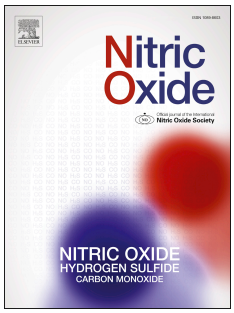
# Accepted Manuscript

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

Raymond N. Allan[a,b], Michael J. Kelso[c], Ardeshir Rineh[c], Nageshwar R. Yepuri[c], Martin Feelisch[a,d], Odel Soren[e], Sanjita Brito-Mutunayagam[a], Rami J. Salib[a,d,f], Paul Stoodley[e,g], Stuart C. Clarke[a,d], Jeremy S. Webb[d,e], Luanne Hall-Stoodley[a,g] & Saul N. Faust[a,b,d]#

[a]Clinical and Experimental Sciences, Faculty of Medicine and Institute for Life Sciences, University of Southampton, Southampton, UK

[b]Southampton NIHR Wellcome Trust Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, Southampton, UK

[c]Illawarra Health and Medical Research Institute, School of Chemistry, University of Wollongong, Wollongong, NSW, Australia

[d]Southampton NIHR Respiratory Biomedical Research Unit, University Hospital Southampton NHS Foundation Trust, Southampton, UK

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

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

[g]Department of Microbial Infection and Immunity, Centre for Microbial Interface Biology, College of Medicine, The Ohio State University, Columbus, Ohio, USA

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

E.mail addresses:

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

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

Ardeshir Rineh (arineh@uow.edu.au)

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

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

Odel Soren (os3c14@soton.ac.uk)

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

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

Paul Stoodley (Paul.Stoodley@osumc.edu)

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

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

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

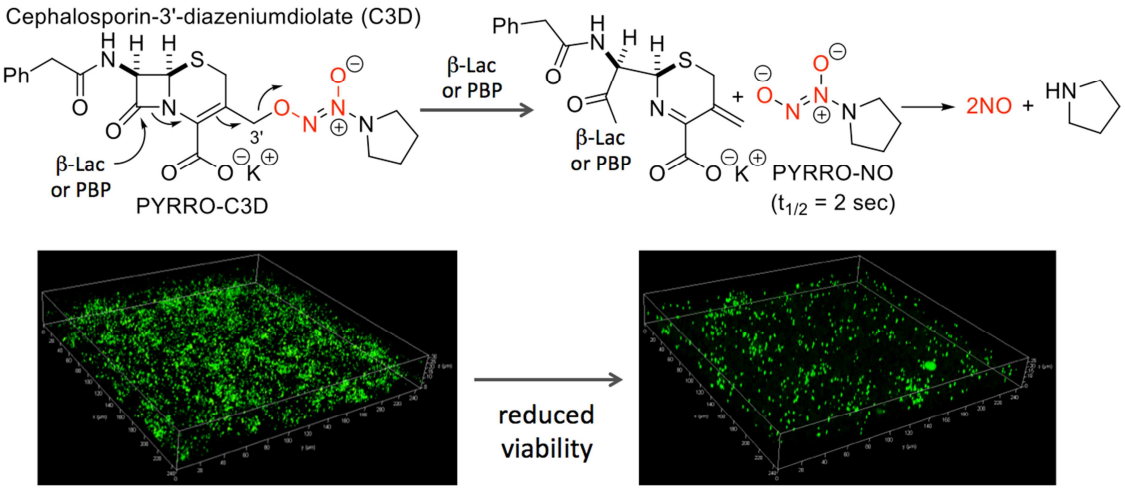
Saul N. Faust (s.faust@soton.ac.uk)

**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.

**Keywords:** *Streptococcus pneumoniae*; biofilm; nitric oxide; antibiotic resistance; cephalosporin-NO-donor.

## 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

combination with conventional antibiotics has thus emerged as a possible anti-biofilm strategy because the NO-mediated transition from biofilm to planktonic states renders bacterial cells more susceptible to antibiotic treatments[7; 8; 9].

