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The antimicrobial activity of a carbon monoxide releasing molecule (EBOR-CORM-1) is shaped by intraspecific variation within *Pseudomonas aeruginosa* populations

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ABSTRACT

Carbon monoxide releasing molecules (CORMs) have been suggested as a new synthetic class of antimicrobials to treat bacterial infections. Here we utilised a novel EBOR-CORM-1 ([NEt$_4$][MnBr$_2$(CO)$_4$]) capable of water-triggered CO-release, and tested its efficacy against a collection of clinical *Pseudomonas aeruginosa* strains that differ in infection-related virulence traits. We found that while EBOR-CORM-1was effective in clearing planktonic and biofilm cells of *P. aeruginosa* strain PAO1 in a concentration dependent manner, this effect was less clear and varied considerably between different *P. aeruginosa* cystic fibrosis (CF) lung isolates. While a reduction in cell growth was observed after 8 hours of CORM application, either no effect or even a slight increase in cell densities and the amount of biofilm was observed after 24 hours. This variation could be partly explained by differences in bacterial virulence traits: while CF isolates showed attenuated *in vivo* virulence and growth compared to strain PAO1, they formed much more biofilm, which could have potentially protected them from the CORM. Even though no clear therapeutic benefits against a subset of isolates was observed in an *in vivo* wax moth acute infection model, EBOR-CORM-1was more efficient at reducing the growth of CF isolate co-culture populations harbouring intraspecific variation, in comparison with efficacy against more uniform single isolate culture populations. Together these results suggest that CORMs could be effective at controlling genetically diverse *P. aeruginosa* populations typical for natural chronic CF infections and that the potential benefits of some antibiotics might not be observed if tested only against clonal bacterial populations.

1. INTRODUCTION

The rapid emergence of multidrug-resistant bacteria is a global problem that is predicted to cause ten million deaths per year by 2050 (O'Neill, 2014). Antibiotic resistance often evolves very quickly via *de novo* mutations and horizontal gene transfer (Normark and Normark, 2002), and as a result, antibiotic discovery has not been able to replace all of the antibiotics that have now become ineffective (Brown and Wright, 2016). New methods and approaches for treating bacterial infections are thus urgently required.

In recent years, carbon monoxide has emerged as a new potential therapeutic due to its properties as a homeostatic and cytoprotective molecule with important signalling capabilities (Motterlini and Otterbein, 2010). Carbon monoxide can be delivered via carbon monoxide releasing molecules (CORMs), which are small molecules that release carbon monoxide in response to certain environmental triggers such as enzymes (Stamellou et al., 2014) or light (Jimenez et al., 2016). Nobre *et al.* first investigated the effect of CORMs on bacteria (Nobre et al., 2007) and found that CORM-2 and CORM-3 reduced the number of colony-forming units of *Escherichia coli* in minimal salts media and *Staphylococcus aureus* in Luria Broth (LB) media (Nobre et al., 2007). The CORM effects were stronger in near-anaerobic conditions and the activation of CORM required direct contact between the molecule and its cellular targets (Nobre et al., 2007). Moreover, the effect of CORM-3 on *Pseudomonas aeruginosa* wild type strain PAO1 was investigated by Desmard *et al.* (Desmard et al., 2009), who found that treatment with the CORM reduced bacterial densities and increased the survival of immunocompromised mice during an infection. It has also previously been found that CORM-2 effectively reduces the densities of *P. aeruginosa* planktonic and biofilm cultures with wild type and clinical strains (Murray et al., 2012). Another study found that manganese-based Trypto-CORM is able to inhibit the growth of *E. coli* when exposed to photochemical stimulus (Ward et al., 2014), while in the dark it is active against *Neisseria gonorrhoeae* (Ward et al., 2017). In both cases, control experiments indicate that the CO liberated from the metal is responsible for the observed behaviour. However, most of the studies thus far have concentrated on exploring CORM effects on relatively short time span (less than 24 hours). Furthermore, although it has been established that many infections are polymicrobial, and that clinical bacterial pathogens can respond differently to CORMs than laboratory strains, no studies have explored CORM effects on bacterial co-cultures.

Cystic fibrosis (CF) is a genetically inherited disease which affects 1 in 2000 to 3000 newborn infants in the EU (who.int, 2010). Patients with CF often develop a thick mucus in the lungs which they are unable to clear (Flume et al., 2010). This mucus makes patients susceptible to frequent and recurring bacterial chest infections and the presence of *P. aeruginosa* is often associated with increasing morbidity and loss of lung function (Pritt et al., 2007). One of the key features of *P. aeruginosa* is its capability to rapidly adapt to the lung environment and to become highly resistant to the antibiotics that are used to treat infections (Smith et al., 2006;Poole, 2011;Folkesson et al., 2012;Winstanley et al., 2016). As a result, *P. aeruginosa* populations show high levels of genetic variation within and between CF patients (Marvig et al., 2013;Williams et al., 2015;O'Brien et al., 2017). This includes phenotypic and genomic heterogeneity within genetically-related populations of *P. aeruginosa* derived from the same clonal lineage (Mowat et al., 2011;Workentine et al., 2013;Williams et al., 2015). This variation might also affect the applicability of potential alternative therapies if it is linked with bacterial life-history traits that relate to potential resistance mechanisms.

Here we synthesised and characterised a water-soluble CORM (EBOR-CORM-1), $[NEt_4][MnBr_2(CO)_4]$, and tested its effectiveness against *P. aeruginosa* strain PAO1 and a selection of *P. aeruginosa* CF isolates originating from a single sputum sample from the lungs of a CF patient, namely patient CF03 from previously published studies (Mowat et al., 2011;Williams et al., 2015). Based on genome sequence data presented in a previous study, these CF isolates were classified into two genetically distinct Liverpool Epidemic Strain (LES) lineages, A and B (Williams et al., 2015;Williams et al., 2016), that differ regarding their virulence traits (O'Brien et al., 2017). These genetically diverged lineages have been shown to commonly coexist within individual patients and to share mutations via homologous recombination that potentially help strains to adapt to the airway during chronic infection (Williams et al., 2015). However, the implications of within-patient genetic variation have been seldom considered in the context of antimicrobial therapies. We hypothesised that effects of EBOR-CORM-1 could vary between different clinical isolates and lineages, and that the susceptibility of isolates could be linked to expression of some other bacterial virulence factors. We found that the CORM was effective in reducing both planktonic and biofilm cells of strain PAO1 in a density-dependent manner. However, CORM effects were more varied and generally weaker against clinical CF isolates. Regardless, CORM efficiently reduced the growth of CF strain lineage co-cultures, which suggest that CORMs could be effective at controlling genetically diverse *P. aeruginosa* infections.

2. MATERIALS AND METHODS

Synthesis and properties of [NEt$_4$][MnBr$_2$(CO)$_4$], EBOR-CORM-1

EBOR-CORM-1 was synthesised as described previously (Angelici, 1964): Mn(CO)$_5$Br (466 mg, 1.69 mmol) and 330 mg (1.57 mmol) of [(C$_2$H$_6$)$_4$N]Br were heated in 18 mL of absolute methanol under a nitrogen atmosphere at 50 °C for 1 hour. The methanol was then evaporated from the orange solution at the above temperature. The remaining yellow solid was dissolved in 40 mL of chloroform, and the solution was filtered under nitrogen. After adding 200 mL of hexane to the filtrate, the cloudy solution was allowed to stand under nitrogen for 2 hours. The air-stable yellow crystals were separated by filtration, washed with hexane, and dried under vacuum giving a yield of 88 % (636 mg). The compound was characterised *via* solid state IR spectroscopy recorded using a KBr disk. Four main bands were seen at 2090, 2001, 1984 and 1942 cm^{-1} and a small shoulder was seen at 1897 cm^{-1}. This is consistent with the literature values (Angelici, 1964). In a chloroform solution of CORM four distinct bands were observed at 2092, 2015, 1987 and 1943 cm^{-1}, again this is similar to previously reported literature values (Angelici, 1964). The change in the number of carbonyl bands between the solid and solution phase measurements typically reflects that different orientations are present in the solid state. The stability of the CORM in the solid state was tested by heating a sample to 50 °C and running ATR IR spectra at 1 hour intervals.

Infrared detection of CO release from EBOR-CORM-1following dissolution in different solvents was conducted by dissolving 12 mg of CORM in 4 mL of solvent in a 25 mL round bottomed flask attached to vacuum evacuated gas IR cell via a closed tap. After 1 h of stirring the flask, the tap was opened to enable gas from the headspace of the flask to enter the IR cell. Carbon monoxide could then be identified via the distinctive gaseous IR signature of a double band, with fine rotational splitting, centred at 2150 cm^{-1} (Klein et al., 2014). The impact of different solvents can be quantified by comparison of the intensity of the CO bands to those from CO$_2$, which is assumed to act as an effective internal standard.

