University of Wollongong
Research Online

Illawarra Health and Medical Research Institute

Faculty of Science, Medicine and Health

2017

Cephalosporin-NO-donor prodrug PYRRO-C3D shows β-lactam-mediated activity against Streptococcus pneumoniae biofilms

Raymond Allan
University Hospital Southampton, University of Southampton

Michael J. Kelso
University of Wollongong, mkelso@uow.edu.au

Ardeshir Rineh
University of Wollongong, ar931@uowmail.edu.au

Nageshwar Rao Yepuri
University of Wollongong, yepuri@uow.edu.au

Martin Feelisch
University of Southampton, University Hospital Southampton

See next page for additional authors

Cephalosporin-NO-donor prodrug PYRRO-C3D shows β-lactam-mediated activity against Streptococcus pneumoniae biofilms

Abstract

Bacterial biofilms show high tolerance towards antibiotics and are a significant problem in clinical settings where they are a primary cause of chronic infections. Novel therapeutic strategies are needed to improve anti-biofilm efficacy and support reduction in antibiotic use. Treatment with exogenous nitric oxide (NO) has been shown to modulate bacterial signaling and metabolic processes that render biofilms more susceptible to antibiotics. We previously reported on cephalosporin-3'-diazeniumdiolates (C3Ds) as NO-donor prodrugs designed to selectively deliver NO to bacterial infection sites following reaction with β-lactamases. With structures based on cephalosporins, C3Ds could, in principal, also be triggered to release NO following β-lactam cleavage mediated by transpeptidases/penicillin-binding proteins (PBPs), the antibacterial target of cephalosporin antibiotics. Transpeptidase-reactive C3Ds could potentially show both NO-mediated anti-biofilm properties and intrinsic (β-lactam-mediated) antibacterial effects. This dual-activity concept was explored using *Streptococcus pneumoniae*, a species that lacks β-lactamases but relies on transpeptidases for cell-wall synthesis. Treatment with PYRRO-C3D (a representative C3D containing the diazeniumdiolate NO donor PYRRO-NO) was found to significantly reduce viability of planktonic and biofilm pneumococci, demonstrating that C3Ds can elicit direct, cephalosporin-like antibacterial activity in the absence of β-lactamases. While NO release from PYRRO-C3D in the presence of pneumococci was confirmed, the anti-pneumococcal action of the compound was shown to arise exclusively from the β-lactam component and not through NO-mediated effects. The compound showed similar potency to amoxicillin against S. *pneumoniae* biofilms and greater efficacy than azithromycin, highlighting the potential of C3Ds as new agents for treating pneumococcal infections.

Disciplines
Medicine and Health Sciences

Authors
Raymond Allan, Michael J. Kelso, Ardeshir Rineh, Nageshwar Rao Yepuri, Martin Feelisch, Odel Soren, Sanjita Brito-Mutunayagam, Rami Salib, Paul Stoodley, Stuart Clarke, Jeremy Webb, Luanne Hall-Stoodley, and Saul Faust

1   **Cephalosporin-NO-donor prodrug PYRRO-C3D shows β-lactam-mediated activity**

2   **against *Streptococcus pneumoniae* biofilms**

3

4   Raymond N. Allan[a,b], Michael J. Kelso[c], Ardeshir Rineh[c], Nageshwar R. Yepuri[c], Martin

5   Feelisch[a,d], Odel Soren[e], Sanjita Brito-Mutunayagam[a], Rami J. Salib[a,d,f], Paul Stoodley[e,g],

6   Stuart C. Clarke[a,d], Jeremy S. Webb[d,e], Luanne Hall-Stoodley[a,g] & Saul N. Faust[a,b,d]#

7

8   [a]Clinical and Experimental Sciences, Faculty of Medicine and Institute for Life Sciences,

9   University of Southampton, Southampton, UK

10   [b]Southampton NIHR Wellcome Trust Clinical Research Facility, University Hospital

11   Southampton NHS Foundation Trust, Southampton, UK

12   [c]Illawarra Health and Medical Research Institute, School of Chemistry, University of

13   Wollongong, Wollongong, NSW, Australia

14   [d]Southampton NIHR Respiratory Biomedical Research Unit, University Hospital

15   Southampton NHS Foundation Trust, Southampton, UK

16   [e]Biological Sciences and Institute for Life Sciences, University of Southampton,

17   Southampton, UK

18   [f]University Hospital Southampton NHS Foundation Trust, Southampton, UK

19   [g]Department of Microbial Infection and Immunity, Centre for Microbial Interface Biology,

20   College of Medicine, The Ohio State University, Columbus, Ohio, USA

21

22   #Address correspondence to Saul Faust, s.faust@soton.ac.uk

23

24   E.mail addresses:

25   Raymond N. Allan (r.allan@soton.ac.uk)

26   Michael J. Kelso (mkelso@uow.edu.au)

27   Ardeshir Rineh (arineh@uow.edu.au)

28   Nageshwar R. Yepuri (nageshwar.yepuri@ansto.gov.au)

29   Martin Feelisch (M.Feelisch@soton.ac.uk)

30   Odel Soren (os3c14@soton.ac.uk)

31   Sanjita Brito-Mutunayagam (sbm1g10@soton.ac.uk)

32   Rami J. Salib (Rami.Salib@uhs.nhs.uk)

33   Paul Stoodley (Paul.Stoodley@osumc.edu)

34   Stuart C. Clarke (S.C.Clarke@soton.ac.uk)

35   Jeremy S. Webb (J.S.Webb@soton.ac.uk)

36   Luanne Hall-Stoodley (Luanne.Hall-Stoodley@osumc.edu)

37   Saul N. Faust (s.faust@soton.ac.uk)

38

39

40

41

42

43

44

45

46

47

48

49

50

51 **Abstract**

52 Bacterial biofilms show high tolerance towards antibiotics and are a significant problem in

53 clinical settings where they are a primary cause of chronic infections. Novel therapeutic

54 strategies are needed to improve anti-biofilm efficacy and support reduction in antibiotic use.

55 Treatment with exogenous nitric oxide (NO) has been shown to modulate bacterial signaling

56 and metabolic processes that render biofilms more susceptible to antibiotics. We previously

57 reported on cephalosporin-3'-diazeniumdiolates (C3Ds) as NO-donor prodrugs designed to

58 selectively deliver NO to bacterial infection sites following reaction with β-lactamases. With

59 structures based on cephalosporins, C3Ds could, in principal, also be triggered to release NO

60 following β-lactam cleavage mediated by transpeptidases/penicillin-binding proteins (PBPs),

61 the antibacterial target of cephalosporin antibiotics. Transpeptidase-reactive C3Ds could

62 potentially show both NO-mediated anti-biofilm properties and intrinsic (β-lactam-mediated)

63 antibacterial effects. This dual-activity concept was explored using *Streptococcus*

64 *pneumoniae*, a species that lacks β-lactamases but relies on transpeptidases for cell-wall

65 synthesis. Treatment with PYRRO-C3D (a representative C3D containing the

66 diazeniumdiolate NO donor PYRRO-NO) was found to significantly reduce viability of

67 planktonic and biofilm pneumococci, demonstrating that C3Ds can elicit direct,

68 cephalosporin-like antibacterial activity in the absence of β-lactamases. While NO release

69 from PYRRO-C3D in the presence of pneumococci was confirmed, the anti-pneumococcal

70 action of the compound was shown to arise exclusively from the β-lactam component and not