Whilst effective in signaling biofilm dispersal and eliciting other anti-biofilm responses *in vitro*, clinical implementation of adjunctive NO therapy with antibiotics in infectious diseases presents several challenges: (a) NO in gaseous form could only be used for a limited range of infections (e.g. body surface and bronchopulmonary infections); (b) use of NO donor compounds that spontaneously release NO in aqueous solution (e.g. sodium nitroprusside, SNP) for internal infections would present significant toxicity risks due to systemic exposure of the host to NO[10]; and (c) developing NO-donor/antibiotic combinations is difficult due to divergent pharmacokinetics and other drug properties of the two molecules. In addition, the lack of specificity towards bacteria and its short half-life make NO treatment of biofilm infections challenging[6]. To address these issues, we are investigating cephalosporin-3´-diazeniumdiolates (C3Ds) as novel, biofilm-activated NO-donor prodrugs.

C3Ds contain a stabilized diazeniumdiolate NO-donor (NONOate) attached at the 3'-position of early generation cephalosporins and were designed to selectively deliver NO to biofilm infection sites following β-lactam ring cleavage mediated by bacterial β-lactamases. It was envisaged that the compounds could be used as targeted NO carriers in combination with conventional antibiotics to treat chronic, β-lactamase expressing, biofilm infections (Figure 1)[11; 12]. We have previously reported that PYRRO-C3D increases the sensitivity of non-typeable *Haemophilus influenzae* biofilms to treatment with azithromycin, a response that was dependent on NO-release following β-lactamase cleavage[13]. It is conceivable, however, that liberation of NO from C3Ds might also be triggered by reaction with transpeptidases/penicillin-binding proteins (PBPs)[11], the molecular target of clinical

cephalosporin antibiotics, since the mechanism of β-lactam hydrolysis (and ensuing elimination of the NONOate) by β-lactamases and transpeptidases would be identical (Figure 1). In addition to releasing NO and triggering anti-biofilm responses (including dispersion in some species), reaction of transpeptidases with the β-lactam of C3Ds should, in principle, also produce direct antibacterial effects. Dual-activity of this type would support thorough exploration of C3Ds in a range of infectious disease indications as "all-in-one" anti-biofilm cephalosporins that don't require co-administered antibiotics (Figure 1).

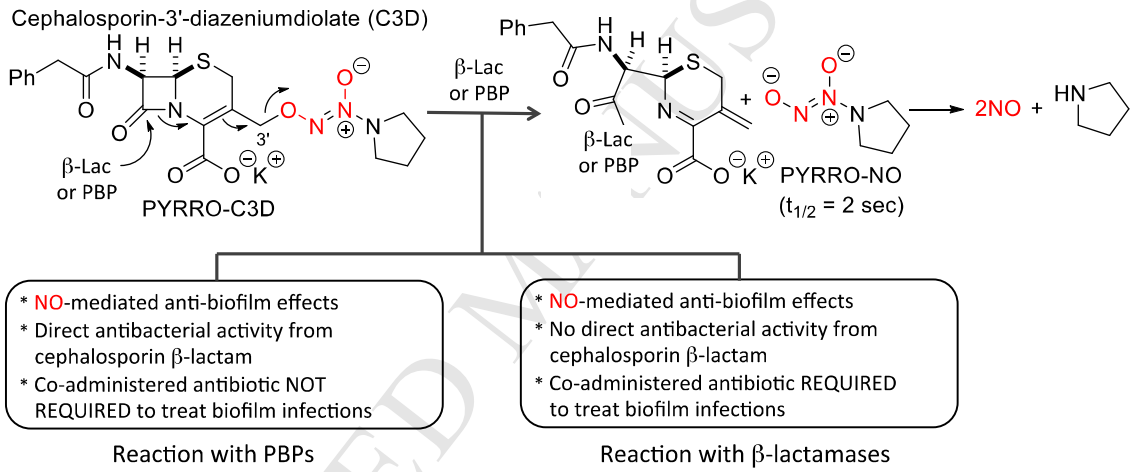



**Figure 1:** Mechanism of NO release from cephalosporin-3'-diazeniumdiolates (C3Ds, e.g. PYRRO-C3D) and proposed effects arising from reaction with PBPs versus β-lactamases.

