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CD4-PP a novel strategy to treat and prevent urinary tract infections

Emelie Alsheim

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Department: Medical Biochemistry and Microbiology

Supervisor: Prof. Annelie Brauner and Dr. Soumitra Mohanty, Karolinska Institute, Stockholm

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Abstract:

Globally, there are 150 million cases of urinary tract infections (UTIs) annually, the majority of which occur in women. Generally, these infections can be treated with antibiotics, but due to increased spread of antimicrobial resistance, UTIs are becoming harder to treat. Antimicrobial peptide (AMP) cathelicidin LL-37 is known to be upregulated in UTIs. The aim of this study was to determine the activity of its synthetic cyclised derivative CD4-PP as a novel treatment. CD4-PP activity was investigated against *Escherichia coli, Enterococcus faecium, E. faecalis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* CD4-PP showed cytotoxicity in uroepithelial cell line T24 at 25µM, similar as LL-37. CD4-PP showed to have a 10-fold lower MIC value compared to LL-37 for several clinical pathogens, inhibiting strains at 0.39-3.125µM. *In vitro* infection of uroepithelial cells treated with 2µM CD4-PP showed to significantly reduce relative survival of *E. coli, E. faecium* and *K. pneumoniae.* CD4-PP showed to upregulate gene expression of tight junction proteins claudin-14 and occludin, as well as the parent peptide LL-37 during treatment in both uninfected and infected uroepithelial cells. Protein expression was upregulated for claudin-14 during treatment both in uninfected and infected uroepithelial cells. Occludin and LL-37 were upregulated during treatment in uninfected and infected states, respectively. CD4-PP showed to be active against several uropathogens, with and without intrinsic resistance to other antibiotics, as well as upregulate factors important for prevention of infection.

Key words:
Cathelicidin, CD4-PP, urinary tract infections, tight junction proteins & innate immunity

Popular summary:

A new treatment strategy for urinary tract infections

Globally, there are a 150 million cases of urinary tract infections (UTIs) annually. A UTI is generally caused by bacteria, with 75% of all cases being caused by *Escherichia coli.* The infection occurs in the urethra, bladder and in serious cases, the kidneys. Most UTIs can be treated with antibiotics, but due to an increase in resistant bacteria, and recurrent infections, UTIs are becoming more difficult to treat. The human body produces its own type of antibiotics, known as antimicrobial peptides (AMP). One such is called LL-37 and can inhibit a wide range of bacteria. A synthetic derivative of LL-37 has been produced, CD4-PP. The aim of this study was to see if CD4-PP is active against bacteria causing UTIs, as well as determining how human cells respond to treatment.

Uroepithelial cells make up the outermost layer of cells covering the urethra. The first step was to establish what the highest concentration of CD4-PP was safe for human uroepithelial cells. Next, the lowest concentration necessary to inhibit bacteria causing UTIs was determined. Following this, uroepithelial cells were infected with eight bacterial strains, and then treated with CD4-PP. Four of these strains carried antibiotic resistance and are clinically difficult to treat. This set-up represents a simplified UTI model. Treating these infected cells, resulted in reduced bacterial presence for six of these strains. CD4-PP was effective against both susceptible, and resistant bacterial strains. This shows promise for future studies involving a clinical application of CD4-PP.

After seeing that CD4-PP successfully inhibited bacteria causing UTIs, the effect of the peptide on human cells was determined. Uroepithelial cells were treated with CD4-PP and infected with *E. coli.* Tight junction proteins are involved in maintaining the structure and integrity of epithelial cells. During CD4-PP treatment their expression was upregulated. This is a positive effect, as during an infection, these proteins are downregulated, allowing the cells to slough off. This can allow bacteria to penetrate further into the cell layers. The AMP LL-37, which CD4-PP is derived from, was also upregulated when infected cells were treated with CD4-PP, which can aid in inhibiting urinary pathogens.

