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Article

Preclinical Evaluation of Nitroxide-Functionalised Ciprofloxacin as a Novel Antibiofilm Drug Hybrid for Urinary Tract Infections

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Abstract: Urinary tract infections (UTIs) are the second most common bacterial infection with high recurrence rates. A major causative agent of UTIs is uropathogenic *Escherichia coli* (UPEC), which can form persistent biofilms on urothelial surfaces and/or urinary catheters. Biofilms are inherently tolerant to antimicrobials, making them difficult to eradicate. Ciprofloxacin hybrids functionalised with nitroxides have previously shown enhanced biofilm eradication activity *in vitro*. Here, we evaluate a ciprofloxacin di-nitroxide hybrid (CDN11), previously reported to have antibiofilm activity against UPEC, as a potential UTI therapeutic in various infection models. We report potent CDN11 activity against mature biofilms formed by cystitis strain UTI89 inside polyethylene catheters. In infected human bladder cell monolayers, CDN11 afforded a 3-log reduction in UPEC colony forming units (CFU) compared to controls, including intracellular bacteria. Infected mouse bladders carrying biofilm-like intracellular UPEC reservoirs had reduced bacterial burdens following treatment with CDN11 *ex vivo*. Activity trends for CDN11 were comparable throughout all models, indicating that our testing pipeline was robust. New antimicrobial development is arduous and requires large investments of time and money. The pipeline described here could be readily used in testing new compounds, fast tracking the development of novel UTI therapeutics.

Keywords: bladder; urinary tract infection; UPEC; bacterial biofilms; catheters; nitroxides; antibiotic resistance; fluoroquinolones; therapeutics

1. Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections with a high rate of recurrence (~30%). UTI clinical syndromes include cystitis, pyelonephritis, urosepsis and asymptomatic bacteriuria (ASB) [1]. Hospital-acquired UTIs represent high-burden nosocomial infections, especially in patients undergoing long periods of urinary catheterisation, known as catheter-associated UTI (CAUTI) [2]. Uropathogenic *Escherichia coli* (UPEC) are the major causative agents of UTIs. UPEC are Gram-negative rod-shaped bacteria that attach to urinary epithelial cells and are capable of invading into bladder cells and persisting intracellularly to evade host responses [3]. UPEC persistence during UTI can be attributed to a number of virulence factors, such as adhesins, flagella, siderophores, and the ability to form biofilms [3]. Biofilms are sessile multicellular communities of bacteria embedded in a self-produced extracellular matrix, capable of forming on both biotic and abiotic surfaces. In UTI, UPEC forms intracellular bacterial communities (IBCs) inside bladder cells during early stages of the infection [4], or in the case of CAUTI, UPEC forms biofilms inside the lumen of indwelling urinary catheters [5,6]. Biofilms are inherently tolerant to environmental stress, antimicrobials, and host immune responses, making them difficult to eradicate

and allowing bacteria to persist [7-10]. UPEC IBCs have similar molecular attributes to biofilms, and their establishment and maintenance in the bladder requires the production of type 1 pili, antigen 43, and a polysaccharide-rich extracellular matrix [4,11-13]. Following IBC formation during acute UTI, UPEC can persist in urinary tissues for longer periods in the form of intracellular quiescent reservoirs (QIRs) or in the case of CAUTI, as residual (persister) bacterial cells remaining attached on the catheter surface after antibiotic therapy; both forms share characteristics with biofilms and are intrinsically difficult to eradicate [14-16].

Nitric oxide (NO) has been reported to have broad-spectrum activity against biofilms (bacterial and fungal) and also in combination with antibiotics providing one of the most promising avenues for clinical therapy [17,18]. However, NO's gaseous and highly reactive nature makes it difficult to work with, or to control the dose and timing of its delivery which can lead to NO-mediated cytotoxicity and off-target effects [17,19]. Recently the evaluation of nitroxides as stable NO mimics has uncovered their potential as alternative biofilm dispersal agents [18]. Similar to NO, nitroxides were shown to reduce biofilm biomass of various Gram-negative species *in vitro* [20]. As nitroxides are stable (unlike NO) and thus more amenable to chemical manipulation, they have been successfully used in the synthesis of functionalised antibiotics and biocides [21-24], ensuring co-delivery with antimicrobials. Some nitroxide-antibiotic hybrids were reported to have improved biofilm eradication activity compared to the parent drug, with promising activity reported against several clinically important Gram-negative and Gram-positive bacteria *in vitro* [21,24]. Biofilm eradication by these hybrid drugs is likely achieved by simultaneous bacterial dispersal and killing (induced by the nitroxide and antibiotic/biocide moieties, respectively) making them highly suitable for clinical applications [25], as using biofilm dispersal agents alone (or even in combination with antibiotics, but unlinked) may pose a risk for the seeding of new infection sites by viable dispersed cells [26].

Here, we evaluate CDN11, a ciprofloxacin-dinitroxide hybrid previously reported to have antibiofilm activity against *E. coli* [24], as a potential UTI therapeutic. Ciprofloxacin is a commonly prescribed fluoroquinolone antibiotic for both uncomplicated and complicated UTIs [27], with diminishing efficacy over the past decade due to the rise of fluoroquinolone resistant UPEC lineages [28,29]. We report that CDN11 displays antibiofilm activity against ciprofloxacin sensitive UPEC in multiple relevant models of UTI, including *in vitro* infected catheters and human bladder cells. Activity was also observed against UPEC IBCs in the murine bladder following *ex vivo* treatment. Our findings showcase nitroxide-antibiotic hybrids as a promising drug co-administration approach targeting common biofilm infections. Moreover, the preclinical pipeline of *in vitro* and *in vivo* UTI models employed in this study, should serve as a robust platform for testing new UTI therapeutics in future studies.