*S. pneumoniae* is a Gram-positive opportunistic pathogen and the causative agent of various invasive infections, such as meningitis and pneumonia, as well as localized mucosal infections (e.g. sinusitis and otitis media). Despite introduction of pneumococcal conjugate vaccines, the clinical incidence of pneumococcal otitis media has stayed largely unchanged due to serotype replacement, and otitis media remains a primary cause of antibiotic prescription in children[14; 15; 16; 17; 18]. *S. pneumoniae* is also a non-β-lactamase-producing organism that uses transpeptidases/PBPs in the construction of its cell wall[19].

Treatment with high concentrations of NO has been shown to produce antibacterial effects on pneumococci when present as *in vitro* biofilms, on the surfaces of adenoid tissue samples *ex vivo*, and in the lungs of mice that develop pneumonia following intranasal infection[7; 20]. We considered *S. pneumoniae* an excellent bacterial model to test whether a representative C3D (i.e. PYRRO-C3D $K^+$ salt, Figure 1) could show direct β-lactam-mediated antibacterial activity (through reaction with PBPs) and NO-mediated anti-biofilm effects without confounding effects from β-lactamases. This dual-activity concept was explored by measuring the direct antibacterial effects of PYRRO-C3D on planktonic and biofilm *S. pneumoniae*, and probing whether the observed responses were mediated by PBP inactivation and/or NO.

## 2. Material and Methods

### 2.1 Bacterial strains and growth conditions.

A *S. pneumoniae* serotype 14 (ST124) clinical isolate[21] and a Serotype 2 strain (D39) containing the plasmid pMV158GFP[22] were used in this study. Strains were subcultured from frozen stocks onto Columbia blood agar (CBA) plates (Oxoid; PB0122), as described previously[21]. Briefly, cultures were incubated at 37 $^{o}$C/5% $CO_2$ and colonies re-suspended in fresh Brain Heart Infusion (BHI) broth (Oxoid; CM1135) for use in experiments.

### 2.2 *In vitro* planktonic experiments.

Flat-bottomed 96-well culture plates (Fisher Scientific) were inoculated with 1.0 x $10^7$ bacteria per well (mid-exponential planktonic cultures) grown in BHI. Stock solutions of PYRRO-C3D, DEA/NO[7], and cephaloram (all 10 mM in dimethyl sulfoxide, DMSO) were diluted in BHI and added to wells at final concentrations ranging from 9 nM − 90 μM. 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 $^{o}$C/5% $CO_2$ and the minimum inhibitory concentration (MIC) obtained by
190 measuring the absorbance ($OD_{595}$) 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 $^{o}$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 $^{o}$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

induce *gfp* expression). Biofilms were then treated with 100 μM PYRRO-C3D or 100 μM DEA/NO in 1:5 diluted BHI + 2 % maltose, or 1:5 diluted BHI + 2 % maltose (untreated control), at 37 $^{o}$C/5% $CO_2$ for 2 h. Treatments/media were removed and the remaining biofilms rinsed twice with HBSS and stained with propidium iodide according to manufacturer's instructions (ThermoFisher Scientific; P3566). Stained biofilms were examined immediately using a Leica SP8 CLSM with inverted stand under a 63x oil immersion lens, performing sequential scanning on 0.5 μm sections. The *gfp* fluorescence intensity threshold was set to that of planktonic pneumococci to remove background extracellular DNA staining. Images were analyzed using Leica LCS Software.

**2.6 Statistical analyses.**

Statistical analyses of *in vitro* planktonic and biofilm data were performed using non-parametric Mann-Whitney t-tests. Comparative data reported as $p<0.05$ were considered statistically different.