The release of CO from EBOR-CORM-1following dissolution in water was also followed via solution phase monitoring of the metal complex's IR bands. In contrast to chloroform, when EBOR-CORM-1was first dissolved in water only two main IR bands were observed at 2050 and 1943 cm^{-1}. In order to investigate activity of EBOR-CORM-1 in liquid culture media, we compared the effects of active and 'inactivated' CORM on the growth of PAO1 strain in LB media as described previously (Murray et al., 2012). Briefly, CORM was inactivated by storing a 2 mM CORM stock LB solution (10% v/v of standard LB concentration, i.e., the same that was used in all the experiments; see below) at room temperature for 24 hours. To

138   estimate the effect of CORM inactivation on PAO1 growth, we added 50 µL of freshly
139   prepared 2 mM CORM, 50 µL of inactivated 2 mM CORM or 50 µL 10% v/v LB (control) to
140   150 µL of PAO1 starter culture on 96-well microplate. All treatments were replicated five
141   times and PAO1 growth monitored for 8 hours at 37 ˚C with spectrophotometer (OD 600 nm;
142   Tecan Infinite).

Bacterial strains and culture media

144   In this study we used *P. aeruginosa* strain PAO1 (ATCC 15692), the earliest archived isolate
145   of the Liverpool Epidemic strain, LESB58 (Winstanley et al., 2009), and 19 clinical *P.*
146   *aeruginosa* LES isolates from the same sputum sample of a chronically infected CF patient
147   (Williams et al., 2015). The CF lung LES isolates originate from the sputum sample of one
148   patient, identified as patient CF03 in previous studies, and consist of two genetically separate
149   lineages A and B (Williams et al., 2015). Lineage A was represented by six isolates, namely
150   isolates: 2, 5, 10, 19, 23 and 25. Lineage B was represented by 13 isolates, namely isolates: 1,
151   6, 8, 17, 24, 26, 28, 32, 33, 34, 35, 36 and 37. Clinical isolates were collected with the
152   consent of the patient and under institutional human investigation approval. All strains and
153   isolates of *P. aeruginosa* were routinely cultured in liquid or solid LB media containing 10.0
154   g tryptone, 5.0 g yeast extract and 10.0 g NaCl in 1 L of ultra-pure water (final pH adjusted to
155   7.0 and 15 g of agar was used for solid media). For all experiments, starter cultures were
156   prepared from cryofrozen stocks by streaking frozen stock culture onto LB plates. After 24
157   hours growth, a single colony was selected and inoculated into 5 mL of liquid LB and grown
158   overnight in a shaking incubator at 37 ˚C in 50 mL centrifuge tubes. Overnight cultures were
159   centrifuged at 4000 rpm (11.5 g) for 15 min (Eppendorf), the resultant pellets were suspended
160   in 10% LB and bacterial densities adjusted to optical density at 600 nm of 0.066 before use
161   (OD 600nm), equalling ~1 x 10^8 cells mL^{-1}.

Measuring the effects of EBOR-CORM-1 concentration on P. aeruginosa PAO1 strain

164   We measured the effect of CORM concentration on *P. aeruginosa* PAO1 in four different
165   ways. First, we examined how EBOR-CORM-1 affects PAO1 growth after both 8 and 24
166   hours of inoculation in 10% LB media (bacteria and CORM inoculated at the same time).
167   Additionally, we measured how effective EBOR-CORM-1 is at clearing both established
168   planktonic and biofilm PAO1 cultures (bacteria pre-grown before adding EBOR-CORM-1).
169   All measurements were conducted on 96-well microplates and each treatment was replicated
170   5 times. A variety of EBOR-CORM-1 concentrations were tested by first preparing a 4 mM
171   CORM stock solution (dissolving EBOR-CORM-1 in 10% LB media by vortexing for 30 s

172   and sonicating for 1.5 min). The stock solution was then sterilised with syringe filtration and

173   serially diluted to result in 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0 mM (control) EBOR-

174   CORM-1 concentrations and 1×10^8 PAO1 cells mL^{-1} with final volume of 200 μl of media.

175   The microplate was then incubated at 37 ˚C for 24 hours.

176   All replicate populations were sampled at 8 and 24 hours after the start of the experiment (20

177   μl of samples) and serially diluted in sterile PBS on microplates to quantify the number of

178   living versus dead cells by flow cytometry. Briefly, DAPI (4',6-diamidino-2-phenylindole for

179   dead and living cells) and PI (Propidium iodide for dead cells) fluorescent stains (both from

180   Sigma-Aldrich) were added to microplate wells with diluted bacterial samples at

181   concentrations of 1 μg/mL and 50 μM, respectively. Plates were then incubated at room

182   temperature for 1 hour before measuring cell densities with a Cytoflex flow cytometer and

183   the CytExpert program. Every well was sampled for 60 s at fast speed setting. Gating of live

184   and dead cells was performed by monitoring DAPI staining on the PB450 channel with the

185   405 nm laser, and PI staining on the ECD channel of the 488 nm laser. Number of living cells

186   was determined as total cells (DAPI) – dead cells (PI).

187   To quantify the effects of EBOR-CORM-1 on established planktonic and biofilm cultures,

188   PAO1 was first grown in the absence of CORM at 37 ˚C for 48 hours. Cell cultures were then

189   inoculated with stock CORM solution to reach the same final concentrations as above: 1

190   mM, 0.5 mM, 0.25 mM, 0.125 mM and 0 mM (control) of CORM. The plate was incubated

191   for four more hours at 37 ˚C before sampling (20 µL), serial dilution and flow cytometry as

192   described above. To quantify effects of EBOR-CORM-1 on biofilm, crystal violet was added

193   to the remaining cell cultures at 10% v/v. After 15 min of incubation, the plate was rinsed

194   with deionised water and solubilised with 228 µL ethanol per well. The biofilm was

195   quantified by measuring absorbance at 600 nm.

196   ***Measuring the effects of EBOR-CORM-1 on clinical P. aeruginosa isolates in***

197   ***mono- and co-cultures***

198   Similar to the PAO1 strain experiments, we measured the effect of EBOR-CORM-1on

199   clinical *P. aeruginosa* isolates after 8 and 24 hours of inoculation in 10% LB media. We also

200   measured the impact of growing the isolates in the absence of EBOR-CORM-1 for 48 hours

201   and then applying EBOR-CORM-1 for 4 hours using both flow cytometry and crystal violet

202   staining. We used only one EBOR-CORM-1 concentration, 0.5 mM, which resulted in clear

203   reduction of PAO1 cultures (see results) alongside control treatment (no CORM).

204 In addition to measuring the effects of EBOR-CORM-1 in monocultures of each clinical
205 isolate, we also quantified the effect of the CORM on mixtures of the CF clinical isolates
206 from patient CF03. First, we prepared the clinical isolate starter cultures as described above,
207 then we mixed the standardised monocultures together in three different ways: as a whole
208 mix (all isolates mixed together in equal proportions), lineage A mix (all isolates classified as
209 lineage A mixed together in equal proportions) and lineage B mix (all isolates classified as
210 lineage B mixed together in equal proportions). All final mixes contained approximately 1 x
211 10^8 cells mL^{-1} before the application of 0.5 mM of EBOR-CORM-1. Each experiment was
212 replicated 5 times. After 24 hours growth at 37 °C, bacterial densities were measured by
213 using a Tecan infinite spectrophotometer: optical density measurements correlate well with
214 the proportion of living cells measured with flow cytometer (Supplementary figure 1).

Characterising bacterial virulence and growth

216 To characterise production of the virulence factors pyocyanin and pyoverdine, all clinical
217 isolates were grown in 200 µL of 10% LB media in round-bottomed 96-well microplates for
218 48 hours at 37 °C (no shaking). After incubation, we measured the bacterial densities (OD
219 600 nm) and centrifuged the microplate for 10 min. at 4000 rpm (11.5 g) in a swing rotor
220 Eppendorf centrifuge. To measure pyocyanin and pyoverdine production, 150 µL of the
221 supernatant of each well was transferred to flat-bottomed 96-well microplates and the
222 absorbance spectrum measured with a spectrophotometer (Tecan infinite). Per capita
223 pyocyanin production was measured for each isolate by measuring the absorbance of
224 supernatant at 691 nm, and then standardizing by bacterial OD (Reszka et al., 2004). Per
225 capita production of the iron-chelating siderophore, pyoverdine, was measured by using
226 excitation-emission assay (O'Brien et al., 2017) where the fluorescence of each supernatant
227 well was measured at 470 nm following excitation at 380 nm, using a Tecan infinite M200
228 pro spectrophotometer. Also, OD was measured at 600 nm to quantify the ratio
229 fluorescence/OD as a quantitative measure of per capita pyoverdine production (O'Brien et
230 al., 2017). The isolate biofilm production was measured as described previously and growth
231 as maximum density and growth rate h^{-1} during 24-hour growth period. Lastly, we also
232 measured the *in vivo* virulence of each isolate by using wax moth model as described
233 previously (O'Brien et al., 2017).