2. Materials and Methods

2.1. Ethics Statement

All UTI mouse work was conducted under ethics approval number A1703-600M issued by the Animal Ethics Committee at the QIMR Berghofer Medical Research Institute.

2.2. Bacterial Strains and Culture Conditions

UPEC strain UTI89 [30] was used in this study as a reference cystitis strain, and EC958 [31] as a multi-drug resistant strain. Unless otherwise stated, all bacteria were routinely maintained and cultured in LB media at 37°C, 200 rpm (1% w/v tryptone (Oxoid, CM0087) 0.5% w/v yeast extract (Gibco, 212750), 0.5% w/v salt) from a single colony on a plate (1.5% w/v agar (BD, DF0140-01-0)), 37°C. For all antimicrobial activity assays, tested was conducted in MH medium (30% w/v dehydrated beef infusion, 1.75% w/v casein hydrolysate, 0.15% w/v starch, Oxoid, CM0405).

2.3. Antimicrobial Compounds

Ciprofloxacin (Sigma, 17850) stocks were routinely made from powder and dissolved in 0.1 M HCl (30 mM, 10 mg/mL). For MIC assays, ciprofloxacin was used at a concentration range of 3000 μ M – 0.0023 μ M. For MBEC assays, ciprofloxacin was used at a concentration range of 800 μ M - 1.57 μ M. For all other assays, ciprofloxacin was used at a concentration of 400 μ M.

CDN11 was synthesised as previously described [24]. Briefly, EDC-promoted amide coupling of ciprofloxacin ethyl ester with Boc-Lys(Cbz)-OH gave the fully protected conjugate which was deprotected by hydrogenolysis at the α -amine and reacted in another EDC amide coupling reaction with Boc-Lys(Cbz)-OH. Deprotection of the two Cbz-protected amines and subsequent amidation with CTEMPO gave the nitroxide functionalised ciprofloxacin, which was deprotected by base mediated ester hydrolysis, followed by Boc-deprotection to give CDN11. Stocks were routinely made from powder and dissolved in 100% DMSO (10.5 mM, 10 mg/mL). For MIC assays, CDN11 was used at a concentration range of 800 μ M – 2.63 μ M. For MBEC assays, CDN11 was used at a concentration range of 800 μ M - 1.57 μ M. For all other assays, CDN11 was used at a concentration of 400 μ M.

2.4. Minimum Inhibitory Concentration (MIC) Assay

MICs for nitroxides, antibiotics, and hybrid compounds were as described in Clinical and Laboratory Standards Institute (CLSI) [32]. Briefly, in a 96-well plate, twofold serial dilutions of each compound were prepared to a final volume of 100 μ L in MH medium (CDN11: 800 μ M - 2.63 μ M, CIP: 3000 μ M – 0.0023 μ M), with a matched DMSO vehicle control for the highest concentration. Each well was inoculated with 100 μ L bacteria suspension in MH containing 5×10^6 bacterial colony forming units (CFUs), prepared from overnight cultures in LB. The MIC for a compound was defined as the lowest concentration that prevented visible bacterial growth after 18 h of static incubation at 37 °C. MICs were performed a minimum of two times with three technical replicates per condition.

2.5. Minimum Biofilm Eradication Concentration (MBEC) Assay

MBEC assays were performed as per the manufacturer's protocol with a Calgary biofilm device (CBD) (Innovotech Inc., Canada). The CBD consists of a 96-well plate and an associated lid consisting of small protrusions (pegs) that are immersed in each well when the lid is covered, facilitating individual biofilm growth on each peg. Briefly, UTI89 grown for 18h in LB were diluted to 1×10^7 CFU/mL in LB and used to inoculate the plate with 130 μ L of culture per well. The CBD was incubated for 24 hours with shaking (150 rpm) at 37°C in 95% relative humidity. Following 24 h of growth, biofilms were washed once in PBS (130 μ L) by incubating statically for 10 s to remove planktonic cells and then the peg lid was transferred to a new 96-well plate containing ciprofloxacin and CDN11 in MH, in concentrations ranging from 800 μ M - 1.57 μ M, with a matched HCl/DMSO vehicle control for the highest concentration, respectively. The plate was then incubated for a further 24 hours at 37°C with shaking (150 rpm), then washed once in PBS (200 μ L, 10 s static incubation) and sonicated in a water bath (Elma Ultrasonic Cleaner S10H) for 20 min at 20°C to suspend the biomass in 200 μ L of PBS. Each well was serially diluted 10-fold and 5 μ L was spotted onto LB agar plates in triplicate to determine CFU recovered from each peg biofilm. Plates were incubated overnight at 37°C with colonies counted the following day to obtain \log_{10} (CFU/mL) values for each treatment condition. Biofilm experiments were performed a minimum of two times, with 3-8 pegs per group. MBEC values are reported as the concentration at which there are no recoverable biofilm CFU (complete eradication).