71 through NO-mediated effects. The compound showed similar potency to amoxicillin against

72 *S. pneumoniae* biofilms and greater efficacy than azithromycin, highlighting the potential of

73 C3Ds as new agents for treating pneumococcal infections.

74

Graphical Abstract
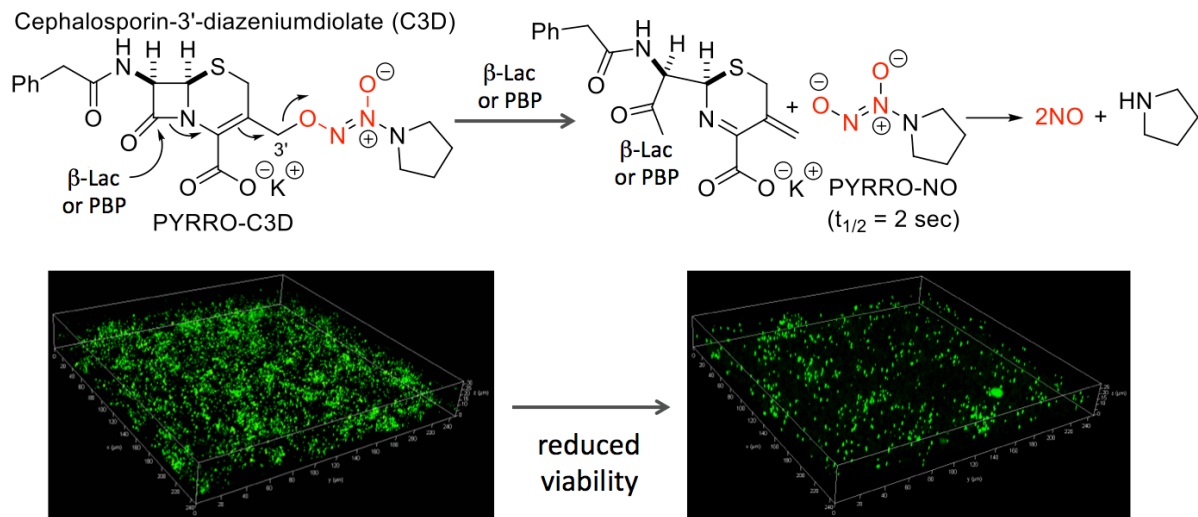
Highlights

- PYRRO-C3D demonstrates direct antibacterial activity against pneumococcal biofilms
- NO release is mediated through interaction with penicillin-binding proteins
- C3Ds are effective against bacteria lacking the capacity for β-lactamase production

1. Introduction

Bacterial biofilms are widely acknowledged as a significant problem in chronic clinical infections due to their increased antibiotic tolerance compared to planktonic (free-living) bacteria and their propensity to acquire antimicrobial resistance (AMR). These diverse bacterial communities have evolved multiple mechanisms that contribute to tolerance. Adaptive responses, including increased expression of efflux pumps and β-lactamases, along with restricted diffusion of antibiotics through the biofilm matrix, all confer tolerance. However, it is the presence of metabolically dormant cells that potentially plays the major role[1; 2; 3]. Nutrient gradients within biofilms can result in a proportion of the bacterial population adopting a metabolically dormant state, creating 'persister' cells that are highly tolerant towards antibiotics targeting bacterial growth and reproduction. Biofilm formation has also been implicated in the development of increased resistance through heightened mutation frequency and horizontal gene transfer[2]. Novel therapeutic strategies that overcome antimicrobial tolerance responses, limit development of AMR and reduce reliance upon conventional antibiotics are needed to create effective new treatments for biofilm-mediated chronic infections.

Nitric oxide (NO) is an ubiquitous signaling molecule across eukaryotic and prokaryotic systems. The presence of low concentrations of exogenous NO has been shown to modulate a range of functions in several bacterial species, such as toxin biosynthesis and protection from oxidative stress[4; 5]. Low NO concentrations also play an important role in bacterial biofilm biology, where they have been shown to signal a dispersal response in a broad range of species, including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli[6]*. In *Streptococcus pneumoniae*, NO treatment of established biofilms was recently shown to influence metabolism and translational activity, modulating both towards levels observed in the planktonic phenotype[7]. Use of NO as adjunctive therapy in

118 combination with conventional antibiotics has thus emerged as a possible anti-biofilm

119 strategy because the NO-mediated transition from biofilm to planktonic states renders

120 bacterial cells more susceptible to antibiotic treatments[7; 8; 9].

121   Whilst effective in signaling biofilm dispersal and eliciting other anti-biofilm

122 responses *in vitro*, clinical implementation of adjunctive NO therapy with antibiotics in

123 infectious diseases presents several challenges: (a) NO in gaseous form could only be used

124 for a limited range of infections (e.g. body surface and bronchopulmonary infections); (b) use

125 of NO donor compounds that spontaneously release NO in aqueous solution (e.g. sodium

126 nitroprusside, SNP) for internal infections would present significant toxicity risks due to

127 systemic exposure of the host to NO[10]; and (c) developing NO-donor/antibiotic

128 combinations is difficult due to divergent pharmacokinetics and other drug properties of the

129 two molecules. In addition, the lack of specificity towards bacteria and its short half-life

130 make NO treatment of biofilm infections challenging[6]. To address these issues, we are

131 investigating cephalosporin-3′-diazeniumdiolates (C3Ds) as novel, biofilm-activated NO-

132 donor prodrugs.

133   C3Ds contain a stabilized diazeniumdiolate NO-donor (NONOate) attached at the 3'-

134 position of early generation cephalosporins and were designed to selectively deliver NO to

135 biofilm infection sites following β-lactam ring cleavage mediated by bacterial β-lactamases.

136 It was envisaged that the compounds could be used as targeted NO carriers in combination

137 with conventional antibiotics to treat chronic, β-lactamase expressing, biofilm infections

138 (Figure 1)[11; 12]. We have previously reported that PYRRO-C3D increases the sensitivity

139 of non-typeable *Haemophilus influenzae* biofilms to treatment with azithromycin, a response

140 that was dependent on NO-release following β-lactamase cleavage[13]. It is conceivable,

141 however, that liberation of NO from C3Ds might also be triggered by reaction with

142 transpeptidases/penicillin-binding proteins (PBPs)[11], the molecular target of clinical

6

143 cephalosporin antibiotics, since the mechanism of β-lactam hydrolysis (and ensuing

144 elimination of the NONOate) by β-lactamases and transpeptidases would be identical (Figure

145 1). In addition to releasing NO and triggering anti-biofilm responses (including dispersion in

146 some species), reaction of transpeptidases with the β-lactam of C3Ds should, in principle,

147 also produce direct antibacterial effects. Dual-activity of this type would support thorough

148 exploration of C3Ds in a range of infectious disease indications as "all-in-one" anti-biofilm

149 cephalosporins that don't require co-administered antibiotics (Figure 1).