Our immune system uses small molecules as signals for attracting cells to help combat pathogens. One of these was upregulated when cells were given CD4-PP in the absence of *E. coli*, but downregulated when CD4-PP was given during an *E. coli* infection. This could potentially cause an issue, as this could result in our cells mounting a too strong immune response. This is something that needs to be further explored.

To summarise, CD4-PP, a synthetic AMP, was shown to successfully inhibit bacteria known to cause UTIs. It also helps in maintaining the cell structure during infection, and by such, aid in prevention of a deeper infection. The exact mechanism of action for CD4-PP is left to be determined, and more information as to how it affects our cells is needed. This study shows for the first time that CD4-PP has the possibility to become a novel treatment for UTIs.

Introduction:

Antimicrobial resistance is a cause for serious global concern, with an increasing spread, leading to more severe morbidity and a higher mortality [1]. This is a consequence of excessive prescription of antibiotics, and the inappropriate use of antibiotics in agriculture and aquaculture [2-4]. Consequently, we now face difficulties in treating common infections due to reduced sensitivity of antibiotics. Urinary tract infections (UTIs) affect 150 million people annually, with an overwhelming majority of these occurring in women [5]. 50-60% of all women are affected at least once during their life, with 25% of these presenting with recurrent urinary tract infections (rUTIs) within 6 months of their first infection [5-7]. Although the infection mainly affects women, it is also an infection which to a great extent affects elderly (>65 years) and children [5, 8-10]. Studies have shown that the median age for a first UTI in boys is at 10 months, and around 4 years for girls, with 1 in 200 infants displaying asymptomatic bacteriuria during their first year [11-12]. Overall, the majority of UTIs (75%) are caused by uropathogenic *Escherichia coli* (UPEC), the rest are generally caused by one of the following: *Klebsiella pneumoniae*, *Enterococcus spp. Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus* and *Candida albicans* [13]. Collectively these species are responsible for 96% of uncomplicated UTIs and 95% of complicated UTIs [5].

Symptoms of UTIs are common frequency and urgency to urinate, dysuria, pelvic and lower back pain, difficulty urinating and blood in urine [14]. A general criterion amongst clinicians for what constitutes as a UTI is the presence of $\geq 10^5$ bacteria per ml [15]. But this has shown to miss nearly half of all UTIs, and a lower threshold of $\geq 10^2$ bacteria per ml have been suggested as more reasonable but does not always require treatment with antibiotics [15-16].

UTIs can be divided up into two general types of UTIs, uncomplicated and complicated. An uncomplicated UTI is due to no functional or anatomical obstructions, renal impairment of other disease that would actively promote a UTI, or risk development of serious complications [17]. Cystitis is the inflammation of the bladder, a lower UTI, or acute uncomplicated cystitis, is acute symptoms such as dysuria and urgency to urinate or voiding [13, 18]. Upper UTI or pyelonephritis is signified by flank pain and persistent fever over 38°C but is not always associated with other common UTI symptoms [13, 18]. There is no certain cause for uncomplicated UTIs, but some risk factors are previous UTIs, sexual activity, diabetes, obesity, genetic susceptibility, and the female gender [5].

Complicated UTIs have associated risk factors compromising the urinary tract or host defence, this includes indwelling catheters [19]. Catheter-associated urinary tract infections (CAUTIs) have been reported to be responsible for up to 80% of all hospital associated infections (HAIs) and is often caused by pathogens carrying antimicrobial resistance [19-20]. Risk of development of CAUTI is associated with prolonged catheterisation, old age, diabetes, and the female gender [21]. Biofilm formation by uropathogens both on and in the catheter is the general origin for CAUTIs, with biofilm formation beginning immediately after catheter insertion [19]. CAUTI is one of the most common sources of urosepticaemia and bacteriaemia in a health care setting [19]. If left untreated a UTI can lead to serious sequelae, such as recurrence, renal damage and pyelonephritis leading to urosepticaemia [5].