2.6. Catheter Biofilm Model

The *in vitro* catheter biofilm assays were performed similar to the MBEC assay. UTI89 grown for 18 h in LB were diluted to 1×10^7 CFU/ml in LB and 400 μ L/well used to inoculate a 48-well plate. Each well contained catheters made from ~3 mm sections of UV sterilised polyethylene tubing (BD, 0.28 mm inner diameter, 0.61 mm outer diameter). Plates were incubated for 24 hours with shaking (150rpm) at 37°C in 95% relative humidity. Following 24 hours of growth, catheters were washed

once in PBS (400 μ L) to remove planktonic bacterial cells and then were transferred to a new plate containing CTEMPO, ciprofloxacin, and CDN11 in MH, at a concentration of 400 μ M each, with matched vehicle controls. Catheters were then incubated for a further 24 hours at 37°C shaking (150 rpm), and then washed once in PBS (400 μ L) and sonicated and processed as above to determine CFU.

2.7. Human Cell Infection Assays

Type 1 fimbriae (T1F) enriched, UTI89 was routinely prepared cultured from highly fimbriated stocks, which were incubated overnight statically at 37°C for 18 h. Bladder epithelial cells T24 (ATCC HTB-4), were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 5% heat-inactivated foetal bovine serum (Invitrogen) at 37°C. Adhesion and invasion assays with UPEC strain UTI89 were performed as previously described [33]. Briefly, T24 cells were seeded at a density of 5×10^5 in 24-well plates, and then grown overnight to form confluent monolayers. Confluent T24 monolayers were infected with UTI89 for 1 hour at 37°C at a multiplicity of infection (MOI) of 10 (5×10^6 CFU/well), then washed with PBS. For adhesion assays, monolayers were then incubated with either RPMI only (untreated), DMSO/0.1M HCl (vehicle controls), 400 μ M ciprofloxacin (antibiotic) or 400 μ M CDN11 (dinitroxide-ciprofloxacin antibiotic hybrid) for 2 hours at 37°C. For invasion assays, infected monolayers as above were first incubated with 200 μ g/mL gentamicin for 1 hour to eliminate extracellular bacteria, then washed three times with PBS. Monolayers were then treated with the same compound groups as the adhesion assays. Post treatment, both monolayers were washed three times with PBS followed by lysis with 500 μ L 0.1% Triton X-100 in PBS for 10 min at 37°C. CFU recovered from each monolayer were determined by serial dilution plating onto LB agar plates. All compound working solutions were made up in RPMI, to a concentration of 400 μ M (40x MIC of CDN11).

2.8. In Vivo UTI Mouse Model and Ex Vivo Bladder Antibiotic Treatment

6–7-week-old female C3H/HeJ mice were catheterised as previously described [34]. Briefly, mice were anaesthetised by isoflurane inhalation and catheterised with $\sim 1-2 \times 10^8$ CFU in 30 μ L of UTI89 in PBS. T1F enriched UTI89 was prepared as above. The prepared inoculum was deposited directly into the bladder using a sterile polyethylene catheter (same material as *in vitro* testing) followed by immediate removal [34]. C3H/HeJ mice were sourced from the ARC (Animal Resource Centre, Western Australia). Mice were sacrificed at 16 hours post inoculation and the bladders were extracted.

Bladders were bisected to expose the lumen and were washed in PBS for 5 min in a well of a 96-well plate (one mouse per well). Bladder halves were then transferred to fresh wells in another plate containing 200 μ g/mL gentamicin in PBS and incubated for 1 hour 37°C to remove extracellular bacteria, then washed again in PBS. Each bladder half was then incubated with either PBS only (untreated), DMSO (vehicle control), 400 μ M ciprofloxacin (antibiotic) or 400 μ M CDN11 (nitroxide functionalised antibiotic) for 6 hours at 37°C. Following treatment, bladders were immediately transferred to tubes containing metal beads for homogenisation. Bladders were homogenised in 50 μ L of PBS using a Mini Bead beater (BioSpec Products) before topping up to 1 mL with PBS and aliquoting 200 μ L of each sample into a 96-well plate. Samples were serially diluted and 5 μ L of each well for each sample was plated onto LB agar in quadruplicate and then incubated overnight at 37°C. The following day, colonies were enumerated, and bacterial CFU was expressed as $\log_{10}(\text{CFU}/0.1\text{g})$ of tissue.

2.9. Statistical Analysis

All statistical analysis was performed in *GraphPad Prism Version 9* software (GraphPad Software). An ordinary one-way ANOVA with Dunn's multiple comparisons correction was used to test for statistically significant differences between CDN11-treated groups and control group means. Statistical significance was set at $p < 0.05$.

3. Results

3.1. CDN11 Eradicates UPEC Biofilms Formed on Urinary Catheters *In Vitro*

Dinitroxide-ciprofloxacin hybrid CDN11 was synthesised as previously described [24]. The minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) of CDN11 against reference cystitis UPEC isolate UTI89 was confirmed as $\leq 10.5 \mu\text{M}$ and $\leq 400 \mu\text{M}$, respectively, in agreement with previous reports [24]. To simulate UPEC biofilm formation in the context of CAUTI, mature biofilms of UTI89 were established at high cell density inside polyethylene catheters and then treated with antibiotics. After 24 hours of treatment with CDN11 at $400 \mu\text{M}$ (MBEC concentration), viable CFU remaining in the biofilm were reduced by >4 logs compared to vehicle treated (DMSO) and untreated biofilm controls (Figure 1). Thus, CDN11 antibiofilm activity in the catheter biofilm treatment model exceeded the 99.9% reduction in CFU cut-off used to define biofilm eradication *in vitro* and was significantly better than ciprofloxacin administered at the same concentration ($p = <0.0001$, Figure 1).