150
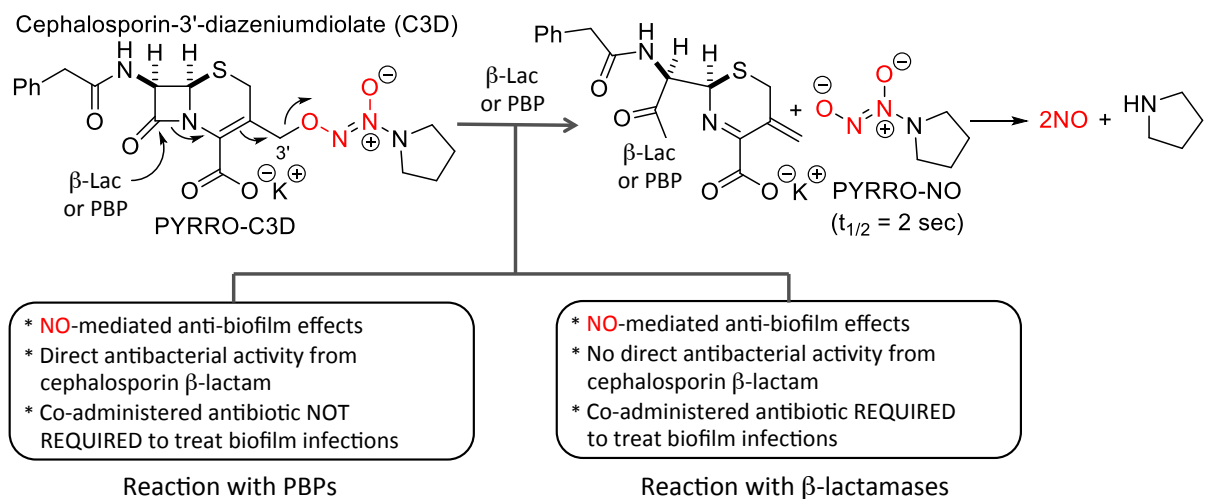
151

152 **Figure 1:** Mechanism of NO release from cephalosporin-3'-diazeniumdiolates (C3Ds, e.g.

153 PYRRO-C3D) and proposed effects arising from reaction with PBPs versus β-lactamases.

154

155   *S. pneumoniae* is a Gram-positive opportunistic pathogen and the causative agent of

156 various invasive infections, such as meningitis and pneumonia, as well as localized mucosal

157 infections (e.g. sinusitis and otitis media). Despite introduction of pneumococcal conjugate

158 vaccines, the clinical incidence of pneumococcal otitis media has stayed largely unchanged

159 due to serotype replacement, and otitis media remains a primary cause of antibiotic

160 prescription in children[14; 15; 16; 17; 18]. *S. pneumoniae* is also a non-β-lactamase-

161 producing organism that uses transpeptidases/PBPs in the construction of its cell wall[19].

162 Treatment with high concentrations of NO has been shown to produce antibacterial effects on

163 pneumococci when present as *in vitro* biofilms, on the surfaces of adenoid tissue samples *ex*

164 *vivo*, and in the lungs of mice that develop pneumonia following intranasal infection[7; 20].

165 We considered *S. pneumoniae* an excellent bacterial model to test whether a representative

166 C3D (i.e. PYRRO-C3D K^{+} salt, Figure 1) could show direct β-lactam-mediated antibacterial

167 activity (through reaction with PBPs) and NO-mediated anti-biofilm effects without

168 confounding effects from β-lactamases. This dual-activity concept was explored by

169 measuring the direct antibacterial effects of PYRRO-C3D on planktonic and biofilm *S.*

170 *pneumoniae*, and probing whether the observed responses were mediated by PBP inactivation

171 and/or NO.

172

173 **2. Material and Methods**

174 **2.1 Bacterial strains and growth conditions.**

175 A *S. pneumoniae* serotype 14 (ST124) clinical isolate[21] and a Serotype 2 strain (D39)

176 containing the plasmid pMV158GFP[22] were used in this study. Strains were subcultured

177 from frozen stocks onto Columbia blood agar (CBA) plates (Oxoid; PB0122), as described

178 previously[21]. Briefly, cultures were incubated at 37 ^{o}C/5% CO_2 and colonies re-suspended

179 in fresh Brain Heart Infusion (BHI) broth (Oxoid; CM1135) for use in experiments.

180

181 **2.2 *In vitro* planktonic experiments.**

182 Flat-bottomed 96-well culture plates (Fisher Scientific) were inoculated with 1.0 x 10^{7}

183 bacteria per well (mid-exponential planktonic cultures) grown in BHI. Stock solutions of

184 PYRRO-C3D, DEA/NO[7], and cephaloram (all 10 mM in dimethyl sulfoxide, DMSO) were

185 diluted in BHI and added to wells at final concentrations ranging from 9 nM − 90 μM.

186 Equivalent BHI volumes with1% DMSO were added in place of treatments for untreated

187   controls. Equivalent concentrations of PYRRO-C3D, DEA/NO and cephaloram alone (i.e. in

188   the absence of bacteria) were used to control for background absorbances. Cultures were

189   incubated at 37 $^{\circ}$C/5% CO_2 and the minimum inhibitory concentration (MIC) obtained by

190   measuring the absorbance (OD595) after 18 hours (EZ Read 400 spectrophotometer,

191   Biochrom) (n=3).

192

193   **2.3 *In vitro* biofilm experiments.**

194   Mid-exponential planktonic cultures grown in BHI were used to inoculate individual wells of

195   untreated polystyrene 6-well plates (1 x 10^8 cells per well) (Corning Incorporated, Costar).

196   Wells were supplemented with fresh BHI diluted 1:5 with distilled H_2O and the cultures

197   incubated at 37 $^{\circ}$C/5% CO_2 under static conditions for 48 h. Spent media was replaced with

198   warm, freshly diluted 1:5 BHI after 24 h. All assays were performed using 2 technical

199   replicates of 2 biological replicates (n=4). Prior to compound treatment, media was removed

200   and the biofilms washed twice with 1:5 diluted BHI. PYRRO-C3D, DEA/NO and

201   cephaloram stock solutions (10 mM in DMSO) were added to wells at final concentrations

202   ranging from 1 μM to 100 μM in 1:5 diluted BHI. Equivalent DMSO concentrations (1%)

203   were maintained for each treatment, including untreated controls. Carboxy-PTIO potassium

204   salt (cPTIO), clavulanic acid and penicillinase (all Sigma; C221, P3494 and P0389

205   respectively) were added at final concentrations of 50 μM, 250 μg/mL and 0.01 U/μL,

206   respectively. For antibiotic co-treatment experiments, amoxicillin and azithromycin (both

207   Sigma, A8523 and PZ0007 respectively) were added at final concentrations of 300 μg/mL

208   and 1 mg/mL, respectively. BHI diluted 1:5 with distilled water and containing an equivalent

209   concentration of DMSO to the treatment solutions (1%) was included as an untreated control.

210   Biofilms were incubated at 37 $^{\circ}$C/5% CO_2 for 2 hours, after which the treatments/media were

211   removed and the remaining biofilms rinsed twice with 1:5 diluted BHI. Biofilms were then

212 resuspended in Hank's balanced salt solution (HBSS), as previously described[23]. In brief,

213 biofilms were scraped and vortexed and the resuspended biofilms and supernatants diluted in

214 HBSS, spot-plated onto CBA plates and incubated at 37 ^{o}C/5% CO_2 for 18 hours before

215 enumerating colony-forming units (CFUs). Biofilm biomass was measured as previously

216 described[7].