234

Testing EBOR-CORM-1 antimicrobial activity in wax moth model in vivo

236 To test the efficacy of EBOR-CORM-1 to constrain bacterial infections *in vivo*, we used a
237 wax moth larvae model (*Galleria mellonella* [Lepidoptera: Pyralidae], Livefood UK Ltd) and
238 followed the infection methodology described previously (O'Brien et al., 2017). We chose
239 three strains for infection experiments: PAO1, LESB58 and isolate 36 (Lineage B) from the
240 clinical sample collection. Before infection, we first grew the selected *P. aeruginosa* isolates
241 for 24 hours at 37 °C and subsequently diluted all cultures to approximately similar densities
242 (equalling approximately 1 x 10^6 cells mL^{-1} in 0.8% w:v NaCl). The virulence of every
243 isolate was then tested in 16 independent wax moth larvae. We also infected 16 larvae with
244 0.8% w/v NaCl salt solution to control for the damage caused by the injection itself. The
245 larvae were injected with either 20 μL of one bacterial solution or NaCl buffer ("non-
246 infected") between the abdominal segments six and seven with a 1 mL Terumo syringe. After
247 2 hours, 8 larvae from each bacterial infection or non-infection group were treated with 20
248 μL injection of 500 μM EBOR-CORM-1, and the other 8 were injected with 0.8% w:v NaCl
249 salt solution (control placebo) in the same location where the bacteria were originally
250 injected. After infection, larvae were placed on individual wells of 24-well cell culture plates
251 and the survival was monitored at 2-hour intervals for 3 days at 37 °C. Larvae were scored as
252 dead when they did not respond to touch with forceps. Larvae that were still alive after 7 days
253 from the infection were given a time of death of 168 hours. Every bacterial isolate was tested
254 for three times. It was concluded that the EBOR-CORM-1 injection alone did not affect
255 larval survival in the absence of bacteria (mortality similar between non-infected CORM-
256 injected larvae and non-infected CORM-free larvae: 5-10%).

Statistical analysis

258 All data were analysed with Generalized Mixed Models (factorial ANOVA) or regression
259 analysis where bacterial densities (Figs. 2, 3 and 4b) or trait values (Fig. 4a; Supplementary
260 figure 4) were explained with the presence and/or concentration of EBOR-CORM-1, CF
261 isolate identity (isolate number) or CF lineage (A or B). All proportional data (%) were
262 arcsine transformed before the analysis to meet the assumptions of parametric models.

3. RESULTS

Chemistry of EBOR-CORM-1

265 The stability of EBOR-CORM-1 in the solid state was demonstrated by heating a sample of
266 solid to 50 °C in air, and showing that there is very little difference in the carbonyl bands
267 observed in ATR IR spectra measured at 1 hour intervals over a 3-hour period (Fig. 1A). In
268 contrast, gas phase infrared analysis proved that CO release from EBOR-CORM-1can be

269 triggered by dissolution in water, phosphate buffer or LB media, or addition of water to a

270 solution of the compound in an organic solvent (Fig. 1B).

271 Solution phase monitoring of the CO stretches of the compound showed that there was no

272 reaction with water over short periods of time, since dissolving EBOR-CORM-1 in water,

273 immediately re-drying it on a vacuum line and then re-dissolving the resultant solid in

274 chloroform yielded an IR spectra which matched that of the as-purified compound in

275 chloroform (Fig. 1C). The only two observed IR bands in the CORM spectrum in water

276 (2050 and 1943 cm^{-1}) were therefore attributed to the molecular symmetry of the hydrated

277 complex, rather than an immediate loss of CO upon contact with water. However, after 90

278 min in water, a loss of these carbonyl bands was observed, and this was attributed to the

279 release of all the CO from the complex (Fig. 1D).

EBOR-CORM-1 activity against planktonic and biofilm cells of P. aeruginosa PAO1

282 We found that applying EBOR-CORM-1 had generally negative effects on *P. aeruginosa*

283 PAO1 growth both after 8 and 24 hours of application ($F_{4, 25} = 50.9$, $p < 0.001$ and $F_{4, 25} =$

284 31.8, $p < 0.001$ for proportion of living cells after 8 and 24 hours, respectively, Fig. 2A) and

285 that these negative effects increased along with the increasing concentration of applied

286 EBOR-CORM-1 (regression analysis: $F_{1, 24} = 43$, $p < 0.001$ and $F_{1, 24} = 35$, $p < 0.001$ for

287 proportion of living cells after 8 and 24 hours, respectively, Fig. 2A). Similarly, EBOR-

288 CORM-1 was highly effective against both established planktonic and biofilm *P. aeruginosa*

289 PAO1 cultures ($F_{4, 25} = 77.5$, $p < 0.001$ and $F_{4, 25} = 39.5$, $p < 0.001$, respectively, Fig. 2A-B)

290 and the antimicrobial activity of CORM increased in a density-dependent manner (regression

291 analysis: $F_{1, 24} = 92$, $p < 0.001$ and $F_{1, 24} = 54$, $p < 0.001$, respectively, Fig. 2A-B).

EBOR-CORM-1 activity against planktonic and biofilm cells of clinical P. aeruginosa cystic fibrosis isolates

294 Similar to strain PAO1, we found that EBOR-CORM-1 had inhibitory effects on all tested

295 clinical *P. aeruginosa* isolates after 8 hours of application of CORM ($F_{1, 152} = 11969$, $p <$

296 0.001, Fig. 3A). While this effect did not depend on the lineage (CORM × lineage: $F_{1, 152} =$

297 1.4, $p < 0.001$), it varied between different clinical isolates (CORM × isolate: $F_{18, 152} = 11969$,

298 $p < 0.001$, Fig. 3A). In contrast, EBOR-CORM-1 had slightly positive effects on *P.*

299 *aeruginosa* growth after 24 hours of application ($F_{1, 152} = 256$, $p < 0.001$, Fig. 3B) and this

300 effect varied between different isolates (CORM × strain: $F_{18, 152} = 2.8$, $p = 0.001$) being

301 slightly stronger (i.e. positive) with isolates belonging to a lineage B (CORM × lineage: $F_{1,}$

302 $_{152} = 24.9$, $p < 0.001$, Fig. 3B). EBOR-CORM-1 also had negative effects when applied to

303 established *P. aeruginosa* cell cultures ($F_{1, 152} = 222$, $p < 0.001$, Fig. 3C). However, these

304 effects depended on the isolate (CORM × isolate: $F_{18, 152} = 2.8$, $p = 0.001$) and the lineage

305 ($F_{1, 152} = 65.2$, $p = 0.001$), reduction being relatively larger with isolates belonging to lineage

306 A (Fig. 3C). In the case of established biofilms, EBOR-CORM-1 had a slightly positive

307 effect ($F_{1, 152} = 9.6$, $p = 0.002$, Fig. 3D) and while this effect varied between different isolates

308 ($F_{18, 152} = 2.0$, $p = 0.01$) it did not differ between the lineages ($F_{1, 152} = 1.2$, $p = 0.265$,

309 respectively, Fig. 3D). Together these results suggest that compared to strain PAO1, EBOR-

310 CORM-1 effects varied more with the clinical *P. aeruginosa* isolates having negative, neutral

311 or positive effects on bacterial growth depending on the isolate identity, lineage and the

312 timing of CORM application.

Linking EBOR-CORM-1 antimicrobial activity with clinical P. aeruginosa isolate virulence and growth

315 We found that all the isolates belonging to a lineage A formed non-mucoid colonies (6 out of

316 6), while most of the isolates belonging to a lineage B formed mucoid (i.e., mucus-like)

317 colonies (11 out of 13) on LB plates (typical mucoid and non-mucoid colonies shown in

318 supplementary figure 3). All clinical isolates differed from the non-mucoid PAO1 strain

319 respective of their virulence and growth (Fig. 4A). More specifically, clinical isolates

320 produced less pyoverdine ($F_{1, 23} = 286$, $p < 0.001$) and pyocyanin ($F_{1, 23} = 170$, $p < 0.001$) and

321 grew slower ($F_{1, 23} = 91$, $p < 0.00$) and reached lower maximum densities in LB medium ($F_{1,}$

322 $_{23} = 15.5$, $p = 0.001$, Fig. 4A). However, clinical isolates produced a considerably larger

323 amount of biofilm ($F_{1, 23} = 21.7$, $p < 0.001$) and showed very low virulence (high time to

324 death) in wax moth larvae *in vivo* ($F_{1, 23} = 1296$, $p < 0.001$, Fig. 4A).