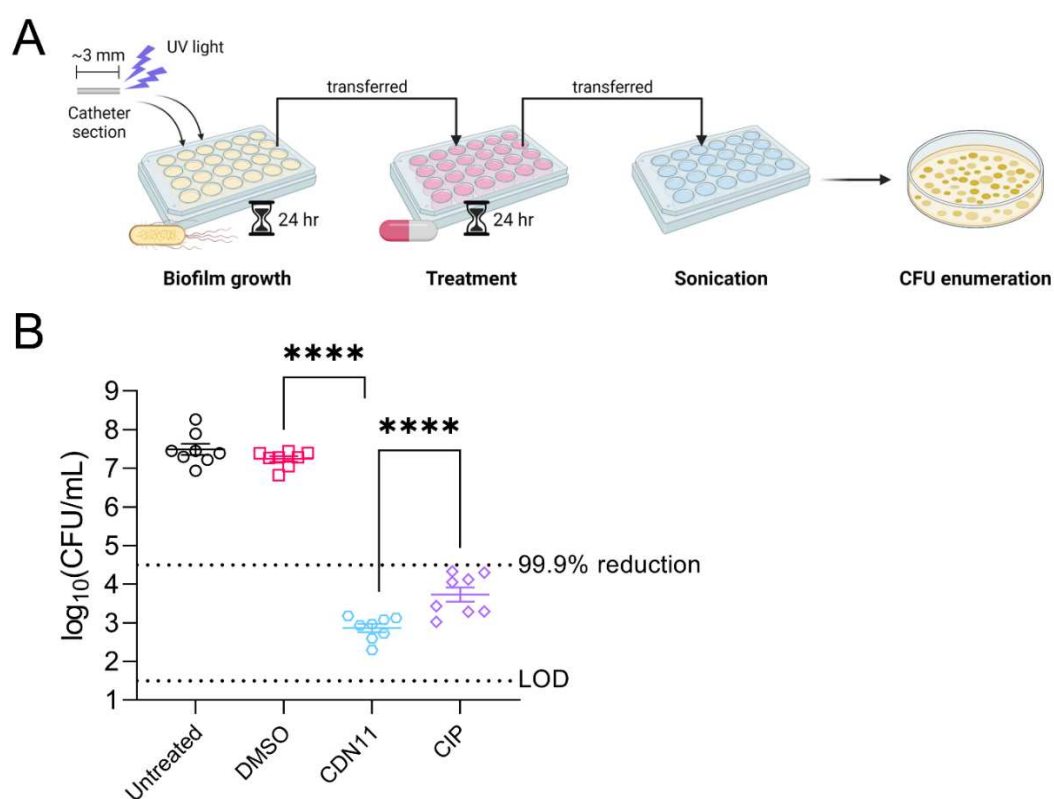


Figure 1. CDN11 has potent antibiofilm activity against UPEC biofilms formed inside urinary catheters *in vitro*. A) Schematic of the *in vitro* catheter biofilm model. ~3 mm sections of UV-sterilised (lightning symbol) polyethylene catheter were incubated with 4×10^6 CFU of UTI89 in LB media for 24 h followed by 24 h of treatment with $400 \mu\text{M}$ CDN11, $400 \mu\text{M}$ ciprofloxacin, 3.5% DMSO (vehicle), or media only (MH, Untreated), before disruption with mild sonication for CFU enumeration. B) Biofilm recovered bacteria from each treatment group were plotted as $\log_{10}(\text{CFU}/\text{mL})$ from 8 biological replicates, with group means and standard deviation shown by horizontal lines and error bars, respectively. Differences between group means were calculated using ordinary one-way ANOVA with Dunn's correction, and significance was shown as **** $p \leq 0.0001$. The technical limit of CFU detection (LOD) and the 99.9% CFU reduction cut-off (compared to vehicle control) are marked by dotted lines.

3.2. *In Vitro* Treatment of Infected Human Bladder Cells with CDN11 Reduces Extracellular and Intracellular UPEC Titers

We next tested if CDN11 could effectively control UPEC infection of human cells *in vitro*. Human T24 bladder epithelial cell monolayers infected with UPEC strain UTI89 for 1 hour at a multiplicity of infection (MOI) of 10 were treated for 2 hours with antibiotics. CDN11 at 400 μ M significantly reduced the total CFU recovered from infected monolayers compared to untreated and vehicle treated controls (mean reduction of ≥ 3.5 logs, Figure 2A). Similarly, ciprofloxacin afforded a significant reduction of the total UPEC titer and cleared infection in one of eight treated monolayers (Figure 2A).

UPEC infection of T24 human bladder cells involves both attachment (extracellular bacteria) and invasion (intracellular bacteria) of bladder cells, resembling UPEC pathogenesis during bladder infection (cystitis) of mice and humans [35]. To specifically test CDN11 activity against intracellular UPEC reservoirs, we performed gentamicin protection assays [33], whereby the infected T24 cell monolayers were first treated with gentamicin to selectively eliminate extracellular UPEC followed by test antibiotics. Despite its large molecular size (>900 Da), CDN11 displayed activity against intracellular UPEC when administered at 400 μ M, reducing the mean intracellular titer by $\sim 40\%$ compared to untreated and vehicle controls and clearing infection in one of five treated monolayers (Figure 2B). Ciprofloxacin-treated monolayers were completely cleared of intracellular UPEC, with no detectable CFU recovered from all monolayers examined (Figure 2B).