217

218 **2.4 Measurements of nitric oxide release.**

219 NO release from PYRRO-C3D was measured using an ISO-NO probe (World Precision

220 Instruments) as per manufacturer's instructions. To quantify the amount of NO released from

221 PYRRO-C3D in the absence of bacterial cells, HBSS (pH 7.4) was maintained at 37 ± 0.5 ^{o}C

222 with stirring in a septum-sealed acrylic chamber and baseline NO levels were monitored over

223 5 min. PYRRO-C3D (100 μM) was then added and the NO signal recorded for 5 min before

224 adding 10 units of *Bacillus cereus* penicillinase (Sigma; P0389) and monitoring NO levels for

225 a further 120 min. To measure release of NO from PYRRO-C3D in the presence of

226 pneumococcal cells, the ISO-NO probe was submerged into the media and positioned directly

227 above 48 h serotype 14 biofilms (grown as described above). NO concentrations were

228 monitored over the ensuing 10 minutes to confirm no endogenous NO production, before

229 adding 100 μM PYRRO-C3D and recording the NO signal for a further 40 minutes.

230

231 **2.5 Confocal Laser Scanning Microscopy (CLSM).**

232 Mid-exponential planktonic cultures of serotype 2 strain D39 (containing the plasmid

233 pMV158GFP) were grown in BHI and used to inoculate 35 mm untreated glass bottom

234 CELLview cell culture dishes (Greiner Bio One). The dishes were supplemented with fresh

235 1:5 diluted BHI and biofilms grown under static conditions at 37 ^{o}C/5% CO_2 for 48 h,

236 replacing spent media with fresh 1:5 diluted BHI supplemented with 2 % maltose at 24 h (to

237   induce *gfp* expression). Biofilms were then treated with 100 μM PYRRO-C3D or 100 μM

238   DEA/NO in 1:5 diluted BHI + 2 % maltose, or 1:5 diluted BHI + 2 % maltose (untreated

239   control), at 37 $^{\circ}$C/5% CO_2 for 2 h. Treatments/media were removed and the remaining

240   biofilms rinsed twice with HBSS and stained with propidium iodide according to

241   manufacturer's instructions (ThermoFisher Scientific; P3566). Stained biofilms were

242   examined immediately using a Leica SP8 CLSM with inverted stand under a 63x oil

243   immersion lens, performing sequential scanning on 0.5 μm sections. The *gfp* fluorescence

244   intensity threshold was set to that of planktonic pneumococci to remove background

245   extracellular DNA staining. Images were analyzed using Leica LCS Software.

246

247   **2.6 Statistical analyses.**

248   Statistical analyses of *in vitro* planktonic and biofilm data were performed using non-

249   parametric Mann-Whitney t-tests. Comparative data reported as $p < 0.05$ were considered

250   statistically different.

251

252   **3. Results**

253   **3.1 PYRRO-C3D treatment reduces viability of planktonic and biofilm *S. pneumoniae*.**

254   NO release from PYRRO-C3D was examined first in the presence of a β-lactamase

255   (penicillinase) using the NO probe. PYRRO-C3D (100 μM) showed low-level release of NO

256   over 5 minutes after being added to HBSS (*p*H 7.4) at 37 ˚C. Subsequent addition of 10 units

257   of penicillinase caused a rapid spike of NO, reaching a peak concentration of 450 nM within

258   5 min, which was followed by a steady decline over 2 h, confirming that PYRRO-C3D

259   efficiently releases NO following β-lactam ring cleavage (Figure 2a).

260
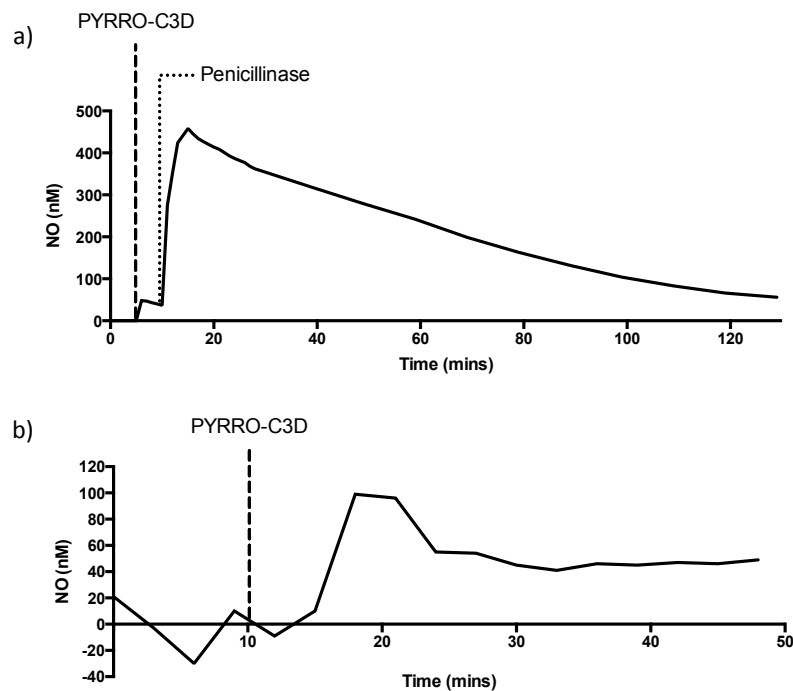
Figure 2: Release of NO from PYRRO-C3D. a) NO release from PYRRO-C3D (100 μM) was monitored following addition to HBSS (*p*H 7.4) at 37 ˚C. After 5 mins, 10 units of penicillinase were added, leading to release of NO from PYRRO-C3D. **b)** 48 h serotype 14 biofilms showed no detectable endogenous NO signal. Addition of 100 μM PYRRO-C3D to the biofilm triggered release of NO.

NO measurements on untreated 48 h serotype 14 pneumococcal biofilms showed no detectable endogenous NO signal (Figure 2b). Treatment with 100 μM PYRRO-C3D produced a peak of NO release (~100 nM) after 8 minutes, which was followed by a steady signal corresponding to ~45 nM NO. Detection of the NO signal in the presence of non-β-lactamase producing *S. pneumoniae* was consistent with PYRRO-C3D undergoing reaction with transpeptidases/PBPs to liberate PYRRO-NO (and NO).

Treatment of planktonic cultures with a range of PYRRO-C3D concentrations (9 nM − 90 μM) identified the MIC as 900 nM, confirming that the compound shows potent antibacterial activity against planktonic *S. pneumoniae* cells (Figure 3a). Equivalent

277 concentrations of cephaloram, the cephalosporin antibiotic closest in structure to PYRRO-

278 C3D whilst lacking an NO donor, showed identical activity (MIC = 900 nM). Treatment with

279 equivalent concentrations of the diazeniumdiolate-based spontaneous NO donor DEA/NO,

280 however, showed no effect on planktonic growth. Collectively, these findings are consistent

281 with PYRRO-C3D eliciting anti-pneumococcal effects through reaction of its cephalosporin

282 β-lactam with PBPs and that, although NO is released from the compound during this

283 process, it does not contribute directly to the antibacterial effect.