## 3. Results

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

NO release from PYRRO-C3D was examined first in the presence of a β-lactamase (penicillinase) using the NO probe. PYRRO-C3D (100 μM) showed low-level release of NO over 5 minutes after being added to HBSS (*p*H 7.4) at 37 ˚C. Subsequent addition of 10 units of penicillinase caused a rapid spike of NO, reaching a peak concentration of 450 nM within 5 min, which was followed by a steady decline over 2 h, confirming that PYRRO-C3D efficiently releases NO following β-lactam ring cleavage (Figure 2a).

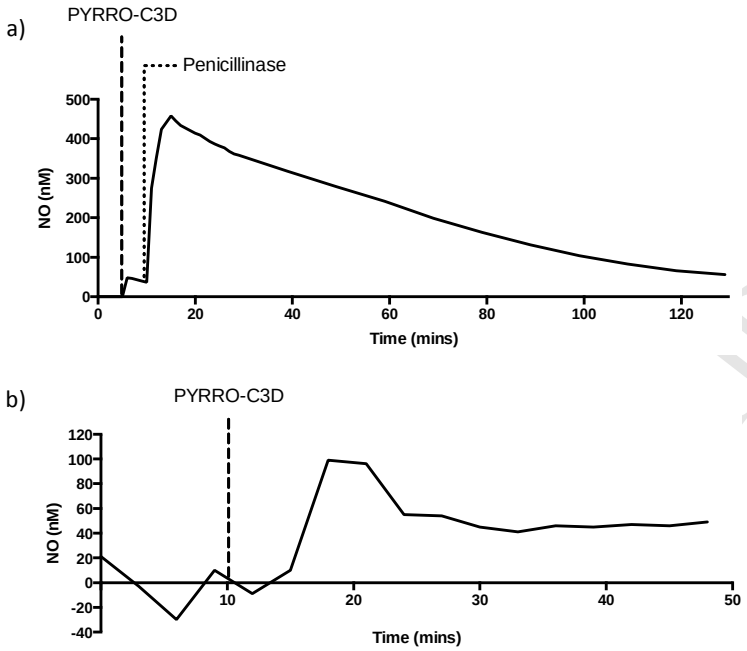
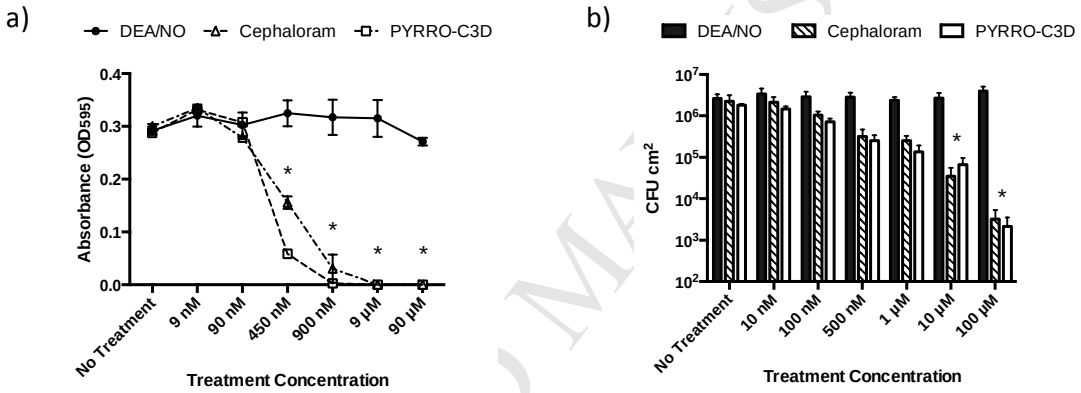



**Figure 2: Release of NO from PYRRO-C3D. a)** NO release from PYRRO-C3D (100 μM) was monitored following addition to HBSS (*p*H 7.4) at 37 ˚C. After 5 mins, 10 units of penicillinase were added, leading to release of NO from PYRRO-C3D. **b)** 48 h serotype 14 biofilms showed no detectable endogenous 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 ($OD_{595}$) 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. *$p \leq 0.05$.
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-