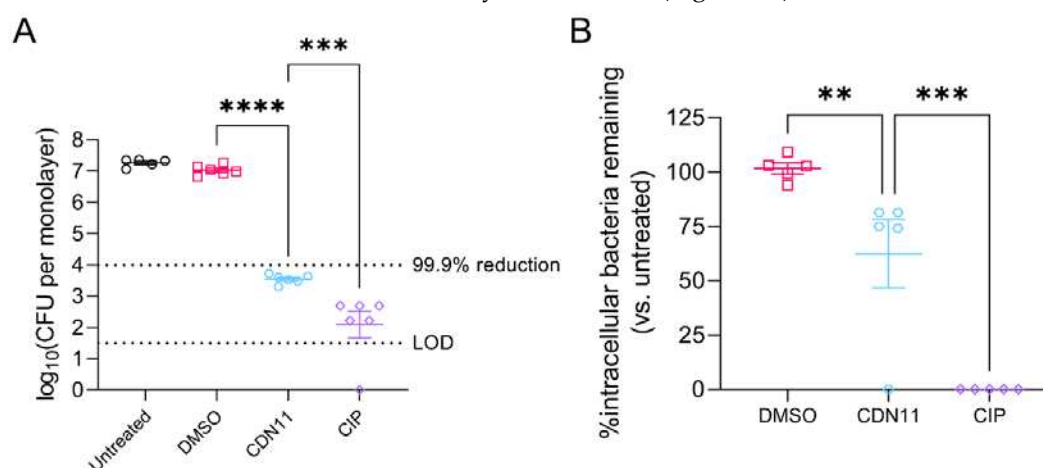


Figure 2. CDN11 controls extracellular and intracellular UPEC during *in vitro* human bladder cell infection. Confluent monolayers of T24 bladder cells were incubated with UPEC strain UTI89 at an MOI of 10 for 1 h followed by A) 2 h of treatment with either 400 μ M CDN11, 400 μ M ciprofloxacin, an equivalent percentage of DMSO instead of CDN11 (vehicle), or media only (RPMI + 5% FCS, Untreated) or B) 1h of treatment with 200 μ g/mL gentamicin to first eliminate extracellular bacteria, followed by 2 h of treatment with either 400 μ M CDN11, 400 μ M ciprofloxacin, an equivalent percentage of DMSO instead of CDN11 (vehicle), or media only (RPMI + 5% FCS). Bacteria recovered from infected monolayers were plotted per treatment group as A) log₁₀(CFU/mL) or B) % intracellular bacteria compared to the media only control group. Data shown are from 5-6 biological repeats, with means and standard deviation shown by horizontal lines and error bars, respectively. Differences between group means were calculated using ordinary one-way ANOVA with Dunn's correction, and significance was shown as ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. The technical limit of CFU detection (LOD) and the 99.9% CFU reduction cut-off (compared to vehicle control) are marked by dotted lines.

3.3. *Ex Vivo* Treatment of Infected Mouse Bladders with CDN11 Reduces Intracellular UPEC Reservoirs

To evaluate early preclinical efficacy for CDN11 as a UTI therapeutic, we next tested its activity against intracellular UPEC reservoirs that are established in the bladder of mice during experimental acute UTI. Infected bladders collected from female C3H/HeJ mice with acute cystitis established by

UPEC strain UTI89 were treated *ex vivo* with gentamicin to eliminate extracellular (luminal) UPEC, followed by treatment with test antibiotics (Figure 3A). CDN11 treatment at 400 μ M completely cleared UPEC from 25% of the infected bladder tissues, and significantly reduced the mean intracellular CFU of the group compared to untreated controls (Figure 3B). In comparison, ciprofloxacin treatment cleared intracellular UPEC from all bladder tissues examined (Figure 3B).