284
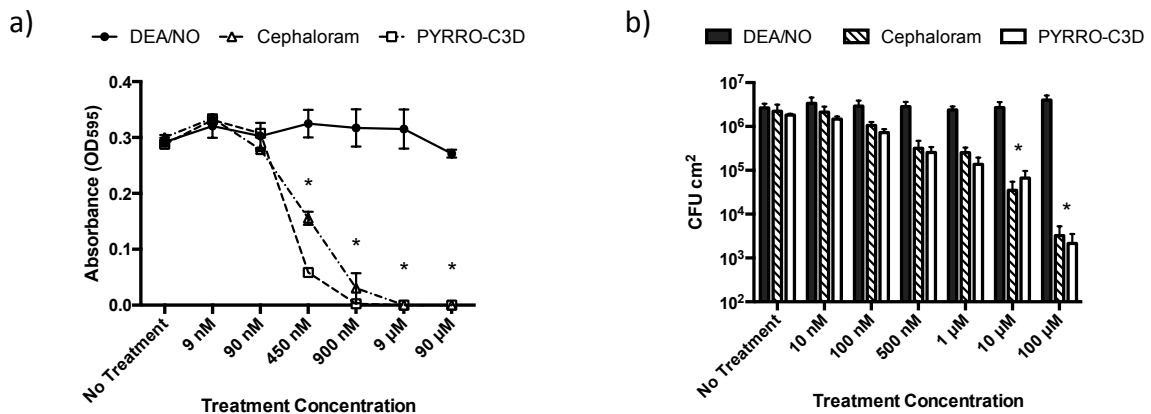
285

286 **Figure 3: Effects of DEA/NO, cephaloram and PYRRO-C3D on the viability of *in vitro***

287 ***S. pneumoniae* planktonic cells and biofilms. a)** Planktonic *S. pneumoniae* serotype 14

288 (ST124) cultures were treated with DEA/NO, cephaloram or PYRRO-C3D for 18 hours and

289 absorbance (OD595) was measured to determine the minimum inhibitory concentration. **b)** 48

290 h serotype 14 biofilms were treated with DEA/NO, cephaloram or PYRRO-C3D for 2 hours

291 before measuring cell viability in the remaining biofilm population. *p≤0.05.

292

293   A range of PYRRO-C3D concentrations (10 nM - 100 μM) were tested next against

294 mature (48 hour) *in vitro S. pneumoniae* biofilms. A two hour treatment time was

295 investigated based on previous studies that demonstrated i) the response of pneumococcal

296 biofilms to exogenous NO[7], and ii) the antimicrobial effect of PYRRO-C3D on non-

297   typeable *H. influenzae* biofilms[13] following 2 hour treatments. The treatment time was also

298   chosen based on the NO release profile of PYRRO-C3D whereby little measurable NO was

299   remaining after 2 hours following activation (Figure 2a). Biofilms were assessed for

300   pneumococcal viability by CFU enumeration showed a concentration-dependent response to

301   PYRRO-C3D, culminating in a 3-log reduction in biofilm CFUs at 100 μM (p=0.014) (Figure

302   3b). As seen in the planktonic phenotype, cephaloram showed identical activity to PYRRO-

303   C3D (3-log reduction in biofilm CFUs at 100 μM, p=0.029) and DEA/NO showed no effect

304   below 100 μM (p=0.49). A 4-log reduction was also observed in CFUs grown from the

305   supernatant surrounding PYRRO-C3D (100 μM) treated cells, compared to untreated controls

306   (p=0.029, data not shown).

307   CLSM imaging and biomass measurements of 48 hour biofilms formed by a GFP-

308   expressing serotype 2 strain (D39) showed no change in maximum biofilm height (p=0.57) or

309   total biomass (p=0.989) following treatment with either 100 μM DEA/NO or PYRRO-C3D

310   (Figure 4a & b), demonstrating that neither compound triggers an NO-mediated dispersal

311   response in pneumococcus. While this is in contrast to the robust dispersal responses seen

312   following C3D treatment of *P. aeruginosa* biofilms and NO treatment of biofilms from other

313   bacteria[8; 9], it is consistent with our recent results showing that NO modulates metabolic

314   activity but not dispersal in *S. pneumoniae* biofilms[7]. Similar to the CFU data, treatment

315   with DEA/NO showed no effect on biofilm viability, whereas a significant reduction in

316   biofilm viability was observed following PYRRO-C3D treatment (Figure 4c, - e).

317
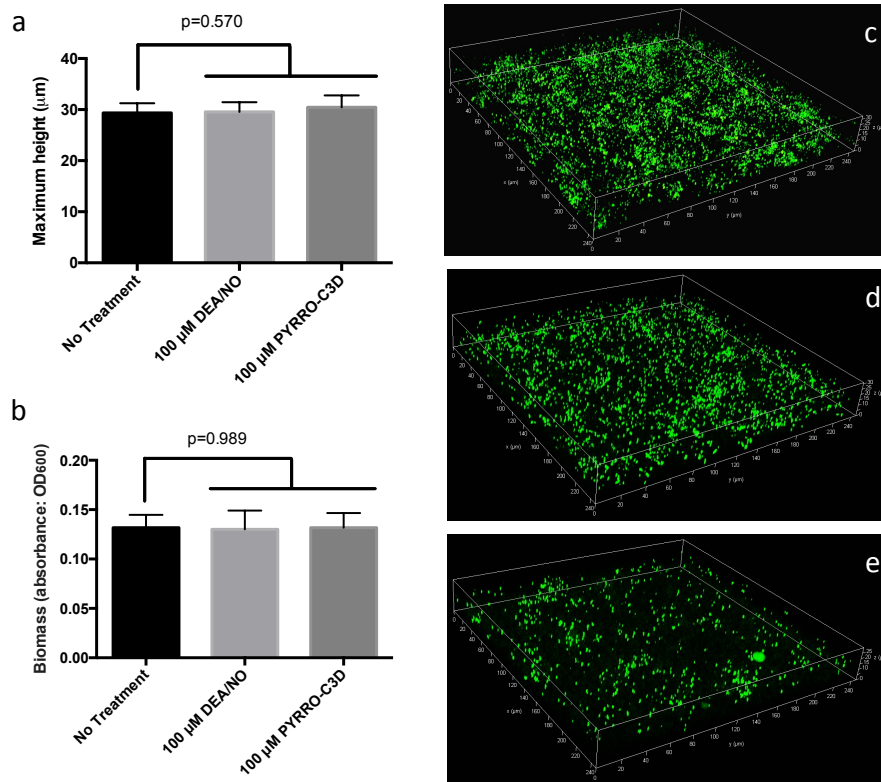
Figure 4: Effects of PYRRO-C3D on *S. pneumoniae* serotype 2 (D39) *in vitro* biofilms.

Established 48 h D39 biofilms expressing GFP were treated with 100 μM PYRRO-C3D or

DEA/NO for 2 hours and imaged using confocal microscopy. Biofilms were counterstained

with propidium iodide to distinguish dead cells from GFP-expressing viable cells (green).

Treatment with DEA/NO and PYRRO-C3D had no effect on either **a)** maximum biofilm

height, or **b)** biofilm biomass compared to untreated controls. Treatment with PYRRO-C3D,

and not DEA/NO, reduced the number of viable bacteria present within the biofilm **(c-e)**.

3.2 Activity of PYRRO-C3D against pneumococcal biofilms is exclusively mediated

through the cephalosporin β-lactam.

