse the effect of CORM-3 on bacterial growth and H_2O_2 production. The antioxidants NAC (1 mM) and ascorbic acid (AA, 100 μM) decreased H_2O_2 levels under basal conditions and in CORM-treated bacteria (Fig. 4*A*), but only NAC could reverse CORM-3-mediated inhibition of bacterial growth (Fig. 4*B*). Both NAC and AA had no effect on the growth of untreated bacteria. Thus, the facts that CORM-3 did not increase H_2O_2 production, and its inhibition of bacterial growth was not reversed by a powerful antioxidant, such as AA, indicate that the bactericidal effect of CORM-3 is unlikely to be mediated by a pathway involved in oxidative stress.

NAC, cysteine, and reduced glutathione inhibit the bactericidal effect of CORM-3

The previous experiments suggest that NAC prevents the effect of CORM-3 on bacterial growth by mechanisms that are independent of its antioxidant properties. A first possible explanation of these results is that NAC could prevent the liberation of CO from CORM-3. However, this possibility can be excluded since the rate of MbCO formation after reacting deoxymyoglobin with CORM-3 is not affected by the presence of NAC (1 mM) (**Fig. 5*A***). In *P. aeruginosa* as in eukaryotic cells, NAC is a donor of cysteine that is essential for glutathione synthesis (27). Therefore, a second possibility is that NAC blocked the effect of CORM-3 through activation of a cysteine-glutathione pathway. We, therefore, assessed whether cysteine and glutathione are able to reverse the inhibition of bacterial growth by CORM-3. Indeed, as shown in Fig. 5*C*, both cysteine (100 μM)