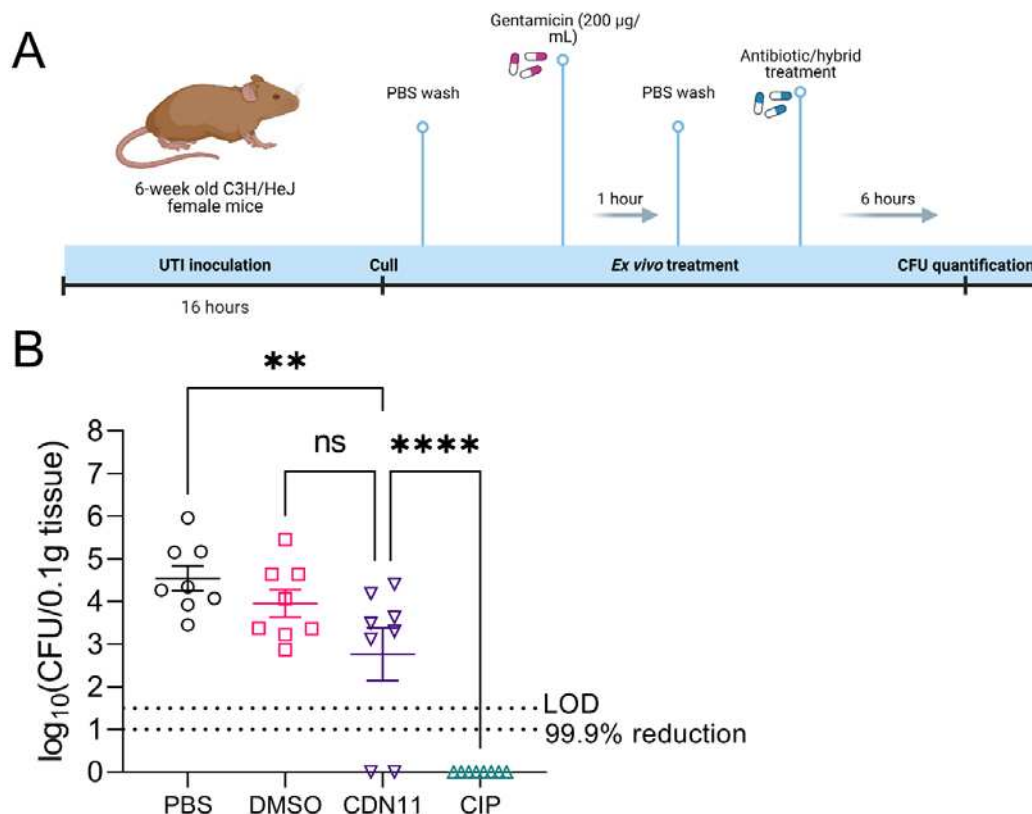


Figure 3. CDN11 reduces intracellular UPEC reservoirs in infected mouse bladders after 6 hours of treatment *ex vivo*. A) Schematic overview of acute UTI mouse model *ex vivo* bladder treatment timeline. 6-week-old female C3H/HeJ mice were inoculated with 1×10^8 CFU of UPEC strain UTI89 and allowed to establish acute UTI for 16 h. Mice were then culled, bladders extracted and bisected, followed by 1 h of treatment with gentamicin, and then 6 h of treatment with either 400 μ M CDN11, ciprofloxacin, or an equivalent percentage of DMSO instead of CDN11 (vehicle), or PBS only.

Bladder halves were homogenised post treatment, serially diluted, and CFU enumerated. B) Bacteria recovered from mouse bladders were plotted per treatment group as $\log_{10}(\text{CFU}/0.1\text{g tissue})$.

Data shown are from 8 mice per group with means and standard deviation shown by horizontal lines and error bars, respectively. Differences between group means were calculated using ordinary one-way ANOVA with Dunn's correction, and significance was shown as **ns** not significant, ****** $p \leq 0.01$, ******** $p \leq 0.0001$. The technical limit of CFU detection (LOD) and the 99.9% CFU reduction cut-off (compared to vehicle control) are marked by dotted lines.

3.4. CDN11 Displays Activity against Biofilms by fluoroquinolone-Sensitive not Fluoroquinolone Resistant UPEC

UTIs are increasingly caused by multidrug resistant UPEC lineages [28]. In particular, *E. coli* sequence type 131 (ST131) is the most recent globally disseminated lineage of multidrug resistant (MDR) UPEC causing UTIs and bloodstream infections, with most ST131 strains being resistant to fluoroquinolones and beta-lactam antibiotics. To investigate whether CDN11 displays antibiofilm activity against MDR UPEC we compared its activity against UTI89 (ciprofloxacin sensitive) and EC958 biofilms, the latter being a representative ST131 MDR UPEC strain (ciprofloxacin resistant) [36]. While CDN11 displayed biofilm CFU reduction against UTI89 in a dose-dependent manner and

at concentrations as low as 12.5 μM , it did not impact established EC958 biofilms even at the highest concentration tested (Figure 4). As EC958 contains both chromosomal and plasmid-encoded ciprofloxacin resistance genes, this finding suggests that CDN11 targets the same enzymes as ciprofloxacin (parent drug) in both planktonic and biofilm bacteria [37].

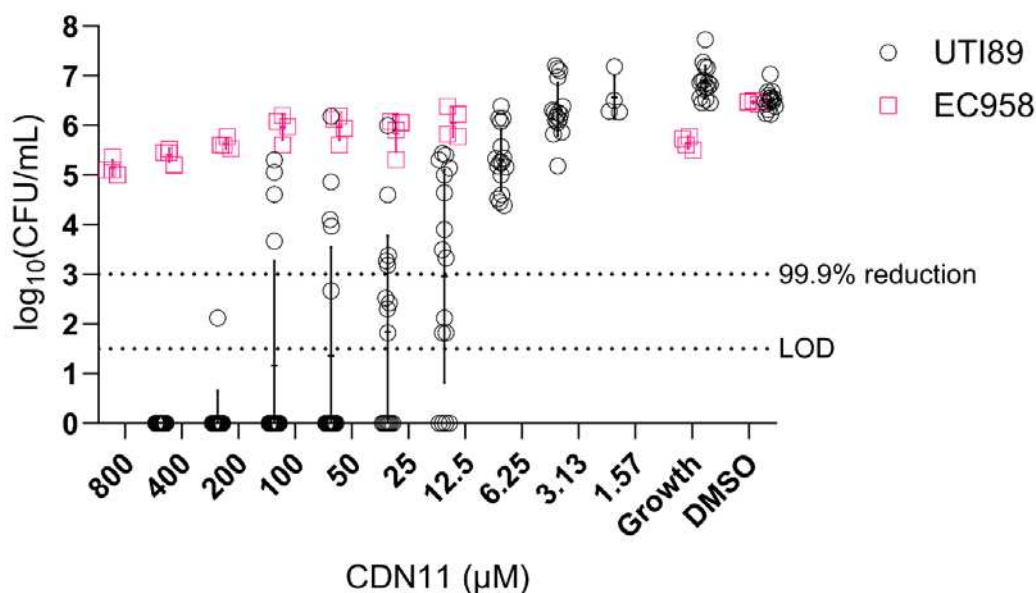


Figure 4. EC958 planktonic and biofilm cells are resistant to CDN11. Minimum biofilm eradication concentration (MBEC) values are reported as complete biofilm eradication (concentration at which there is no recoverable viable biofilm CFU). UTI89 and EC958 biofilms were grown for 24 h using the Calgary Biofilm Device at 37°C with shaking, followed by treatment for 24 h in CDN11 at 37°C with shaking. Biofilms were disrupted with mild sonication for CFU enumeration. Enumerated bacterial CFU was plotted as $\log_{10}(\text{CFU/mL})$. Data represents $n = 16$ (UTI89) and $n = 4$ (EC958) biological repeats with means and standard deviation shown. The technical limit of CFU detection (LOD) and the 99.9% CFU reduction cut-off (compared to vehicle control) are marked by dotted lines.