325 When comparing the two CF lineages, we found that isolates belonging to a lineage B

326 consistently outperformed the isolates belonging to a lineage A by producing more

327 pyoverdine ($F_{1, 18} = 6.06$, $p = 0.025$), biofilm ($F_{1, 18} = 15.08$, $p = 0.001$) and by growing faster

328 ($F_{1, 18} = 22.35$, $p < 0.001$) and to higher maximum densities ($F_{1, 18} = 6.27$, $p = 0.023$) in LB

329 medium (Fig. 4A; Supplementary figure 4). However, lineages did not differ in pyocyanin

330 production ($F_{1, 18} = 1.99$, $p = 0.176$) or virulence ($F_{1, 18} = 1.03$, $p = 0.324$; Fig. 4A;

331 Supplementary figure 4). Across all clinical isolates, density reduction by CORM correlated

332 negatively with biofilm formation ($F_{1, 18} = 4.8$, $p = 0.042$). Together these results suggest that

333 clinical isolates differed from PAO1 and from each other respective to various life-history

334 traits important for establishing an infection.

EBOR-CORM-1 activity against clinical P. aeruginosa CF isolate co-cultures

336　Despite the observed isolate-specific variation in *P. aeruginosa* monocultures, EBOR-

337　CORM-1 was effective in reducing the growth of *P. aeruginosa* co-cultures after 24 hours of

338　application (CORM: $F_{1, 24}$ = 132, p < 0.001, Fig. 4B). Moreover, this reduction was the same

339　regardless of whether the mix contained only one lineage or both lineages (CORM × co-

340　culture: $F_{2, 24}$ = 0.5, p = 0.612). These results suggest that intraspecific *P. aeruginosa*

341　population heterogeneity makes the bacteria more susceptible to EBOR-CORM-1 treatment.

EBOR-CORM-1 activity against P. aeruginosa strains in wax moth model

343　We found that *P. aeruginosa* isolates differed in their virulence (time to death) from each

344　other ($F_{2, 24}$ = 12.2, p < 0.001): PAO1 and LESB58 strains were equally virulent, and both

345　exhibited higher virulence than the clinical isolate 36 (killing larvae approximately in 17

346　hours [PAO1], 36 hours [LESB58] and 92 hours [clinical isolate 36]; values averaged over

347　both non-CORM and CORM treatments, Fig. 5). In contrast to *in vitro* results, application of

348　EBOR-CORM-1 did not increase the survival of infected larvae ($F_{1, 24}$ = 1.3, p = 0.257) with

349　any of the infected strains (CORM × strain: $F_{2, 24}$ = 1.4, p = 0.273, Fig. 5). All larvae became

350　highly pigmented (black throughout) during the infection regardless of the *P. aeruginosa*

351　isolate.

4. DISCUSSION

353　Here we set out to study the antimicrobial activity of $[NEt_4][MnBr_2(CO)_4]$, EBOR-CORM-1,

354　against clinical *P. aeruginosa* isolates *in vitro*. This CORM was chosen as a suitable

355　representative of this class of molecule based on the aqueous solubility, facile synthesis

356　(Angelici, 1964), content of a non-toxic metal core, and simple architecture which makes it

357　akin to a "parent compound" for CORMs that have been engineered to possess sophisticated

358　CO release mechanisms. In contrast to more complex CORMs, the molecule was shown to

359　have a water activated mechanism of CO release, as seen in previous studies of $[MX(CO)_5]^-$

360　species, where X is a halide (Zhang et al., 2009). Such water induced degradations are

361　believed to proceed via a two-step pathway whereby water causes loss of the halide followed

362　by formation of a dimer species; from which the CO is released. This may explain the

363　changes in the IR spectra recorded in water when compared to chloroform, although the data

364　do not directly match those for $[Mn_2Br_2(CO)_8]$ (El-Sayed and Kaesz, 1963), the product

365　expect on loss of Br^- from EBOR-CORM-1. We found that while EBOR-CORM-1showed

366　density-dependent antimicrobial activity against both planktonic and biofilm cells of the

367　widely studied laboratory-adapted strain PAO1, these effects were more varied and weaker

368　against clinical CF lung isolates. Regardless, EBOR-CORM-1 was efficient at reducing the

369   growth of CF isolate lineage mixes, which suggests that it could have therapeutic potential in

370   controlling heterogeneous *P. aeruginosa* infections. Solutions of inactivated EBOR-CORM-1

371   were essentially inactive against *P. aeruginosa* strain PAO1 (Supplementary figure 2)

372   implying that, at least in this case, the observed activity was due to CO released from the

373   complex rather than the residual metal salts (or indeed $[NEt_4]^+$).

374   Similar to a study published by Murray *et al.* (2012), we found considerable variation in

375   CORM antimicrobial activity between different clinical CF isolates, which depended whether

376   we explored EBOR-CORM-1 effects on relatively short (8 hours) or long timescales (24

377   hours) and if we compared CORM antimicrobial activity on actively growing and established

378   cell cultures (after 48 hours of bacterial growth). Our results after 8 hours of EBOR-CORM-1

379   application are very similar to a previous study (Murray et al., 2012) showing clear reduction

380   in bacterial densities. However, this effect vanished by the 24 hour time point, and

381   surprisingly, some bacterial isolate cultures reached higher optical densities in the presence

382   compared to absence of CORM, which could have been due to increase in number of cells or

383   expression of exoproducts that were picked up by OD600 nm (e.g. pyocyanin or alginate).

384   The most likely explanation for this is that CORM effects were short-lived (Fig. 1), which

385   allowed bacteria to recover and grow to high densities during 24 hours after application of

386   CORM. However, when CORM effects were measured after 4 hours of application to

387   established cell cultures, we could still observe clear reduction in mean bacterial densities.

388   Together these results suggest that CORM effects could be seen up to 4 hours post

389   application and that CORM could eradicate bacterial cells whether they are at exponential or

390   stationary phase of their growth. Interestingly, CORM effects varied between clinical isolates

391   and were clearer with the isolates belonging to lineage A. While Murray *et al.* (2012) did not

392   observe clear variation in CORM effects against planktonic cell cultures, they found

393   differences in CORM efficiency in eradicating bacterial biofilms. This is also consistent with

394   our data and reinforces the hypothesis that *P. aeruginosa* clinical isolates are likely to

395   respond differently to CORM therapies.

396   To explore clinical isolate variation in more detail, we compared differences in bacterial

397   virulence and growth traits between the PAO1 and clinical CF lung isolates. We found that

398   relative to strain PAO1, clinical CF isolates grew slower, had lowered virulence and

399   produced lower amounts of pyoverdine and pyocyanin, which are important virulence factors

400   (O'Brien et al., 2017). This is consistent with previous research and typical for *P. aeruginosa*

401   isolates retrieved from chronic lung infections (Smith et al., 2006;Folkesson et al.,

402   2012;Marvig et al., 2013;Williams et al., 2015). The clinical isolates produced much more

403 biofilm compared to strain PAO1 and biofilm formation was the highest in the isolates
404 belonging to lineage B. Biofilms could potentially provide a protective function against
405 CORMs. Biofilms often have much higher antibiotic resistance than their aquatic
406 counterparts (Stewart and William Costerton, 2001) and there are multiple reasons for this.
407 First, antibiotics might be ineffective because the biofilm acts as a diffusion barrier (de Beer
408 et al., 1997). Second, subpopulations within the biofilm can sometimes differentiate into a
409 highly protected phenotypes that can repopulate the biofilms (Cochran et al., 2000). Third,
410 the biofilm might change the chemical microenvironment, forming zones of nutrient and
411 oxygen depletion or waste accumulation that prevents the antibiotics from functioning
412 optimally (de Beer et al., 1994). Although we did not explore this specifically, clinical
413 isolates belonging to a lineage A were more susceptible to CORMs and produced relatively
414 less biofilm compared to strains belonging to a lineage B. Thus, overall a negative correlation
415 was found between density reduction by CORM and biofilm formation. Our results therefore
416 suggest that biofilm might provide a protective function against the CORM.