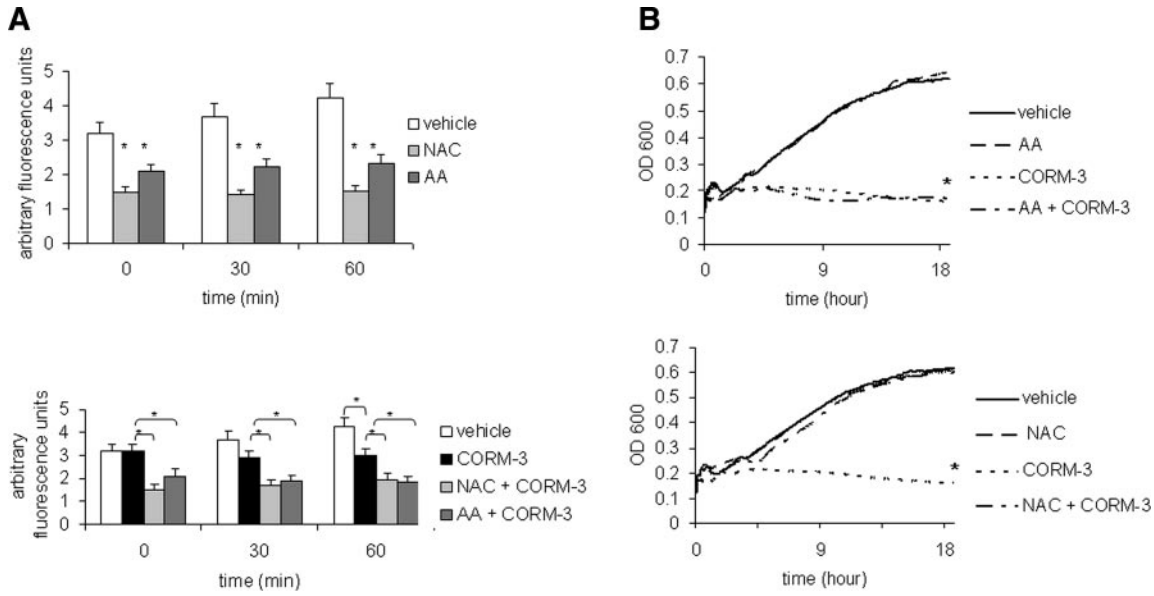

Figure 4. Role of redox status on antibacterial effect of CORM-3. *A*) Bacterial production of H_2O_2 was measured fluorometrically by the fluorochrome DCFH-DA. Histograms represent means ± SE of 6 independent experiments. Top panel: effect of *N*-acetylcysteine (NAC, 1 mM) and ascorbic acid (AA, 100 μM) on H_2O_2 production by PAO1 in basal conditions (no CORM-3). Bottom panel: effect of CORM-3 (10 μM) in the presence or absence of NAC (1 mM) or AA (100 μM) on H_2O_2 production by PAO1. *$P < 0.05$. *B*) Effect of CORM-3 (10 μM) in the presence or absence of NAC (1 mM) or AA (100 μM) on PAO1 growth. Growth curves followed for 18 h were assessed spectrophotometrically; curves represent means of 6 independent experiments. Initial PAO1 inoculum was 4×10^6 CFU/ml. Error bars are omitted for clarity. Top panel: vehicle and AA curves are superimposed. CORM-3 and AA + CORM-3 growth curves are superimposed. Bottom panel: vehicle, NAC, and NAC + CORM-3 curves are superimposed. *$P < 0.05$ *vs.* vehicle at 18 h. All comparisons by nonparametric Kruskal-Wallis test with Dunn's posttest.

and reduced glutathione (50 μM), but not oxidized glutathione (50 μM), completely abolished the antimicrobial action of CORM-3. We then hypothesized that CORM-3 could cause an acute drop in bacterial glutathione content, which serves as a detoxification system against antimicrobial drugs. If this hypothesis were correct, then NAC, cysteine, and reduced glutathione could act in restoring the intracellular glutathione pool. However, we found that the content of reduced glutathione in PAO1 was not modified at 30 and 60 min after treatment with CORM-3 or vehicle (Fig. 5*D*). These experiments strongly suggest that the mechanism through which CORM-3 inhibits bacterial growth is blocked by exogenous cysteine and reduced-glutathione but is independent of increases in the free intracellular glutathione pool.

Finally, we considered the possibility that this unidentified mechanism could involve binding of CO to the respiratory chain. We incubated PAO1 strain with NAC (1 mM) before measuring oxygen consumption and observed that the inhibition of PAO1 oxygen consumption induced by CORM-3 (100 μM) is completely reversed by NAC (Fig. 5*B*). Similar results were obtained with cysteine and reduced but not oxidized glutathione (unpublished data).

CORM-3 prolongs host survival by a direct bactericidal effect in a model of PAO1 bacteremia in mice

To test whether the bactericidal properties of CORM-3 against *P. aeurginosa* infection *in vitro* can be translated into a protective effect *in vivo*, we used a lethal model of PAO1 bacteremia in BALB/c mice. Mice were treated with an intraperitoneal injection of CORM-3 (7.5 mg/kg), which was administered 5 min after the bacterial inoculation. We first evaluated whether CORM-3 could prolong survival 72 h after the onset of the infection. Indeed, CORM-3 was effective against PAO1-induced bacteremia and reduced significantly the mortality of mice from 80% to 0. No mice died after the 72 h time point (**Fig. 6*A***). Moreover, infected animals exhibited less interest in their surroundings and developed piloerection. These signs were greatly attenuated in CORM-3-treated animals. Additional experiments demonstrated that CORM-3 treatment in noninfected mice did not induce any mortality nor any sign of illness during the 2 wk of the observational period (unpublished data). We next determined whether the protective effect of CORM-3 involved a bactericidal activity *in vivo* by counting bacteria in spleen. Bacterial counts in spleen, measured at 1 and 3 h after bacterial inoculation, was significantly reduced by the treatment with CORM-3 (Fig. 6*B*).

To confirm that CORM-3 has a direct *in vivo* bactericidal effect rather than stimulating bacterial phagocytosis by the host (9), we examined the bactericidal effect of CORM-3 in a model of PAO1 bacteremia in cyclophosphamide-induced leukopenic BALB/c mice. Cyclophosphamide-treated mice had a leukocyte count 80% lower than normal mice (see supplemental information). As shown with immunocompetent mice, the bacterial count in the