4. Discussion

This study evaluated a new hybrid antibiotic as a UTI therapeutic in various preclinical models that replicate clinically important aspects of the disease. Currently, the only therapeutics that treat the causative agents of UTI are antibiotics. Biofilms formed on patient catheters however are difficult to treat with antibiotics alone, and are often hard to eradicate due to high antibiotic tolerance [38]. During UTI, the formation of biofilms and intracellular bacterial reservoirs contribute to disease persistence and recurrence. Greater emphasis should be placed on targeting biofilms for treating high-burden infections like UTIs. CDN11 is an antibiotic conjugate designed to target biofilm bacteria through its nitroxide moieties that were previously demonstrated to display biofilm dispersal activity [20,39]. Here we report that administration of CDN11 can effectively control UPEC infection in relevant preclinical models, significantly reducing the overall bacterial burden associated with infection on both biotic and abiotic surfaces, and in some cases even clearing UPEC from infected cells and tissues. Our study is the first to showcase the potential of this hybrid antibiotic as a new UTI therapeutic.

The Calgary biofilm device (biofilms cultured on pegs) is widely used as a simple high-throughput assay to assess antibiofilm activity of new potential therapeutics [40]. Using this assay, we determined the MBEC value of CDN11 as the minimum concentration required to fully eradicate UPEC biofilms (i.e. kill 100% of cells), not reduce 99.9% of the cells compared to growth controls that is defined as the biofilm bactericidal concentration (BBC) and is often used interchangeably with MBEC [41]. This concentration guided our downstream testing in biofilm models of UPEC in the context of UTI. We could translate our findings from the peg model to the catheter model, which

more closely replicates aspects of UTI disease, such as biofilm formation on patient catheters in CAUTI. Using concentrations and treatment durations derived from the peg model, we noted similar trends between *in vitro* models. While catheter biofilms were not completely eradicated by CDN11 treatment at 1 × MBEC, the observed reduction in biofilm CFU was significant (over 4-logs), bringing remaining CFU very close to the limit of detection for this assay, which is characteristically low (~35 CFU/peg). The *in vitro* catheter model simulates some clinically relevant factors of CAUTI, such as replicating bacterial titers seen on patient catheters and physicochemical interactions with catheter materials. The latter makes it an especially useful model for testing different catheter coatings that may contain antimicrobial compounds to limit biofilm formation or disrupt established biofilms [42]. However, the catheter model has limitations in representing important host factors during CAUTI, such as fibrinogen deposition and recruitment of inflammatory immune cells [16,43].

In order to replicate more host-pathogen interactions during *in vitro* testing, we included a human cell infection model. UPEC *in vitro* infection of human bladder cells is commonly employed in UPEC pathogenesis studies and has been shown to recapitulate the key initial steps in UPEC pathogenesis in the urinary tract: initial FimH-mediated adherence to epithelial cells followed by UPEC uptake into the cell and intracellular persistence [33,44,45]. Important host factors necessary for this initial adhesion step are the mannosylated uroplakins present on the surface of bladder epithelial cells [46], and proinflammatory cytokines IL-6 and IL-8, which are responsible for the recruitment of immune cells to the bladder [47,48], both of which can be recapitulated using the T24 bladder cell line [49,50]. During UTI, UPEC strains such as UTI89 are able to persist inside host cells, so by using the gentamicin protection assay model [33], we can test for compound activity specifically against intracellular bacteria. In these models, UPEC are mostly planktonic, but have been observed to form IBC-like structures [51]. While these have largely been observed in mouse UTI models and patient urine [4,52], several *in vitro* culture models have reported the presence of structures similar to IBCs [33,53]. CDN11 showed antimicrobial activity against both planktonic and biofilm-like UPEC in the human cell infection model, suggesting that it can penetrate mammalian cells to access intracellular bacteria, albeit less effectively than ciprofloxacin.

Cell culture models can also discern potential compound cytotoxicity, an important step before progressing to *ex vivo* and *in vivo* testing. In a previous study, CDN11 exhibited no cytotoxicity to T24 bladder cells even up to a concentration of 500 μM [24], which exceeds the concentration used here to treat infected T24 cells. We did however note some morphological changes to T24 monolayers (cell rounding) that appears to be due partly to the vehicle (DMSO) used, which has been previously observed in other studies using bladder and other cell lines [54,55]. While commonly used cytotoxicity assays like the lactate dehydrogenase (LDH) assay can inform about a compound's mammalian cell toxicity, they are not indicative of alterations to cell-cell interactions. These are important in the context of many mucosal infections, including UTI, and have important implications for example in maintaining bladder tissue integrity during infection, which is important for limiting extensive UPEC invasion during UTI [56,57]. Investigating drug effects on cell-cell interactions in this model could screen out compounds that may disrupt the bladder architecture, before progressing them to *in vivo* testing.