typeable *H. influenzae* biofilms[13] following 2 hour treatments. The treatment time was also chosen based on the NO release profile of PYRRO-C3D whereby little measurable NO was remaining after 2 hours following activation (Figure 2a). Biofilms were assessed for pneumococcal viability by CFU enumeration showed a concentration-dependent response to PYRRO-C3D, culminating in a 3-log reduction in biofilm CFUs at 100 μM ($p=0.014$) (Figure 3b). As seen in the planktonic phenotype, cephaloram showed identical activity to PYRRO-C3D (3-log reduction in biofilm CFUs at 100 μM, $p=0.029$) and DEA/NO showed no effect below 100 μM ($p=0.49$). A 4-log reduction was also observed in CFUs grown from the supernatant surrounding PYRRO-C3D (100 μM) treated cells, compared to untreated controls ($p=0.029$, data not shown).

CLSM imaging and biomass measurements of 48 hour biofilms formed by a GFP-expressing serotype 2 strain (D39) showed no change in maximum biofilm height ($p=0.57$) or total biomass ($p=0.989$) following treatment with either 100 μM DEA/NO or PYRRO-C3D (Figure 4a & b), demonstrating that neither compound triggers an NO-mediated dispersal response in pneumococcus. While this is in contrast to the robust dispersal responses seen following C3D treatment of *P. aeruginosa* biofilms and NO treatment of biofilms from other bacteria[8; 9], it is consistent with our recent results showing that NO modulates metabolic activity but not dispersal in *S. pneumoniae* biofilms[7]. Similar to the CFU data, treatment with DEA/NO showed no effect on biofilm viability, whereas a significant reduction in biofilm viability was observed following PYRRO-C3D treatment (Figure 4c, - e).

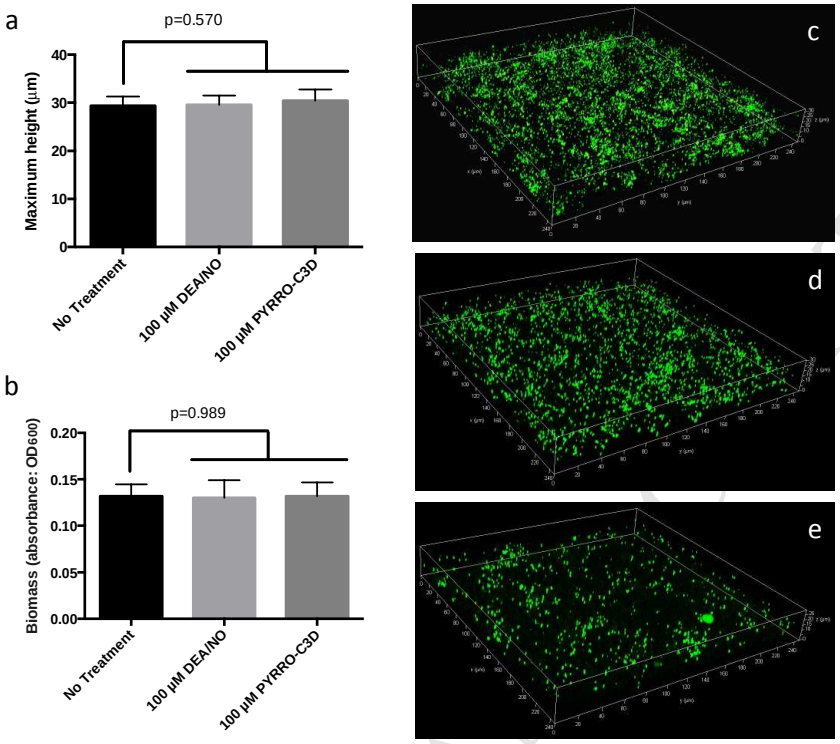



**Figure 4: Effects of PYRRO-C3D on *S. pneumoniae* serotype 2 (D39) *in vitro* biofilms.** Established 48 h D39 biofilms expressing GFP were treated with 100 μM PYRRO-C3D or DEA/NO for 2 hours and imaged using confocal microscopy. Biofilms were counterstained with propidium iodide to distinguish dead 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 □g.mL$^{-1}$). No change in the response to PYRRO-C3D was observed (p=0.929) (Fig. 5a), confirming that β-lactamases were playing