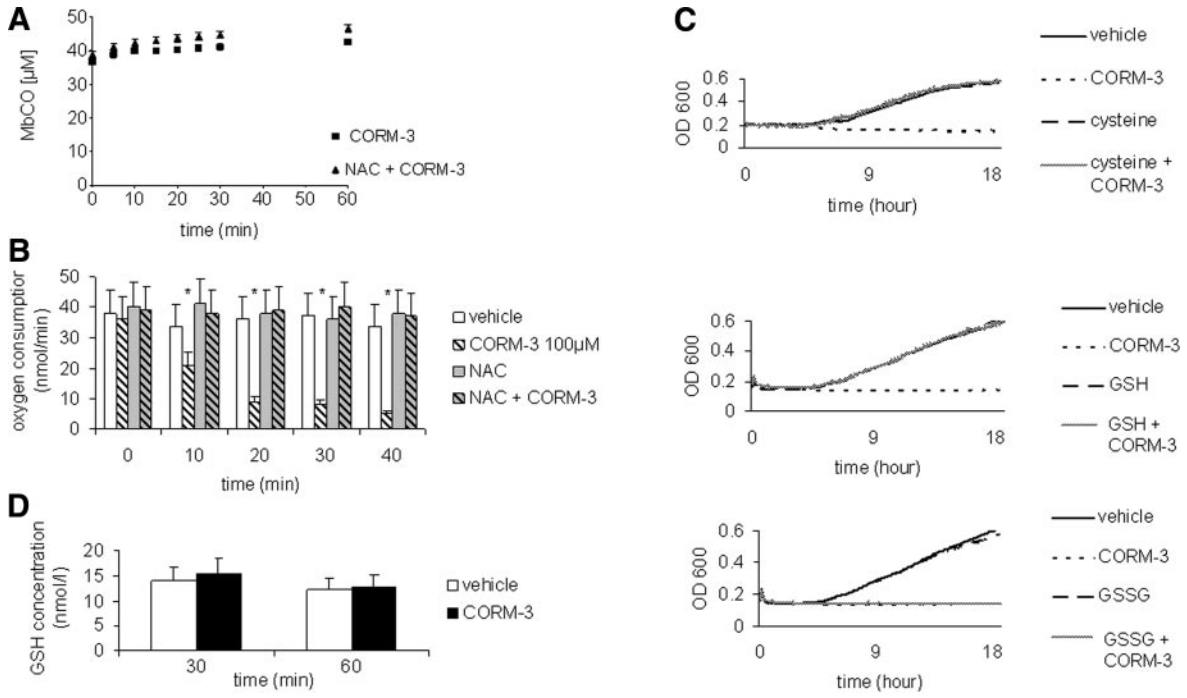

Figure 5. Role of thiols on antibacterial effect of CORM-3. *A*) CO release from CORM-3 in presence or absence of NAC (1 mM), assessed spectrophotometrically by measuring conversion of deoxymyoglobin to MbCO. Data points represent means ±SE of 4 independent experiments. Differences are nonsignificant; nonparametric Mann-Whitney *U* test. *B*) Oxygen consumption was measured with a Clark-type oxygen electrode. PAO1 strain (5×10^8 CFU/ml) was initially pretreated with NAC (1 mM) or vehicle and then treated with CORM-3 (100 µM) or vehicle. Measurements were performed at 0, 10, 20, 30, and 40 min after treatment. Histograms represent means ±SE of 4 independent experiments. $*P < 0.05$ *vs.* CORM-3; nonparametric Kruskal-Wallis test with Dunn's posttest. *C*) Effect of 100 µM cysteine (top graph), 50 µM reduced glutathione (GSH) (middle graph), or 50 µM oxidized glutathione (GSSG) (bottom graph) on PAO1 growth in presence of CORM-3 or vehicle. Thiols were added 1 h prior to CORM-3 or vehicle; growth was followed for 18 h. Curves represent means of 6 independent experiments. Initial PAO1 inoculum was 4×10^6 CFU/ml. Top graph: vehicle, cysteine, and cysteine + CORM-3 curves are superimposed. Middle graph: vehicle, GSH, and GSH + CORM-3 curves are superimposed. Bottom graph: vehicle and GSSG curves as CORM-3 and GSSG + CORM-3 curves are superimposed. $*P < 0.05$ *vs.* vehicle at 18 h; nonparametric Kruskal-Wallis test with Dunn's posttest. *D*) Effect of CORM-3 (10 µM) on bacterial GSH production measured spectrophotometrically by reduction of DTNB at 30 and 60 min after treatment with CORM-3 or vehicle. Histograms represent means ±SE of 6 independent experiments. Differences are nonsignificant; nonparametric Mann-Whitney *U* test.