Having established that 100 μM PYRRO-C3D was effective in reducing

pneumococcal viability in biofilms (Figure 3), the treatment was repeated in the presence of

the β-lactamase inhibitor clavulanic acid (250 mg.mL^{-1}). No change in the response to

PYRRO-C3D was observed (p=0.929) (Fig. 5a), confirming that β-lactamases were playing

333   no part in the compound's activity. Treatment of biofilms with 100 μM PYRRO-C3D was

334   next repeated in the presence of the NO-scavenger cPTIO. Addition of 50 μM cPTIO, which

335   showed no effect on its own, did not change the activity of PYRRO-C3D (p=0.829, Fig. 5b),

336   confirming that the NO being released from PYRRO-C3D was having no effect. The effect of

337   PYRRO-C3D on *S. pneumoniae* viability was then assessed in the presence of 0.01 U/μL

338   penicillinase, the same β-lactamase shown to cleave the β-lactam of PYRRO-C3D and

339   liberate NO (Figure 2). Addition of penicillinase significantly reduced the activity of

340   PYRRO-C3D (p=0.0286, Fig. 5c). Together these data provide compelling evidence that

341   PYRRO-C3D produces direct activity against biofilm pneumococci via reaction of its

342   cephalosporin β-lactam with transpeptidases/PBPs only, and that subsequent release of NO

343   from the compound produces no measurable effect on cell viability.

344
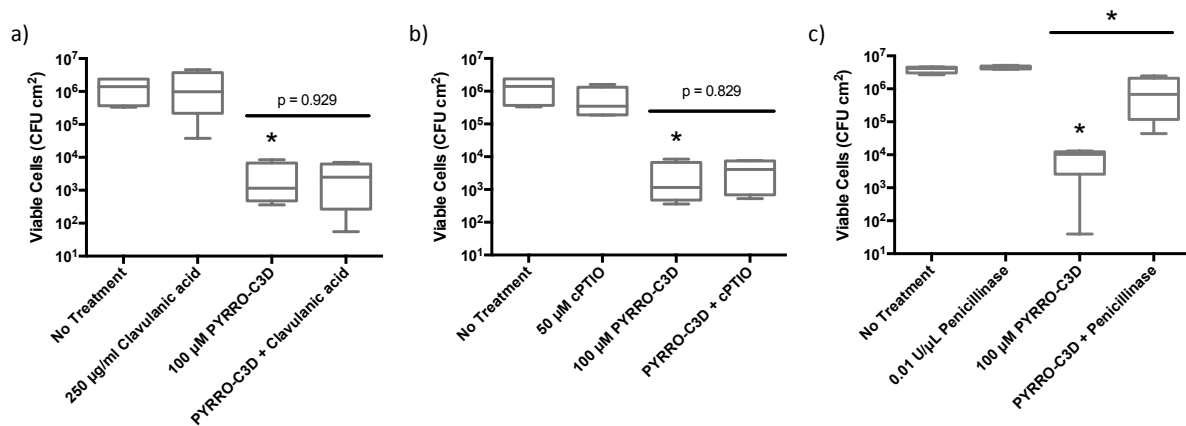
345

346   **Figure 5: Response of *S. pneumoniae* serotype 14 (ST124) *in vitro* biofilms to PYRRO-**

347   **C3D treatment in the presence of clavulanic acid, cPTIO and penicillinase.** 48 h *S.*

348   *pneumoniae* biofilms were treated with 100 μM PYRRO-C3D for 2 h in the presence of **a)**

349   250 μg/mL clavulanic acid, **b)** 50 μM cPTIO and **c)** 0.01 unit/μL penicillinase. Pneumococcal

350   viability in biofilms following treatment was assessed by CFU enumeration. *p≤0.05.

351

3.3 PYRRO-C3D shows similar activity to amoxicillin and is more active than azithromycin against pneumococcal biofilms.

The anti-biofilm activity of PYRRO-C3D was next compared to amoxicillin and azithromycin, two antibiotics commonly prescribed for the treatment of *S. pneumoniae* infections. Established pneumococcal serotype 14 and serotype 2 biofilms were treated for 2 hours with 100 μM PYRRO-C3D, supra-MIC concentrations of amoxicillin (300 μg/mL) or azithromycin (1 mg/mL), and bacterial viability was assessed by CFU enumeration. PYRRO-C3D and amoxicillin both produced 3-log reductions in viability against serotype 14 and 2-log reductions against serotype 2 (Fig. 6a & b). Treatment with azithromycin, a non-β-lactam (macrolide) antibiotic, showed no significant effects on serotype 14 (p=0.582) or serotype 2 (p=0.829) viability.
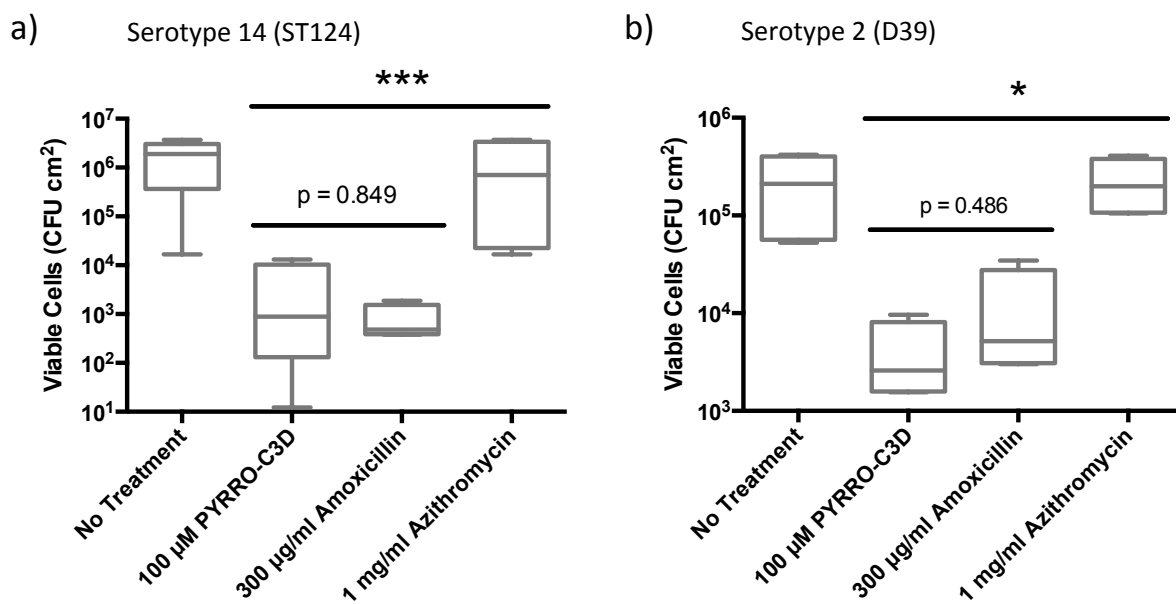
Figure 6: Comparison of the antibacterial activities of PYRRO-C3D, amoxicillin and azithromycin against *S. pneumoniae* serotype 14 (ST124) and serotype 2 (D39) *in vitro* biofilms. Established 48 h **a)** serotype 14 and **b)** serotype 2 biofilms were treated with 100

368 μM PYRRO-C3D, 300 μg/mL amoxicillin or 1 mg/mL azithromycin for 2 hours and assessed

369 for pneumococcal viability by CFU enumeration. *p≤0.05; ***p≤0.001.

370

371 **4. Discussion**

372   Previous studies showed that low levels of NO were released from our prototype C3D

373 (DEA-CP) in the presence of non-β-lactamase producing *E. coli* cell extracts[11] and it was

374 postulated that the NO release resulted from reaction of the compound with PBPs, the

375 enzymes responsible for cross-linking peptidoglycan chains during bacterial cell wall

376 synthesis. It is well known that cephalosporins and other β-lactam antibiotics elicit

377 antibacterial effects by covalently binding to the active sites of PBPs in a process that also

378 results in β-lactam ring cleavage[24]. This led us to speculate that reactions between PBPs

379 and C3Ds might elicit a direct, β-lactam-mediated antibacterial effect and in the process

380 liberate the NONOate (and NO) (Fig. 1). Since anti-biofilm effects of NO are now well

381 documented, we postulated that NO released from C3Ds following reaction with PBPs might

382 confer additional anti-biofilm activity.