no part in the compound's activity. Treatment of biofilms with 100 μM PYRRO-C3D was next repeated in the presence of the NO-scavenger cPTIO. Addition of 50 μM cPTIO, which showed no effect on its own, did not change the activity of PYRRO-C3D (p=0.829, Fig. 5b), confirming that the NO being released from PYRRO-C3D was having no effect. The effect of PYRRO-C3D on *S. pneumoniae* viability was then assessed in the presence of 0.01 U/μL penicillinase, the same β-lactamase shown to cleave the β-lactam of PYRRO-C3D and liberate NO (Figure 2). Addition of penicillinase significantly reduced the activity of PYRRO-C3D (p=0.0286, Fig. 5c). Together these data provide compelling evidence that PYRRO-C3D produces direct activity against biofilm pneumococci via reaction of its cephalosporin β-lactam with transpeptidases/PBPs only, and that subsequent release of NO from the compound produces no measurable effect on cell viability.

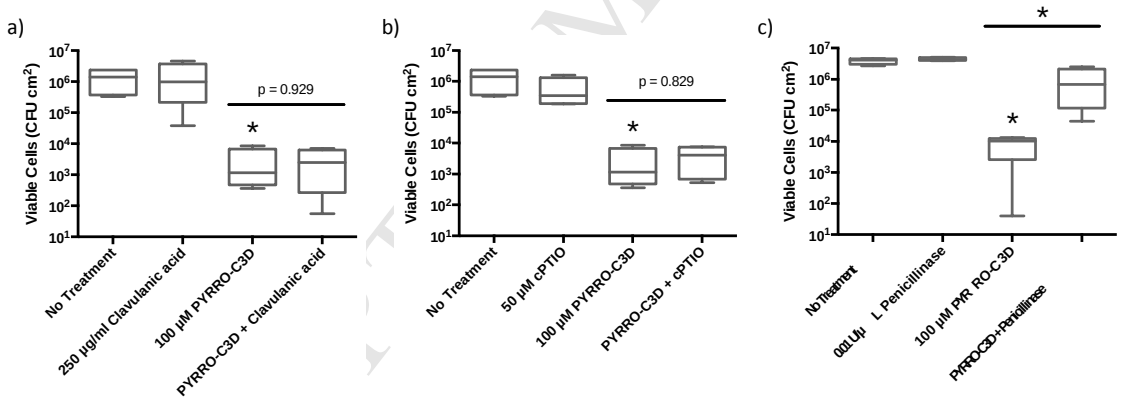



**Figure 5: Response of *S. pneumoniae* serotype 14 (ST124) *in vitro* biofilms to PYRRO-C3D treatment in the presence of clavulanic acid, cPTIO and penicillinase.** 48 h *S. pneumoniae* biofilms were treated with 100 μM PYRRO-C3D for 2 h in the presence of **a)** 250 μg/mL clavulanic acid, **b)** 50 μM cPTIO and **c)** 0.01 unit/μL penicillinase. Pneumococcal viability in biofilms following treatment was assessed by CFU enumeration. *p≤0.05.