spleen of cyclophosphamide-treated mice was significantly reduced by CORM-3 1 and 3 h after bacterial inoculation (Fig. 6*C*). Furthermore, the prolonged survival and the bactericidal effect were observed also in C57/BL6 mice, which have a different immunological profile than BALB/c animals (unpublished data). Collectively, these results strongly support a direct bactericidal action of CORM-3 *in vivo*, an

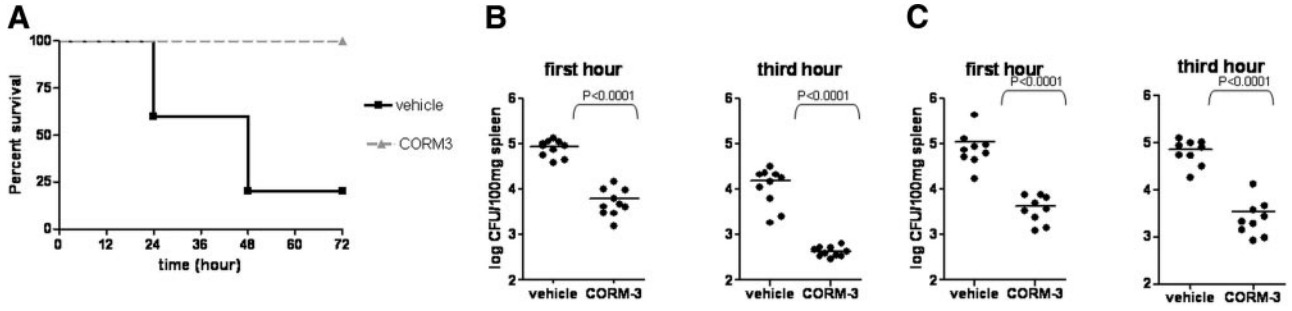

Figure 6. *In vivo* effects of CORM-3 against PAO1 bacterial infection. *A*) Kaplan-Meier curves representing survival of BALB/c mice in a model of PAO1 bacteremia (see text for details) after treatment by a single intraperitoneal injection of CORM-3 (7.5 mg/kg) or vehicle. Black curve, vehicle group; gray curve, CORM-3-treated group; $n = 40$ mice/group. $*P < 0.0005$; log-rank test. *B*) Bacterial count in spleen of BALB/c mice 1 and 3 h after PAO1 inoculation (n=10 mice/group) (vehicle or 7.5 mg/kg CORM-3) at each time. Nonparametric Mann-Whitney *U* test. *C*) Bacterial count in spleen of leukopenic BALB/c mice 1 and 3 h after PAO1 inoculation (n=10 mice/group) (vehicle or 7.5 mg/kg CORM-3) at each time. Nonparametric Mann-Whitney *U* test.

effect that is not related to the ability of the host's leukocytes to phagocytize the pathogens.

DISCUSSION

To the best of our knowledge, the results presented here are the first demonstration of the potent bactericidal activity of a water-soluble CORM (CORM-3), against the pathogenic activities of *P. aeruginosa*. The fact that the antimicrobial action of CORM-3 observed on a laboratory strain of *P. aeruginosa* (PAO1) was also reproduced against 3 antibiotic-resistant strains isolated from hospitalized patients and the use of clinical pertinent bacterial concentrations (from 10^6 to 5×10^8 CFU/ml) are indicative of the therapeutic potential of this prototypic CO-based pharmaceutical (28). This is very important because the strains of *P. aeruginosa* resistant to antibiotics represent a major public health problem (3) and no new antibiotics have been developed to fight this nosocomial infection in the past 2 decades. Moreover, our data indicate that the bactericidal activity of CORM-3 (10 μM) was as potent as the antibacterial action displayed by amikacine, an antibiotic currently used in clinical practice to treat *P. aeruginosa* infections.