In vivo infection models provide a powerful preclinical model for testing UTI therapeutics that replicate interactions between multiple tissues/cell types, UPEC (extracellular and intracellular reservoirs), the candidate compound, and host physiology. The mouse UTI model is widely used in the field to investigate host-pathogen interactions and potential drug candidates [34]. Here we treated infected mouse bladders for 6 hours with CDN11, to simulate the half-life of ciprofloxacin in the human body, while the dosage (1xMBEC; 400 μM) roughly equated to half the therapeutic ciprofloxacin dose commonly prescribed to adults for acute UTI [58,59]. The mouse UTI model can be adapted to study both acute and chronic UTI, and allows for many measurable outcomes, such as bacterial load in urine and urinary organs, immune responses, and tissue morphology changes. In the mouse UTI model, UPEC can be found as free-living (planktonic) extracellular bacteria, in an IBC (biofilm), or as filamentous cells upon exiting infected urothelial cells [4,60]. Using a model that incorporated filamentous UPEC, QIRs, recruited immune cells (which release nitric oxide), and the

bladder's tight cell layer architecture would provide insights into how the compound might interact with host responses and penetrate tissue layers, which would provide clinicians and researchers alike with more detailed information about a candidate compound's activity [14,60,61]. We noted that throughout all our testing of CDN11, it retained antibiofilm activity in *in vitro* and *ex vivo* treatment models.

We observed that CDN11 is a potent antibiofilm agent in *in vitro* models, outperforming ciprofloxacin in both peg and catheter models, despite its lowered antibiotic activity against planktonic UTI89. We further probed CDN11's antibiofilm activity by testing its activity against the ciprofloxacin-resistant strain EC958. Both planktonic and biofilm EC958 were resistant to high concentrations of CDN11 (>800 μM for MIC and MBEC), suggesting that CDN11's bactericidal mode of action is the same against planktonic and biofilm bacteria. Loss of activity against ciprofloxacin resistant strains also suggests CDN11 still targets the same enzymes as ciprofloxacin. Given that nitroxides alone are not bactericidal [39], it is most likely the dual action of the nitroxide and antibiotic that is responsible for the enhanced activity seen against UTI89 biofilms, which are ciprofloxacin sensitive.

We observed less CDN11 activity in models where biofilms formed on biotic surfaces, and in particular intracellularly (human cell infection assays and infected mouse bladder *ex vivo* treatment). Comparing CDN11 to ciprofloxacin, CDN11 has a much higher molecular weight (952.20 g mol^{-1} vs. 331.34 g mol^{-1}), suggesting that it may have reduced cell permeability of both bacterial and mammalian cells [62,63]. Other factors known to reduce cell permeability and tissue penetration include a higher polar surface area and bond flexibility [62], both of which are increased for CDN11 compared to ciprofloxacin. Ciprofloxacin's bactericidal activity results from its binding and inhibition of DNA gyrase and topoisomerase IV [64], and so is most potent when inside bacterial cells. Despite a possible decrease in cell permeability, CDN11 was still able to reduce bacterial numbers in all models tested. Therefore, in chemically linking an antibiotic to nitroxide(s), there may be some loss in activity against planktonic bacteria but an enhancement of activity against biofilms formed on abiotic surfaces, as reported here. We also speculate that CDN11 might have reduced activity against UTI89 biofilm-like cells (IBC and filamentous) present in cell infection assays and murine bladders as a result of its reduced mammalian cell permeability.

A challenge in designing effective antimicrobials for UTI is catering to the different niches and forms of UPEC during pathogenesis. Drugs that can access intracellular UPEC would be desirable, as extracellular UPEC can be more readily accessed by antibiotics and immune cells, and also voided through urine. Additionally, drugs with antibiofilm activity would be beneficial for targeting catheter biofilms and IBCs. CDN11's reduced activity in cellular infection models lends itself to being better purposed as a topical antibiofilm agent, as it had better activity in the catheter biofilm model. However, CDN11 could be further chemically modified to attempt to address these shortcomings, given its ability to reduce recoverable bacteria in all models. Alternatively, first-line antibiotics such as fosfomycin and nitrofurantoin [27] have yet to be hybridised with nitroxides and present an unexplored avenue for UTI antibiofilm agents. To date, several other antimicrobial agents have been hybridised to nitroxides, and have shown similar trends in antibiofilm activity against their target species [21,65,66]. Thus paired with our data, nitroxide antibiotic hybridisation is a promising strategy for eradicating biofilms, especially in infections that involve biofilms or biofilm-like structures. However in order to design and synthesise candidates with a high chance of therapeutic success, more fundamental investigations need to be conducted into the mode of action of nitroxides. Understanding how nitroxides impact biofilms will be key to designing better therapeutics that will reflect better efficacy in preclinical models, allowing for faster translation into human trials.

5. Conclusions

In conclusion, we present here a robust pipeline for preclinical testing of drugs targeting bacterial biofilms in urinary tract infections, using the nitroxide ciprofloxacin hybrid CDN11 as a candidate. New antibiotic development is arduous and requires large investments of time and money [67]. As new compounds and antibiotics become available, streamlining their testing through

relevant preclinical pipelines that incorporate different aspects of the disease represents an efficient and time-saving approach to selecting lead candidates for clinical development.

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Data Availability Statement: We encourage all authors of articles published in MDPI journals to share their research data. In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Where no new data were created, or where data is unavailable due to privacy or ethical restrictions, a statement is still required. Suggested Data Availability Statements are available in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>.

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