### 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.

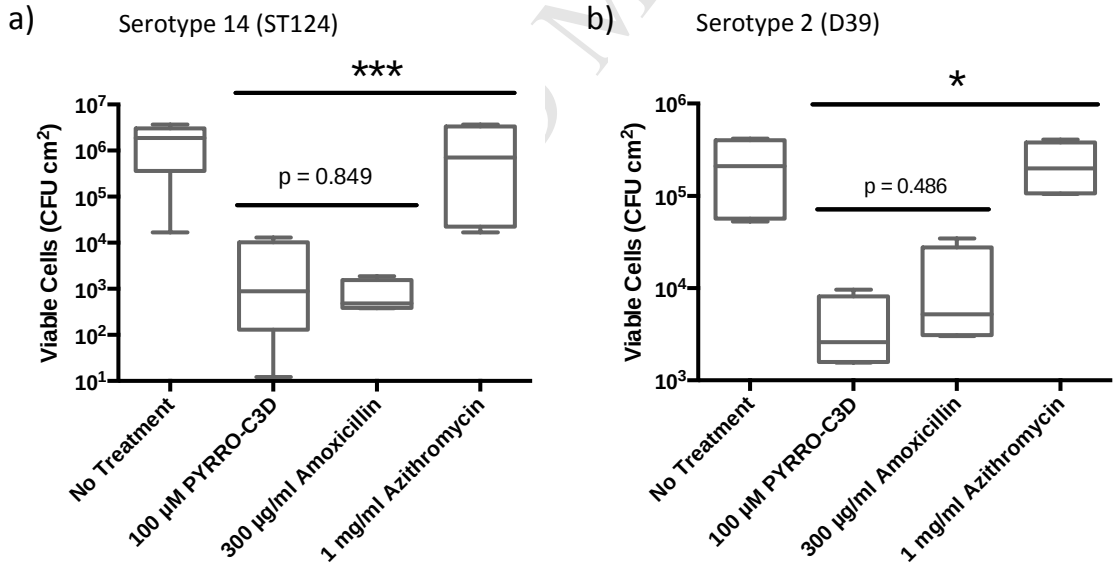



**Figure 6: Comparison of the antibacterial activities of PYRRO-C3D, amoxicillin and azithromycin against *S. pneumoniae* serotype 14 (ST124) and serotype 2 (D39) *in vitro* biofilms.** Established 48 h **a)** serotype 14 and **b)** serotype 2 biofilms were treated with 100

μM PYRRO-C3D, 300 μg/mL amoxicillin or 1 mg/mL azithromycin for 2 hours and assessed for pneumococcal viability by CFU enumeration. *p≤0.05; ***p≤0.001.

## 4. Discussion

Previous studies showed that low levels of NO were released from our prototype C3D (DEA-CP) in the presence of non-β-lactamase producing *E. coli* cell extracts[11] and it was postulated that the NO release resulted from reaction of the compound with PBPs, the enzymes responsible for cross-linking peptidoglycan chains during bacterial cell wall synthesis. It is well known that cephalosporins and other β-lactam antibiotics elicit antibacterial effects by covalently binding to the active sites of PBPs in a process that also results in β-lactam ring cleavage[24]. This led us to speculate that reactions between PBPs and C3Ds might elicit a direct, β-lactam-mediated antibacterial effect and in the process liberate the NONOate (and NO) (Fig. 1). Since anti-biofilm effects of NO are now well documented, we postulated that NO released from C3Ds following reaction with PBPs might confer additional anti-biofilm activity.

We tested this dual-activity hypothesis using non-β-lactamase producing *S. pneumoniae* strains that express five high molecular weight PBPs (1a, 1b, 2a, 2b and 2x) and one low molecular weight PBP3[19]. The absence of β-lactamases ensured that NO released from the compound must arise from an alternative mechanism, most likely PBP-mediated β-lactam cleavage. The representative C3D selected for the study was PYRRO-C3D, a close structural analogue of DEA-CP that carries PYRRO/NO ($t_{1/2}$ = 2 secs) as the NONOate instead of DEA/NO ($t_{1/2}$ = 2 min)[25]. PYRRO-C3D was chosen for its faster NO release, which we believe would be important for C3D use *in vivo* since diffusion of an expelled NONOate away from infection sites (before releasing the NO cargo) would reduce effectiveness and raise NO-mediated safety concerns.

The ability of PYRRO-C3D to release NO following β-lactam cleavage was confirmed first by treating the compound with penicillinase and directly observing NO. Release of NO from PYRRO-C3D in the presence of *S. pneumoniae* cells lacking β-lactamase was demonstrated, consistent with *S. pneumoniae* PBPs hydrolysing the compound's β-lactam and triggering release of NO. PYRRO-C3D was then shown to reduce viability of both planktonic and biofilm *S. pneumoniae*, confirming that the compound shows direct antibacterial activity against this bacterium. The level of activity was consistent with the known tendency of biofilms to be less susceptible to antimicrobial treatments than their planktonic counterparts[26; 27; 28], since treatment with 900 nM PYRRO-C3D completely inhibited planktonic growth, whereas a significant reduction in biofilm viability (3-log) required more than 100-fold higher concentrations.