417 Despite the isolate variations observed in bacterial monocultures, EBOR-CORM-1was
418 effective at reducing the growth of *P. aeruginosa* clinical isolate mixed cultures. One
419 explanation for this is that, in addition to CORM, *P. aeruginosa* growth was limited by
420 antagonistic intraspecific species interactions in co-cultures. *P. aeruginosa* has been shown to
421 exert both facilitative and antagonistic effects on each other via siderophore (Harrison et al.,
422 2008) and bacteriocin (Ghoul et al., 2015) production. In our case, all the clinical isolates
423 were derived from the same Liverpool Epidemic Strain clonal lineage and therefore likely
424 carried the same siderophore and bacteriocin genes. Additionally, resource competition is
425 likely to further limit *P. aeruginosa* growth both in CF lungs and simplified laboratory
426 microcosms. As a result, even though some clinical strains were relatively insensitive to
427 EBOR-CORM-1, their growth could have been constrained by competition with the other
428 strains in co-cultures. We found that this was the case for all strain mixes regardless if the
429 strains belonged to a lineage A, B or them both. This suggests that the susceptibility of the
430 lineages measured in monocultures did not predict the susceptibility of isolate mixes within
431 or between lineages. However, such antagonism was not observed in the absence of EBOR-
432 CORM-1, which suggests that CORM-triggered antagonistic intraspecific interactions in *P.*
433 *aeruginosa* co-cultures. Mechanistically, this could have been driven by competition sensing
434 in response to CORM-mediated cell damage in *P. aeruginosa* populations (Cornforth and
435 Foster, 2013). However, this needs to be confirmed in future experiments. Interestingly, all
436 the clinical strains we used originated from a single CF patient and interactions between them
437 thus reflect the realistic ecology of CF lungs. In the future, it would be useful to determine

438   pairwise interactions between these CF strains and look at CORM effects on other coexisting

439   bacterial species observed in CF infections (Folkesson et al., 2012).

440   We found that EBOR-CORM-1 had no clear therapeutic benefits in the wax moth infection

441   model. There are several potential explanations for this. First, EBOR-CORM-1 had limited

442   long-term activity when in contact with water. As a result, the bactericidal effect may only

443   have elicited lag in the initial phase of bacterial growth and proliferation within the wax

444   moths. Second, insect tissue is not homogeneous and it is possible that we failed to deliver

445   the CORM to the specific area of infection, or that bacteria were able to colonise new areas

446   that were not exposed to the CORM. Third, insects differ from laboratory media (such as LB)

447   as a bacterial growth environment, which could also affect pathogen virulence. For example,

448   it has been recently demonstrated that plant versus animal based growth media can have

449   physiological effects on bacterial virulence (Ketola et al., 2016) and that LB media does not

450   adequately reflect *P. aeruginosa* growth on lung tissue (Harrison et al., 2014;Harrison and

451   Diggle, 2016). Hence, the wax moth injection model might not reliably reflect the virulence

452   of CF isolates derived from chronic infections. However, it is also the case that many of the

453   affordable and available CF infection animal models do not truly reflect the real CF lung

454   disease environment. It remains to be established whether CORM therapy could be applied in

455   the context of CF lung infections. It is possible, for example, that it might be more suitable

456   for treating topical infections such as burn wounds, for which better animal models are

457   available (Rumbaugh et al., 2012).

458   Further work is also needed to understand the mode of action of EBOR-CORM-1. While

459   respiratory oxidases and globins at heme targets are generally considered the prime targets of

460   CO and CORMs (Wareham et al., 2015), it has been demonstrated that CORMs can have

461   multiple different other targets (Wilson et al., 2015). For example, CO also binds to the di-

462   iron site in bacterial NO reductases and to iron, copper, and nickel sites in certain microbial

463   proteins such as CO dehydrogenase (Lu et al., 2004;Wasser et al., 2005). In some cases,

464   CORMs might have intracellular targets but their accumulation within the cells can be very

465   weak (Tinajero-Trejo et al., 2016). Moreover, in the future it would be important to test if

466   EBOR-CORM-1 is cytotoxic to eukaryotic cells. The concentration we used are in line with

467   previously published work where no, or very mild, cytotoxic effects were observed (Murray

468   et al., 2012). We are currently conducting experiments to validate this independently and to

469   understand how EBOR-CORM-1interacts with bacterial cells. While, our wax moth assays

470   show that the concentrations we used had no negative effects on short-term insect viability,

471   more detailed cytotoxicity assays are needed in the future. Lastly, the low solubility of

472 EBOR-CORM-1 in water, and its activation in this medium, is problematic for delivery and
473 activation at specific sites within patients. In addition to chemically increasing the molecule
474 stability, CORMs could be enclosed in microvesicles (van Dommelen et al., 2012) to ensure
475 more efficient antimicrobial activity and drug delivery.

476 In conclusion, our results show that EBOR-CORM-1 shows antimicrobial activity against
477 both planktonic and biofilm cells of *P. aeruginosa* strain PAO1 but that these effects are
478 more varied and less pronounced against clinical CF lung isolates in monocultures. In
479 contrast, more heterogeneous *P. aeruginosa* populations comprising intraspecific phenotypic
480 variants were more susceptible to CORM treatment. This potentially has wider implications
481 in the testing of novel therapeutics. At present, this is done almost exclusively using clonal *P.*
482 *aeruginosa* populations. Our observations suggest that testing carried out on more
483 heterogeneous populations of *P. aeruginosa*, more closely resembling those found in the CF
484 lung, may give different and sometimes more promising results.

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ACKNOWLEDGEMENTS

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FIGURES AND FIGURE LEGENDS

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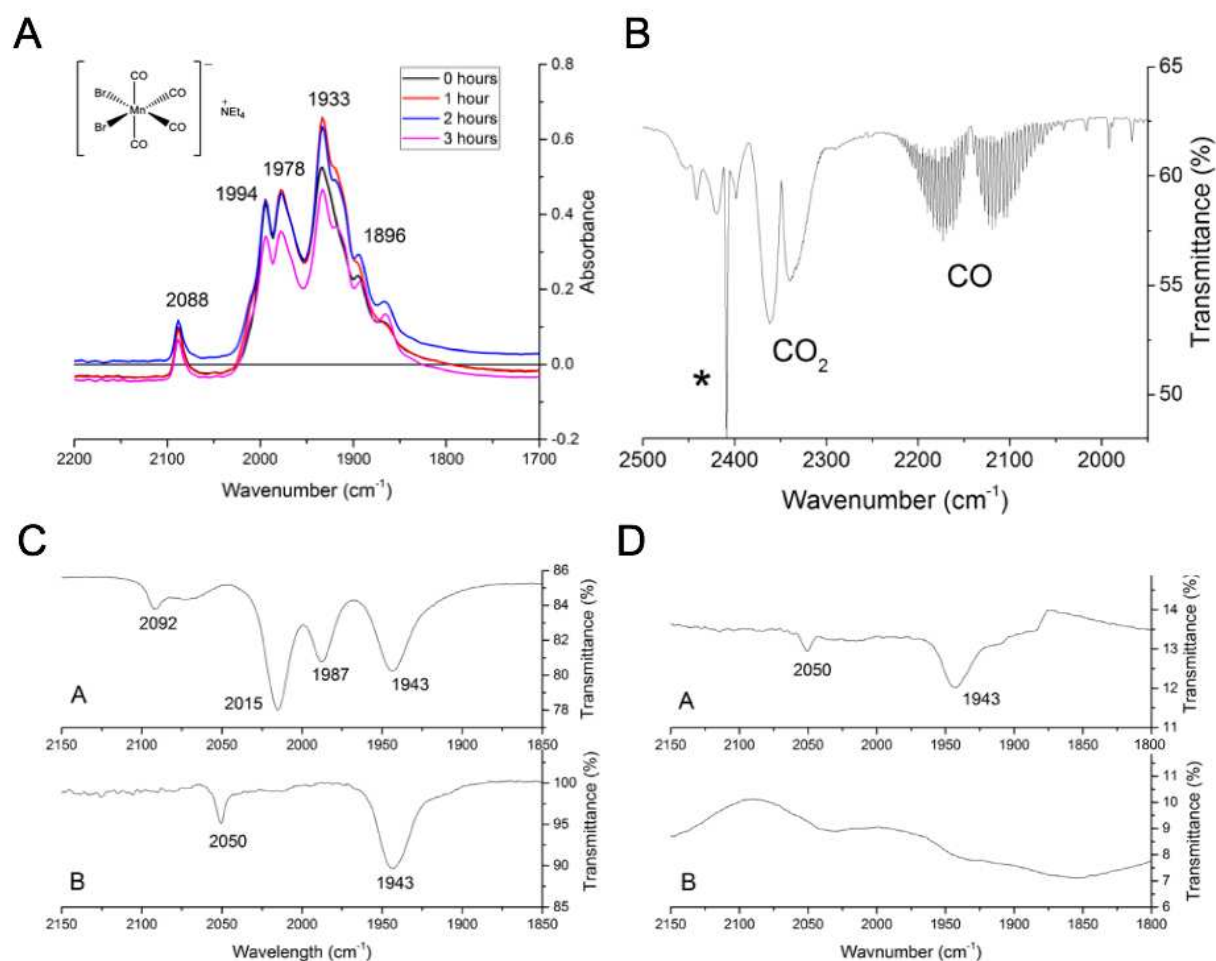
Figure 1. The stability of1 EBOR-CORM-1. Panel A shows the IR spectra of EBOR-CORM-1 upon heating at 50° C for 0 hours (black line), for 1 hours (red line), for 2 hours (blue) and for 3 hours (pink). The structure of the EBOR-CORM-1 is shown in inset on the left. Panel B shows the gas phase IR spectra of EBOR-CORM-1 in chloroform with added water where the * indicates a band from chloroform. Panel C shows the IR spectra of EBOR-CORM-1 in chloroform (top) and water (bottom) and panel D the IR spectra of CO in water after 1 min (top) and 90 min (bottom) dissolution. All frequencies given are in cm⁻¹.