383   We tested this dual-activity hypothesis using non-β-lactamase producing *S.*

384 *pneumoniae* strains that express five high molecular weight PBPs (1a, 1b, 2a, 2b and 2x) and

385 one low molecular weight PBP3[19]. The absence of β-lactamases ensured that NO released

386 from the compound must arise from an alternative mechanism, most likely PBP-mediated β-

387 lactam cleavage. The representative C3D selected for the study was PYRRO-C3D, a close

388 structural analogue of DEA-CP that carries PYRRO/NO ($t_{1/2}$ = 2 secs) as the NONOate

389 instead of DEA/NO ($t_{1/2}$ = 2 min)[25]. PYRRO-C3D was chosen for its faster NO release,

390 which we believe would be important for C3D use *in vivo* since diffusion of an expelled

391 NONOate away from infection sites (before releasing the NO cargo) would reduce

392 effectiveness and raise NO-mediated safety concerns.

393   The ability of PYRRO-C3D to release NO following β-lactam cleavage was

394   confirmed first by treating the compound with penicillinase and directly observing NO.

395   Release of NO from PYRRO-C3D in the presence of *S. pneumoniae* cells lacking β-

396   lactamase was demonstrated, consistent with *S. pneumoniae* PBPs hydrolysing the

397   compound's β-lactam and triggering release of NO. PYRRO-C3D was then shown to reduce

398   viability of both planktonic and biofilm *S. pneumoniae*, confirming that the compound shows

399   direct antibacterial activity against this bacterium. The level of activity was consistent with

400   the known tendency of biofilms to be less susceptible to antimicrobial treatments than their

401   planktonic counterparts[26; 27; 28], since treatment with 900 nM PYRRO-C3D completely

402   inhibited planktonic growth, whereas a significant reduction in biofilm viability (3-log)

403   required more than 100-fold higher concentrations.

404   β-lactamases were confirmed as playing no role in PYRRO-C3D's activity since no

405   difference was seen in the presence of the β-lactamase inhibitor clavulanic acid. Absence of

406   antibacterial activity when planktonic and biofilm cultures were treated with the spontaneous

407   NO-donor DEA/NO provided evidence that the effects of PYRRO-C3D against

408   pneumococcus are exclusively due to its cephalosporin β-lactam core and are not NO

409   mediated. We further observed that the cephalosporin equivalent of PYRRO-C3D lacking a

410   NONOate (i.e. cephaloram) showed identical activity to PYRRO-C3D, and that addition of

411   the NO-scavenger cPTIO failed to change PYRRO-C3D activity. Moreover, PYRRO-C3D

412   significantly reduced (4-log) the number of viable planktonic bacteria remaining in the

413   surrounding media, likely due to the direct antibacterial effect of PYRRO-C3D. Confocal

414   imaging and measurements of biomass showed that no significant reduction in biofilm

415   maximum height or biomass occurred following PYRRO-C3D treatment, but there was a

416   significant reduction in the number of viable bacteria remaining within biofilms, validating

417   the reduction in CFUs. These findings together were consistent with PYRRO-C3D acting

418   directly as a cephalosporin-like β-lactam antibiotic, a notion further supported by its reduced

419   activity in the presence of penicillinase.

420   Finally, the antibacterial activity of PYRRO-C3D towards pneumococcal biofilms

421   was compared with that of antibiotics commonly used to treat pneumococcal infections. We

422   found that PYRRO-C3D possessed similar antibacterial efficacy to amoxicillin against both

423   serotype 2 and 14 biofilms, which is perhaps not surprising given that the compounds are

424   structurally and functionally very similar, with both targeting PBP-mediated cell wall

425   synthesis. PYRRO-C3D was found to be much more effective than azithromycin, an

426   antibiotic that targets protein biosynthesis.

427   The findings presented here are consistent with our recent study, which showed that

428   high concentrations of NO (1 mM) are needed to elicit bactericidal effects or enhance

429   antibiotic efficacy against four different serotypes of pneumococcal *in vitro* biofilms[7]. The

430   current study demonstrated that PYRRO-C3D at 100 µM liberates maximum NO

431   concentrations of ~450 nM and 100 nM upon contact with penicillinase and pneumococcal

432   cells, respectively. It therefore seems likely that PYRRO-C3D does not release sufficient NO

433   when activated by PBPs to modulate pneumococcal biofilm metabolism towards the

434   planktonic phenotype *in vitro*. This may, however, not be the case in the upper respiratory

435   tract, for example, where the constitutive release of NO by host cells could have an

436   augmentative effect, as observed in our recent study where an anti-pneumococcal response to

437   100 µM NO was seen on host adenoid tissue[7].

438   In summary, this study demonstrated that a representative C3D (PYRRO-C3D)

439   releases NO and shows direct antibacterial effects against planktonic and biofilm forms of

440   non-β-lactamase producing *S. pneumoniae*. The activity was confirmed to arise exclusively

441   from β-lactam mediated reactions with *S. pneumoniae* PBPs, with no measurable contribution

442   coming from the released NO. In the treatment of pneumococcal biofilms, PYRRO-C3D was

443 found to be equally as effective as amoxicillin and more effective than azithromycin when

444 used alone.

445

446 **5. Conclusions**

447   Introduction of a diazeniumdiolate at the cephalosporin 3'-position was shown for the

448 first time to be structurally compatible with binding to the molecular target of β-lactam

449 antibiotics, PBPs. Medicinal chemistry tuning of the cephalosporin aminoacyl side chain and

450 diazeniumdiolate portions may identify C3Ds with PBP-mediated activity against other

451 species, and perhaps even broad-spectrum activity. While the study did not demonstrate that

452 PYRRO-C3D produces combined NO and β-lactam based anti-biofilm effects against *S.*

453 *pneumoniae,* it is possible that such dual-effects might be observed with C3Ds in other

454 species and with other analogues. Non-β-lactamase producing bacteria that undergo NO-

455 mediated biofilm dispersion would be of particular interest for future study.