Uncomplicated UTIs can generally be treated with antibiotics. However, due to the increase spread and persistence of antibiotic resistance the treatment options are becoming increasingly limited [3]. Presence of naturally occurring endogenous antibiotics is the origin of antibiotic resistance genes, the introduction of antibiotics in a health care setting has driven this evolutionary process further [3]. Bacteria have shown to be able to share plasmids carrying genes for antibiotic resistance or develop resistance due to spontaneous mutations [3, 22].

Resistance can also be selected for, during treatment with high concentrations of antibiotics, as well as due to low concentrations present in the environment [23]. Pathogens can also be resistant to more than one class of antibiotics, where secondary resistance can increase the efficiency of the first [24].

Antibiotic resistance in Europe alone has been estimated to cost €1.5 billion, with 40% of this being due to loss of work productivity [2]. The availability of purchasing antibiotics over the counter without prescription in some countries or via the internet, allows individuals to treat ailments with antibiotics which are not effective, or for an insufficient period [25]. Ultimately several factors contribute to common infections becoming more difficult to treat, and treatments can be extensive, with the patient being required to take several different antibiotics with a multitude of side effects. As mentioned, rUTIs are common amongst women who have already had at least one UTI and requires repeated treatment, these infections are often caused by resistant pathogens [26]. There is an increased incidence in UTIs caused by resistant strains such as extended-spectrum β-lactam (ESBL) *E. coli,* multidrug resistant (MDR) *K. pneumoniae* and *E. coli,* and vancomycin resistant Enterococci (VRE) [2]. Not to mention that several urinary pathogens already carry intrinsic resistance to several classes of antibiotics [27]. Such as *P. aeruginosa* which is resistant to β-lactams and fluoroquinolones [28-29].

Our immune system itself has several methods for combating infections. One such method is the production of antimicrobial peptides (AMPs), broad spectrum oligopeptides that are a part of our innate immune defence [30]. These peptides are produced by several types of cells, such as leukocytes and epithelial cells [31-32]. Discovering new alternatives to conventional antibiotics are paramount due to the rapid spread of bacterial resistance, especially for treatment of UTIs [33]. A problem with development of new antibiotics is that drug companies are aware that a new drug will possibly only be effective for a few years, until resistance is developed. Unfortunately, this makes the search for new antibiotics very expensive, causing a lack of investment, with few of the proposed drugs ever making it to market [4]. From start to finish, production of a new antibiotic has been estimated to cost over $1.5 billion [34]. With the target-to-hit, hit-to-lead and lead optimisation taking approx. 4.5 years, costing alone close to $700 million [35].

Our immune system produces several AMP's, successful in combating infections by bacteria, viruses, and fungi [31]. By utilising this, we can create synthetic derivatives which are more potent than the ones we produce ourselves [36]. Antimicrobial peptides have a broad spectrum of efficacy, being bactericidal against both Gram positive and negative bacteria, regulating inflammatory response and promoting re-epithelization [32]. This shortens the time spent on finding a substance that could potentially work as an antimicrobial and saves millions of dollars in production [32, 37]. As well as there being only a few reports of resistance developed against AMPs, making them the perfect starting points for development of new antimicrobials [37].

Human AMP Cathelicidin LL-37 is upregulated in response to UTIs by uroepithelial cells [38]. LL-37 has an α-helical structure, and is 37 amino acids long, with two leading leucines residues [32]. The function of LL-37 has previously been described by the carpet model on negatively charged membranes [39-40]. Through aggregating on the bacterial surface, and then disrupting the bacterial lipid bilayer by formation of toroidal, or lipid pores. Where LL-37 inserts itself perpendicular to the bilayer, to form a pore, with the bacterial membrane exhibiting positive curvature inwards, to form a pore [41-42].

LL-37 has been truncated to a smaller linear peptide known as KR-12, comprising of residues 18-29 [43]. Research working on developing a synthetic derivative of LL-37 utilising the KR-12 portion has produced several synthetic derivatives by the Department of Pharmacognosy at

Uppsala University [44]. The most successful derivative came from cyclisation of two KR-12 peptides with linkers connecting each, creating CD4-PP, a synthetic cyclised derivative of the parent peptide LL-37. The backbone cyclisation of KR-12 to form CD4-PP showed to increase the antimicrobial activity and make it more proteolytically stable [44]. This method increased the efficacy against a broad spectrum of pathogens, as well as further increase the stability when compared to the linear KR-12 [44].