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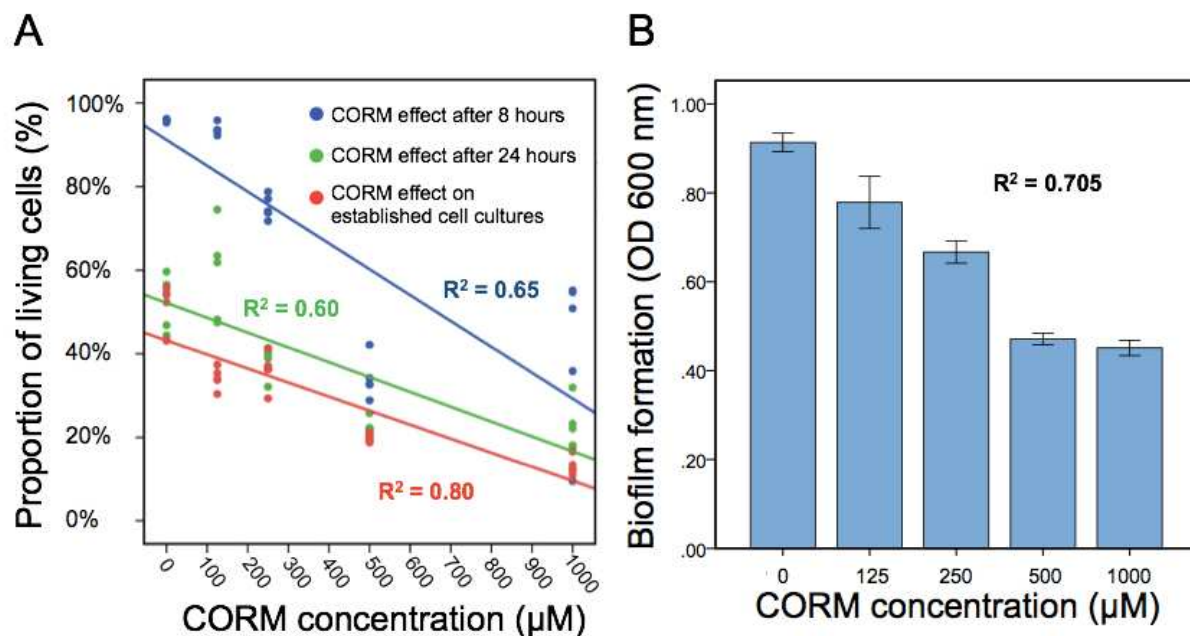
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503 **Figure 2.** The EBOR-CORM-1 effects on planktonic and biofilm cells of *P. aeruginosa*
504 PAO1. In panel A, different lines denote for cell densities after 8 hours (blue line) and 24
505 hours (green line) of EBOR-CORM-1 application and EBOR-CORM-1 effects on established
506 cell cultures (red line) in different CORM concentrations. Panel B shows EBOR-CORM-1
507 effects on PAO1 biofilms in different CORM concentrations. The R^2 denotes for the fit of
508 regression with our data, and in panel B, bars denote for ± 1 s.e.m.

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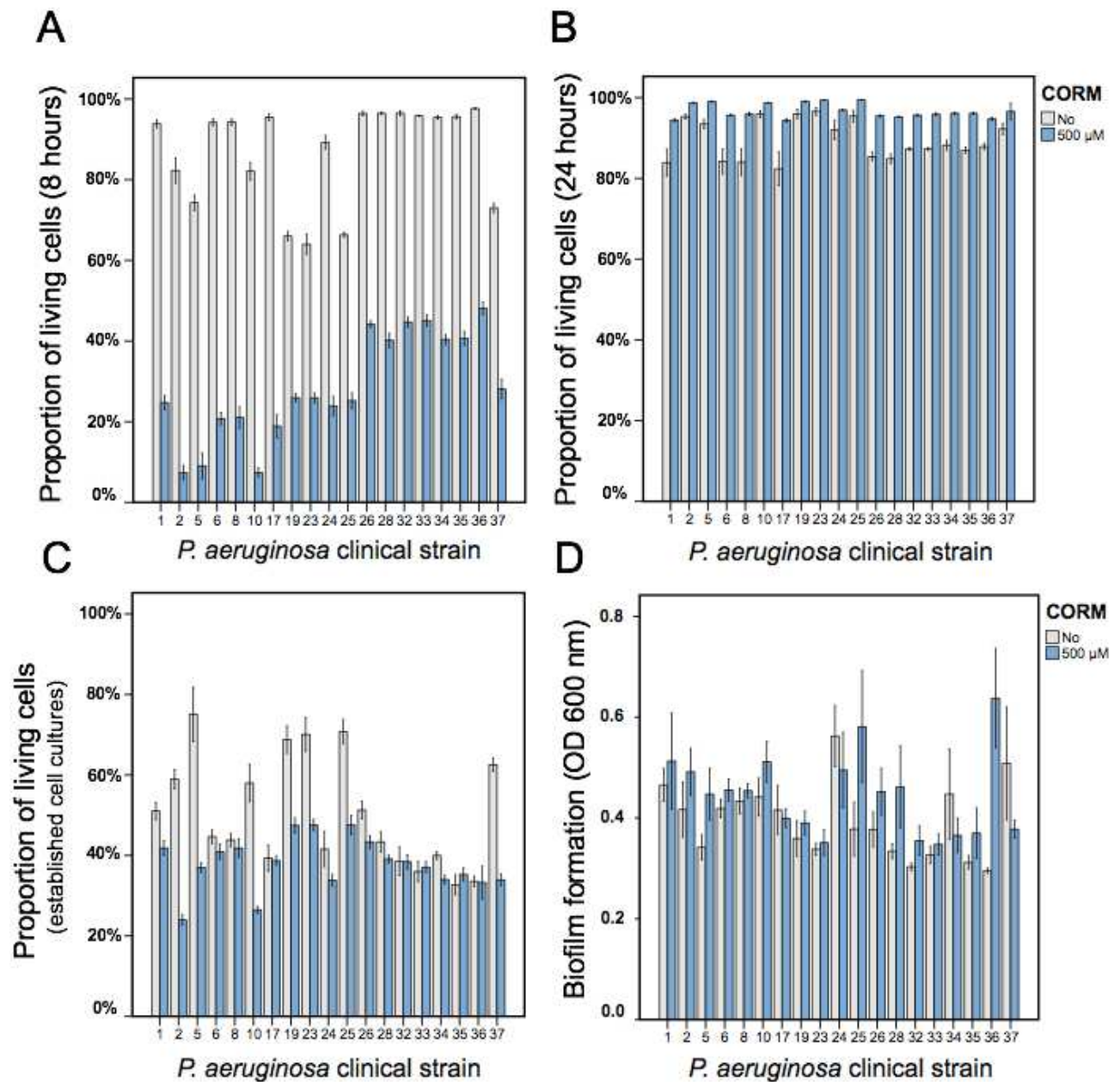
Figure 3. The EBOR-CORM-1 effects on planktonic and biofilm cells of clinical *P. aeruginosa* CF isolates. Panels A and B show the proportion of living cells after 8 hours and 24 hours of EBOR-CORM-1 application, respectively. Panel C and D show the EBOR-CORM-1 effects on established cell cultures and biofilms, respectively. In all panels, bars denote for ±1 s.e.m.