456

466

467 **References**

468 [1] C.A. Fux, J.W. Costerton, P.S. Stewart, P. Stoodley, Survival strategies of infectious
469   biofilms, Trends Microbiol 13 (2005) 34-40.
470 [2] N. Hoiby, T. Bjarnsholt, M. Givskov, S. Molin, O. Ciofu, Antibiotic resistance of
471   bacterial biofilms, Int J Antimicrob Agents 35 (2010) 322-32.
472 [3] K. Lewis, Persister cells, dormancy and infectious disease, Nat Rev Microbiol 5
473   (2007) 48-56.
474 [4] B.R. Crane, J. Sudhamsu, B.A. Patel, Bacterial nitric oxide synthases, Annu Rev
475   Biochem 79 (2010) 445-70.
476 [5] I. Gusarov, E. Nudler, NO-mediated cytoprotection: instant adaptation to oxidative
477   stress in bacteria, Proc Natl Acad Sci U S A 102 (2005) 13855-60.
478 [6] N. Barraud, M.J. Kelso, S.A. Rice, S. Kjelleberg, Nitric oxide: a key mediator of biofilm
479   dispersal with applications in infectious diseases, Curr Pharm Des 21 (2015) 31-
480   42.
481 [7] R.N. Allan, S. Morgan, S. Brito-Mutunayagam, P. Skipp, M. Feelisch, S.M. Hayes, W.
482   Hellier, S.C. Clarke, P. Stoodley, A. Burgess, H. Ismail-Koch, R.J. Salib, J.S. Webb,
483   S.N. Faust, L. Hall-Stoodley, Low Concentrations of Nitric Oxide Modulate
484   Streptococcus pneumoniae Biofilm Metabolism and Antibiotic Tolerance,
485   Antimicrob Agents Chemother 60 (2016) 2456-66.
486 [8] N. Barraud, D.J. Hassett, S.H. Hwang, S.A. Rice, S. Kjelleberg, J.S. Webb, Involvement of
487   nitric oxide in biofilm dispersal of Pseudomonas aeruginosa, J Bacteriol 188
488   (2006) 7344-53.
489 [9] N. Barraud, M.V. Storey, Z.P. Moore, J.S. Webb, S.A. Rice, S. Kjelleberg, Nitric oxide-
490   mediated dispersal in single- and multi-species biofilms of clinically and
491   industrially relevant microorganisms, Microb Biotechnol 2 (2009) 370-8.
492 [10] L.A. Ridnour, D.D. Thomas, D. Mancardi, M.G. Espey, K.M. Miranda, N. Paolocci, M.
493   Feelisch, J. Fukuto, D.A. Wink, The chemistry of nitrosative stress induced by
494   nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful
495   biological situations, Biol Chem 385 (2004) 1-10.
496 [11] N. Barraud, B.G. Kardak, N.R. Yepuri, R.P. Howlin, J.S. Webb, S.N. Faust, S. Kjelleberg,
497   S.A. Rice, M.J. Kelso, Cephalosporin-3'-diazeniumdiolates: targeted NO-donor
498   prodrugs for dispersing bacterial biofilms, Angew Chem Int Ed Engl 51 (2012)
499   9057-60.
500 [12] N.R. Yepuri, N. Barraud, N.S. Mohammadi, B.G. Kardak, S. Kjelleberg, S.A. Rice, M.J.
501   Kelso, Synthesis of cephalosporin-3'-diazeniumdiolates: biofilm dispersing NO-
502   donor prodrugs activated by beta-lactamase, Chem Commun (Camb) 49 (2013)
503   4791-3.
504 [13] S.A. Collins, M.J. Kelso, A. Rineh, N.R. Yepuri, J. Coles, C.L. Jackson, G.D. Halladay,
505   W.T. Walker, J.S. Webb, L. Hall-Stoodley, G.J. Connett, M. Feelisch, S.N. Faust, J.S.
506   Lucas, R.N. Allan, Cephalosporin-3' -diazeniumdiolate NO-donor prodrug PYRRO-
507   C3D enhances azithromycin susceptibility of Non-typeable Haemophilus
508   influenzae biofilms, Antimicrob Agents Chemother (2016).
509 [14] K.L. Moffitt, T.M. Gierahn, Y.J. Lu, P. Gouveia, M. Alderson, J.B. Flechtner, D.E.
510   Higgins, R. Malley, T(H)17-based vaccine design for prevention of Streptococcus
511   pneumoniae colonization, Cell Host Microbe 9 (2011) 158-65.
512 [15] J.R. Casey, D.G. Adlowitz, M.E. Pichichero, New patterns in the otopathogens causing
513   acute otitis media six to eight years after introduction of pneumococcal
514   conjugate vaccine, Pediatr Infect Dis J 29 (2010) 304-9.
515 [16] A. Vergison, R. Dagan, A. Arguedas, J. Bonhoeffer, R. Cohen, I. Dhooge, A. Hoberman,
516   J. Liese, P. Marchisio, A.A. Palmu, G.T. Ray, E.A. Sanders, E.A. Simoes, M. Uhari, J.

517   van Eldere, S.I. Pelton, Otitis media and its consequences: beyond the earache,
518   Lancet Infect Dis 10 (2010) 195-203.
519 [17] M.T. van den Aardweg, A.G. Schilder, E. Herkert, C.W. Boonacker, M.M. Rovers,
520   Adenoidectomy for otitis media in children, Cochrane Database Syst Rev (2010)
521   CD007810.
522 [18] C.W. Boonacker, M.M. Rovers, G.G. Browning, A.W. Hoes, A.G. Schilder, M.J. Burton,
523   Adenoidectomy with or without grommets for children with otitis media: an
524   individual patient data meta-analysis, Health Technol Assess 18 (2014) 1-118.
525 [19] R. Hakenbeck, T. Grebe, D. Zahner, J.B. Stock, beta-lactam resistance in
526   Streptococcus pneumoniae: penicillin-binding proteins and non-penicillin-
527   binding proteins, Mol Microbiol 33 (1999) 673-8.
528 [20] A.R. Kerr, X.Q. Wei, P.W. Andrew, T.J. Mitchell, Nitric oxide exerts distinct effects in
529   local and systemic infections with Streptococcus pneumoniae, Microb Pathog 36
530   (2004) 303-10.
531 [21] R.N. Allan, P. Skipp, J. Jefferies, S.C. Clarke, S.N. Faust, L. Hall-Stoodley, J. Webb,
532   Pronounced metabolic changes in adaptation to biofilm growth by Streptococcus
533   pneumoniae, PLoS One 9 (2014) e107015.
534 [22] C. Nieto, M. Espinosa, Construction of the mobilizable plasmid pMV158GFP, a
535   derivative of pMV158 that carries the gene encoding the green fluorescent
536   protein, Plasmid 49 (2003) 281-5.
537 [23] L. Hall-Stoodley, L. Nistico, K. Sambanthamoorthy, B. Dice, D. Nguyen, W.J. Mershon,
538   C. Johnson, F.Z. Hu, P. Stoodley, G.D. Ehrlich, J.C. Post, Characterization of biofilm
539   matrix, degradation by DNase treatment and evidence of capsule downregulation
540   in Streptococcus pneumoniae clinical isolates, BMC Microbiol 8 (2008) 173.
541 [24] P. Macheboeuf, C. Contreras-Martel, V. Job, O. Dideberg, A. Dessen, Penicillin
542   binding proteins: key players in bacterial cell cycle and drug resistance
543   processes, FEMS Microbiol Rev 30 (2006) 673-91.
544 [25] L.K. Keefer, Progress toward clinical application of the nitric oxide-releasing
545   diazeniumdiolates, Annu Rev Pharmacol Toxicol 43 (2003) 585-607.
546 [26] H. Ceri, M.E. Olson, C. Stremick, R.R. Read, D. Morck, A. Buret, The Calgary Biofilm
547   Device: new technology for rapid determination of antibiotic susceptibilities of
548   bacterial biofilms, J Clin Microbiol 37 (1999) 1771-6.
549 [27] M.R. Parsek, P.K. Singh, Bacterial biofilms: an emerging link to disease
550   pathogenesis, Annu Rev Microbiol 57 (2003) 677-701.
551 [28] N. Cerca, S. Martins, F. Cerca, K.K. Jefferson, G.B. Pier, R. Oliveira, J. Azeredo,
552   Comparative assessment of antibiotic susceptibility of coagulase-negative
553   staphylococci in biofilm versus planktonic culture as assessed by bacterial
554   enumeration or rapid XTT colorimetry, J Antimicrob Chemother 56 (2005) 331-
555   6.
556
557
558
559
560