β-lactamases were confirmed as playing no role in PYRRO-C3D's activity since no difference was seen in the presence of the β-lactamase inhibitor clavulanic acid. Absence of antibacterial activity when planktonic and biofilm cultures were treated with the spontaneous NO-donor DEA/NO provided evidence that the effects of PYRRO-C3D against pneumococcus are exclusively due to its cephalosporin β-lactam core and are not NO mediated. We further observed that the cephalosporin equivalent of PYRRO-C3D lacking a NONOate (i.e. cephaloram) showed identical activity to PYRRO-C3D, and that addition of the NO-scavenger cPTIO failed to change PYRRO-C3D activity. Moreover, PYRRO-C3D significantly reduced (4-log) the number of viable planktonic bacteria remaining in the surrounding media, likely due to the direct antibacterial effect of PYRRO-C3D. Confocal imaging and measurements of biomass showed that no significant reduction in biofilm maximum height or biomass occurred following PYRRO-C3D treatment, but there was a significant reduction in the number of viable bacteria remaining within biofilms, validating the reduction in CFUs. These findings together were consistent with PYRRO-C3D acting

directly as a cephalosporin-like β-lactam antibiotic, a notion further supported by its reduced activity in the presence of penicillinase.

Finally, the antibacterial activity of PYRRO-C3D towards pneumococcal biofilms was compared with that of antibiotics commonly used to treat pneumococcal infections. We found that PYRRO-C3D possessed similar antibacterial efficacy to amoxicillin against both serotype 2 and 14 biofilms, which is perhaps not surprising given that the compounds are structurally and functionally very similar, with both targeting PBP-mediated cell wall synthesis. PYRRO-C3D was found to be much more effective than azithromycin, an antibiotic that targets protein biosynthesis.

The findings presented here are consistent with our recent study, which showed that high concentrations of NO (1 mM) are needed to elicit bactericidal effects or enhance antibiotic efficacy against four different serotypes of pneumococcal *in vitro* biofilms[7]. The current study demonstrated that PYRRO-C3D at 100 µM liberates maximum NO concentrations of ~450 nM and 100 nM upon contact with penicillinase and pneumococcal cells, respectively. It therefore seems likely that PYRRO-C3D does not release sufficient NO when activated by PBPs to modulate pneumococcal biofilm metabolism towards the planktonic phenotype *in vitro*. This may, however, not be the case in the upper respiratory tract, for example, where the constitutive release of NO by host cells could have an augmentative effect, as observed in our recent study where an anti-pneumococcal response to 100 µM NO was seen on host adenoid tissue[7].

In summary, this study demonstrated that a representative C3D (PYRRO-C3D) releases NO and shows direct antibacterial effects against planktonic and biofilm forms of non-β-lactamase producing *S. pneumoniae*. The activity was confirmed to arise exclusively from β-lactam mediated reactions with *S. pneumoniae* PBPs, with no measurable contribution coming from the released NO. In the treatment of pneumococcal biofilms, PYRRO-C3D was

found to be equally as effective as amoxicillin and more effective than azithromycin when used alone.

## 5. Conclusions

Introduction of a diazeniumdiolate at the cephalosporin 3'-position was shown for the first time to be structurally compatible with binding to the molecular target of β-lactam antibiotics, PBPs. Medicinal chemistry tuning of the cephalosporin aminoacyl side chain and diazeniumdiolate portions may identify C3Ds with PBP-mediated activity against other species, and perhaps even broad-spectrum activity. While the study did not demonstrate that PYRRO-C3D produces combined NO and β-lactam based anti-biofilm effects against *S. pneumoniae*, it is possible that such dual-effects might be observed with C3Ds in other species and with other analogues. Non-β-lactamase producing bacteria that undergo NO-mediated biofilm dispersion would be of particular interest for future study.

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**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