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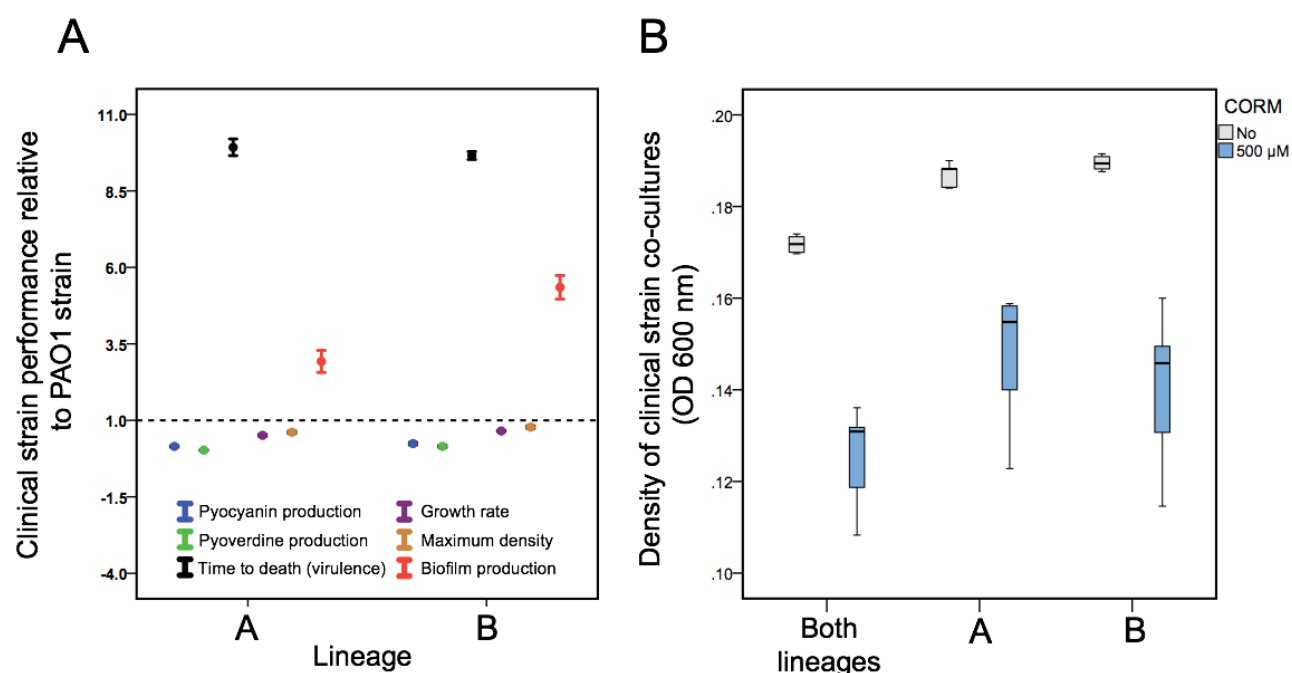
519   **Figure 4.** Differences in *P. aeruginosa* growth and virulence trait variation between PAO1
520   and clinical CF isolates (panel A) and EBOR-CORM-1 effects on clinical CF isolate lineage
521   mixes (panel B). In panel A, different colours denote for pyocyanin (blue) and pyoverdine
522   (green) production, time to death (black), growth rate (purple), maximum density (yellow)
523   and biofilm production for clinical isolates belonging to lineages A and B. The dashed line
524   shows the mean performance of PAO1 strain. Panel B shows EBOR-CORM-1 effect on
525   clinical CF isolate mixes after 24 hours of CORM application. In panel A, bars denote for ±1
526   s.e.m., and in panel B, extreme values around lower and upper quartile (black line shows the
527   median).

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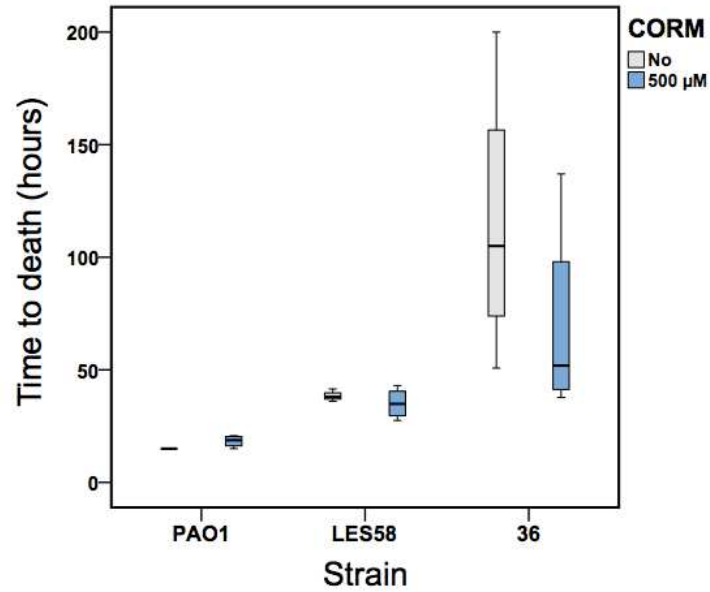
Figure 5. The EBOR-CORM-1 activity against three *P. aeruginosa* strains in wax moth
model. Boxplots show larval survival in the absence (light grey) and presence (blue) of
EBOR-CORM-1 for PAO1, LESB58 and clinical isolate #36 (lineage B). Bars show extreme
values around lower and upper quartile and black lines show the median.

REFERENCES

Angelici, R.J. (1964). Preparation, Characterization, and Reactions of the cis-Dihalotetracarbonylmanganate (I) Anions. *Inorganic Chemistry* 3**,** 1099-1102.

Brown, E.D., and Wright, G.D. (2016). Antibacterial drug discovery in the resistance era. *Nature* 529**,** 336-343.

Cochran, W., Mcfeters, G., and Stewart, P. (2000). Reduced susceptibility of thin Pseudomonas aeruginosa biofilms to hydrogen peroxide and monochloramine. *Journal of applied microbiology* 88**,** 22-30.

Cornforth, D.M., and Foster, K.R. (2013). Competition sensing: the social side of bacterial stress responses. *Nature Reviews Microbiology* 11**,** 285-293.

De Beer, D., Stoodley, P., and Lewandowski, Z. (1997). Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy. *Biotechnology and Bioengineering* 53**,** 151-158.

De Beer, D., Stoodley, P., Roe, F., and Lewandowski, Z. (1994). Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnology and bioengineering* 43**,** 1131-1138.

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., and Boczkowski, J. (2009). 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.

El-Sayed, M., and Kaesz, H. (1963). Infrared Spectra and Structure of the Tetracarbonyl Halide Dimers of Manganese, Technetium, and Rhenium. *Inorganic Chemistry* 2**,** 158-162.

Flume, P.A., Mogayzel, P.J., Robinson, K.A., Rosenblatt, R.L., Quittell, L., and Marshall, B.C. (2010). Cystic Fibrosis Pulmonary Guidelines. *American Journal of Respiratory and Critical Care Medicine* 182**,** 298-306.

Folkesson, A., Jelsbak, L., Yang, L., Johansen, H.K., Ciofu, O., Hoiby, N., and Molin, S. (2012). Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. *Nature Reviews Microbiology* 10**,** 841-851.

Ghoul, M., West, S.A., Johansen, H.K., Molin, S., Harrison, O.B., Maiden, M.C., Jelsbak, L., Bruce, J.B., and Griffin, A.S. (2015). Bacteriocin-mediated competition in cystic fibrosis lung infections. *Proc Biol Sci* 282.

Harrison, F., and Diggle, S.P. (2016). An ex vivo lung model to study bronchioles infected with Pseudomonas aeruginosa biofilms. *Microbiology* 162**,** 1755-1760.

Harrison, F., Muruli, A., Higgins, S., and Diggle, S.P. (2014). Development of an ex vivo porcine lung model for studying growth, virulence, and signaling of Pseudomonas aeruginosa. *Infection and immunity* 82**,** 3312-3323.

Harrison, F., Paul, J., Massey, R.C., and Buckling, A. (2008). Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. *ISME J* 2**,** 49-55.

Jimenez, J., Chakraborty, I., Carrington, S.J., and Mascharak, P.K. (2016). Light-triggered CO delivery by a water-soluble and biocompatible manganese photoCORM. *Dalton Trans* 45**,** 13204-13213.

Ketola, T., Mikonranta, L., Laakso, J., and Mappes, J. (2016). Different food sources elicit fast changes to bacterial virulence. *Biol Lett* 12**,** 20150660.

Klein, M., Neugebauer, U., Gheisari, A., Malassa, A., Jazzazi, T.M., Froehlich, F., Westerhausen, M., Schmitt, M., and Popp, J.R. (2014). IR spectroscopic methods for the investigation of the CO release from CORMs. *The Journal of Physical Chemistry A* 118**,** 5381-5390.

582   Lu, S., Suharti, De Vries, S., and Moënne-Loccoz, P. (2004). Two CO Molecules Can Bind
583   Concomitantly at the Diiron Site of NO Reductase from Bacillus a zotoformans.
584   *Journal of the American Chemical Society* 126**,** 15332-15333.