Our results shed some light on the mechanisms of action of CORM-3 against *P. aeruginosa*. We found that CO liberated from CORM-3 targets at least two spectroscopically distinct CO-binding cytochromes, presumably terminal oxidases, crucial enzymes present in *P. aeruginosa*'s respiratory chain. Indeed, the inhibitory action of CORM-3 was associated with a decreased bacterial oxygen consumption, which preceded the antimicrobial effect, strongly suggesting a direct link between these two effects. We also found that inhibition of oxygen consumption by CORM-3 does not involve an overproduction of ROS by the respiratory chain because both H_2O_2 levels and the glutathione pool were unchanged after incubation of *P. aeruginosa* with CORM-3. In addition, AA did not reverse the bactericidal effect of CORM-3, despite reducing substantially the production of H_2O_2 by bacteria. It is intriguing that the effects of CORM-3 on oxygen consumption and bacterial growth were abolished by thiol donors. Thus, we postulate that inhibition of oxygen consumption by CORM-3 is likely to involve cysteine moieties strategically located in the enzymes of the respiratory chain. A binding of CO to cysteine residues cannot be envisioned from a chemical perspective, but it is worth noting that other amino acids such as histidine or aspartic acid in protein channels have been identified recently as crucial for transducing the signal mediated by CO gas or CORMs (29, 30). Clearly, the involvement of cysteines in the findings presented here deserves further investigation. It is also interesting that although ruthenium chloride did not affect bacterial growth, CORM-3 elicited a more intense and long-lasting inhibition of bacterial growth than CO gas. Indeed, CO gas behaved more as a bacteriostatic agent, whereas CORM-3 exerted a bactericidal effect. The reason for this difference between CO gas and CORM-3 is not clear at present, but certainly, the data point to a more efficient way of the ruthenium-containing compound to deliver CO and consequently mediate a more robust pharmacological action. Moreover, it has to be noted that the effects of CO should be interpreted in the light of a ratio $CO:O_2$. In this context, the apparent paradox of the CO-releasing agent being more effective than CO gas should be explained by a relatively greater concentration of CO *vs.* O_2 in the microdomain of the CORM-3/target complex. The alteration of the $CO:O_2$ ratio could have profound effects on O_2 metabolism, production of free radicals, and energetics, all important elements in restoring cellular homeostasis.

Our results show that CORM-3 significantly increased survival and reduced bacterial count in the spleen of mice subjected to lethal PAO1 bacteremia. The antimicrobial action of CORM-3 was evident in 2 strains of mice (C57BL/6 and BALB/c) with different immunological profiles. Moreover, and perhaps most importantly, treatment with CORM-3 was able to reduce bacterial count in the spleen of immunosupressed mice in a similar fashion as seen with immunocompetent mice, strongly suggesting a direct, rather than host-mediated, antibacterial effect of CORM-3. We did not observe any toxicity of CORM-3 against host either *in vitro* (where cytotoxicity against macrophages was significant only at concentrations of CORM-3 50-fold higher than the bactericidal concentrations) or *in vivo* (in mice treated with increasing doses of CORM-3 from 7.5 to 22.5 mg/kg and observed during 2 wk, no mortality and no apparent adverse effect were found, unpublished data). The high sensitivity of *P. aeruginosa* toward CORM-3 as compared to macrophages may be due to a greater accessibility of CO to the respiratory chain enzymes in bacterium. In fact, the respiratory chain of *P. aeruginosa* is located in the bacterial membrane as opposed to eukaryotic cells where the respiratory chain is located in the membrane of intracellular mitochondria.

In conclusion, CORM-3 possesses *in vitro* and *in vivo* bactericidal properties against laboratory PAO1 and antibiotic-resistant strains of *P. aeruginosa*. This effect is mediated by inhibition of bacterial respiration, which is, to the best of our knowledge, a stratagem not yet utilized by existing antibiotics. Considering these results and the lack of toxicity against the host, CORM-3 and other CORMs could be considered as very promising new pharmaceutical tools. FJ

The study was performed under grants from Assistance Publique-Hopitaux de Paris, France (as a Contrat d'Interface to J.B.) and the French Cystic Fibrosis Association Vaincre La Mucoviscidose.

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Received for publication September 29, 2008.
Accepted for publication November 6, 2008.