585   Marvig, R.L., Johansen, H.K., Molin, S., and Jelsbak, L. (2013). Genome analysis of a
586   transmissible lineage of pseudomonas aeruginosa reveals pathoadaptive mutations
587   and distinct evolutionary paths of hypermutators. *PLoS Genet* 9**,** e1003741.

588   Motterlini, R., and Otterbein, L.E. (2010). The therapeutic potential of carbon monoxide. *Nat*
589   *Rev Drug Discov* 9**,** 728-743.

590   Mowat, E., Paterson, S., Fothergill, J.L., Wright, E.A., Ledson, M.J., Walshaw, M.J.,
591   Brockhurst, M.A., and Winstanley, C. (2011). Pseudomonas aeruginosa population
592   diversity and turnover in cystic fibrosis chronic infections. *Am J Respir Crit Care*
593   *Med* 183**,** 1674-1679.

594   Murray, T.S., Okegbe, C., Gao, Y., Kazmierczak, B.I., Motterlini, R., Dietrich, L.E., and
595   Bruscia, E.M. (2012). The carbon monoxide releasing molecule CORM-2 attenuates
596   Pseudomonas aeruginosa biofilm formation. *PLoS One* 7**,** e35499.

597   Nobre, L.S., Seixas, J.D., Romao, C.C., and Saraiva, L.M. (2007). Antimicrobial action of
598   carbon monoxide-releasing compounds. *Antimicrob Agents Chemother* 51**,** 4303-
599   4307.

600   Normark, B.H., and Normark, S. (2002). Evolution and spread of antibiotic resistance.
601   *Journal of internal medicine* 252**,** 91-106.

602   O'brien, S., Williams, D., Fothergill, J.L., Paterson, S., Winstanley, C., and Brockhurst, M.A.
603   (2017). High virulence sub-populations in Pseudomonas aeruginosa long-term cystic
604   fibrosis airway infections. *BMC Microbiol* 17**,** 30.

605   O'neill, J. (2014). "Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of
606   Nations", in: *Review on Antimicrobial Resistance.* HM Government & Wellcome
607   Trust).

608   Poole, K. (2011). Pseudomonas aeruginosa: resistance to the max. *Front Microbiol* 2**,** 65.

609   Pritt, B., O'brien, L., and Winn, W. (2007). Mucoid Pseudomonas in cystic fibrosis. *Am J*
610   *Clin Pathol* 128**,** 32-34.

611   Reszka, K.J., O'malley, Y., Mccormick, M.L., Denning, G.M., and Britigan, B.E. (2004).
612   Oxidation of pyocyanin, a cytotoxic product from Pseudomonas aeruginosa, by
613   microperoxidase 11 and hydrogen peroxide. *Free Radic Biol Med* 36**,** 1448-1459.

614   Rumbaugh, K.P., Trivedi, U., Watters, C., Burton-Chellew, M.N., Diggle, S.P., and West,
615   S.A. (2012). Kin selection, quorum sensing and virulence in pathogenic bacteria. *Proc*
616   *Biol Sci* 279**,** 3584-3588.

617   Smith, E.E., Buckley, D.G., Wu, Z., Saenphimmachak, C., Hoffman, L.R., D'argenio, D.A.,
618   Miller, S.I., Ramsey, B.W., Speert, D.P., Moskowitz, S.M., Burns, J.L., Kaul, R., and
619   Olson, M.V. (2006). Genetic adaptation by Pseudomonas aeruginosa to the airways of
620   cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the*
621   *United States of America* 103**,** 8487-8492.

622   Stamellou, E., Storz, D., Botov, S., Ntasis, E., Wedel, J., Sollazzo, S., Kramer, B.K., Van
623   Son, W., Seelen, M., Schmalz, H.G., Schmidt, A., Hafner, M., and Yard, B.A. (2014).
624   Different design of enzyme-triggered CO-releasing molecules (ET-CORMs) reveals
625   quantitative differences in biological activities in terms of toxicity and inflammation.
626   *Redox Biol* 2**,** 739-748.

627   Stewart, P.S., and William Costerton, J. (2001). Antibiotic resistance of bacteria in biofilms.
628   *The Lancet* 358**,** 135-138.

629   Tinajero-Trejo, M., Rana, N., Nagel, C., Jesse, H.E., Smith, T.W., Wareham, L.K., Hippler,
630   M., Schatzschneider, U., and Poole, R.K. (2016). Antimicrobial Activity of the
631   Manganese Photoactivated Carbon Monoxide-Releasing Molecule [Mn (CO) 3 (tpa-
632   κ3 N)]+ Against a Pathogenic Escherichia coli that Causes Urinary Infections.
633   *Antioxidants & redox signaling* 24**,** 765-780.

Van Dommelen, S.M., Vader, P., Lakhal, S., Kooijmans, S., Van Solinge, W.W., Wood, M.J., and Schiffelers, R.M. (2012). Microvesicles and exosomes: opportunities for cell-derived membrane vesicles in drug delivery. *Journal of Controlled Release* 161**,** 635-644.

Ward, J.S., Lynam, J.M., Moir, J., and Fairlamb, I.J. (2014). Visible‑Light‑Induced CO Release from a Therapeutically Viable Tryptophan‑Derived Manganese (I) Carbonyl (TryptoCORM) Exhibiting Potent Inhibition against E. coli. *Chemistry-A European Journal* 20**,** 15061-15068.

Ward, J.S., Morgan, R., Lynam, J.M., Fairlamb, I.J., and Moir, J.W. (2017). Toxicity of tryptophan manganese (I) carbonyl (Trypto-CORM), against Neisseria gonorrhoeae. *MedChemComm* 8**,** 346-352.

Wareham, L.K., Poole, R.K., and Tinajero-Trejo, M. (2015). CO-releasing Metal Carbonyl Compounds as Antimicrobial Agents in the Post-antibiotic Era. *J Biol Chem* 290**,** 18999-19007.

Wasser, I.M., Huang, H.-W., Moënne-Loccoz, P., and Karlin, K.D. (2005). Heme/non-heme diiron (II) complexes and O2, CO, and NO adducts as reduced and substrate-bound models for the active site of bacterial nitric oxide reductase. *Journal of the American Chemical Society* 127**,** 3310-3320.

Who.Int (2010). *WHO | Genes and human disease* [Online]. Available: http://www.who.int/genomics/public/geneticdiseases/en/index2.html [Accessed 16-02 2017].

Williams, D., Evans, B., Haldenby, S., Walshaw, M.J., Brockhurst, M.A., Winstanley, C., and Paterson, S. (2015). Divergent, coexisting Pseudomonas aeruginosa lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med* 191**,** 775-785.

Williams, D., Paterson, S., Brockhurst, M.A., and Winstanley, C. (2016). Refined analyses suggest that recombination is a minor source of genomic diversity in Pseudomonas aeruginosa chronic cystic fibrosis infections. *Microbial Genomics* 2.

Wilson, J.L., Wareham, L.K., Mclean, S., Begg, R., Greaves, S., Mann, B.E., Sanguinetti, G., and Poole, R.K. (2015). CO-Releasing Molecules Have Nonheme Targets in Bacteria: Transcriptomic, Mathematical Modeling and Biochemical Analyses of CORM-3 [Ru(CO)3Cl(glycinate)] Actions on a Heme-Deficient Mutant of Escherichia coli. *Antioxid Redox Signal* 23**,** 148-162.

Winstanley, C., Langille, M.G., Fothergill, J.L., Kukavica-Ibrulj, I., Paradis-Bleau, C., Sanschagrin, F., Thomson, N.R., Winsor, G.L., Quail, M.A., Lennard, N., Bignell, A., Clarke, L., Seeger, K., Saunders, D., Harris, D., Parkhill, J., Hancock, R.E., Brinkman, F.S., and Levesque, R.C. (2009). Newly introduced genomic prophage islands are critical determinants of in vivo competitiveness in the Liverpool Epidemic Strain of Pseudomonas aeruginosa. *Genome Res* 19**,** 12-23.

Winstanley, C., O'brien, S., and Brockhurst, M.A. (2016). Pseudomonas aeruginosa Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. *Trends Microbiol* 24**,** 327-337.

Workentine, M.L., Sibley, C.D., Glezerson, B., Purighalla, S., Norgaard-Gron, J.C., Parkins, M.D., Rabin, H.R., and Surette, M.G. (2013). Phenotypic heterogeneity of Pseudomonas aeruginosa populations in a cystic fibrosis patient. *PLoS One* 8**,** e60225.

Zhang, W.-Q., Atkin, A.J., Thatcher, R.J., Whitwood, A.C., Fairlamb, I.J., and Lynam, J.M. (2009). Diversity and design of metal-based carbon monoxide-releasing molecules (CO-RMs) in aqueous systems: revealing the essential trends. *Dalton Transactions***,** 4351-4358.