As mentioned, all forms of UTIs are becoming more difficult to treat with the antibiotics available today. Development of new antibiotics which show low cytotoxicity in human cells, and are effective against the most common pathogens, including those with established resistance, are paramount to successfully treat these types of infections and comprise some of the early steps of drug development [4]. In the aspect of UTIs, an additional benefit would be if new treatment could prevent the exfoliation of uroepithelial cells. As during an infection exfoliation of uroepithelial cells can allow for invasion of pathogens deeper into the tissue [5]. Upregulation of tight junction proteins such as claudins and occludin which are involved in maintaining the structure and integrity of uroepithelial cells could help to prevent this occurrence. Maintenance of the uroepithelium during infection could also allow for the host innate defence to combat the infection by expressing intrinsic AMPs, such as LL-37.

Aims:

The aim of this study was to see if CD4-PP would be active against treating common urinary pathogens *in vitro* and evaluate the host cell response of tight junction proteins claudin-14 and occludin, AMP LL-37 and chemokine IL-8 when uroepithelial cells are treated with CD4-PP in both uninfected and infected states. Common urinary tract pathogens *E. coli, E. faecalis, E. faecium, K. pneumoniae* and *P. aeruginosa* has here been of main interest. These pathogens are involved in UTIs ranging from uncomplicated, to severe cases leading to serious sequelae and in some cases death [45-46]. To make this study more applicable to clinical infection biology, patients samples collected at the Karolinska University Hospital, Stockholm, have been included. This to expand on the possible effect of CD4-PP, beyond type strains, and puts the peptide in a clinical perspective.

Materials and methods:

Antimicrobial peptides (AMPs):

1mM stock of antimicrobial peptides (AMPs) cathelicidin LL-37, linear derivative KR-12 and cyclic derivative CD4-PP all diluted in micellar water was produced and kindly provided by Dr. Taj Muhammad and Prof. Ulf Göransson, at the Department of Pharmacognosy, Uppsala University.

Cell lines:

Human uroepithelial cell lines T24 (ATCC HTB-4) and 5637 (ATCC HTB-9) were used. Cell line T24 was cultured using McCoy's 5A (ref. 26600-023, Gibco) and 5637 with RPMI-1640 (ref. 11879-020, Gibco) in 10% heat inactivated foetal bovine serum (FBS) (ref. 10270106, Gibco, country of origin Brasil). Cells were continuously grown in the absence of antibiotics.

Bacterial isolates:

ATCC and CCUG type strains used can be seen in *Table 1*. Clinical isolates used for minimal inhibitory concentration (MIC) were isolated at the Department of Clinical Microbiology at the Karolinska University Hospital, Stockholm.

Table 1. **Type strains used in this study and relevant information.**

Bacterial species:	Type strain:	Additional information:
E. coli	CFT073	Uropathogenic (UPEC)
E. coli	CCUG 55197	Extended Spectrum β-Lactamase (ESBL)
E. faecalis	ATCC 29212	
E. faecium	CCUG 33829	
E. faecium	CCUG 36804	Penicillin and Vancomycin resistant
K. pneumoniae	ATCC 25955	
K. pneumoniae	CCUG 58547	ESBL, Carbapenem, cephalosporin, quinolone, and trimethoprim resistant (MDR)
P. aeruginosa	ATCC 27853	
P. aeruginosa	CCUG 51971	VIM-4 metallo-beta-lactamase, carbapenem resistant

Table 1 show the ATCC and CCUG codes for each type strain used in this study, with any associated additional information available for each strain.

Cell cytotoxicity assay:

Cell line T24 was grown in a 96-well plate and when reached 80% confluency, were treated with 2-fold dilutions starting at 100µM of LL-37, 200µM of KR12 and 100µM of CD4-PP. Cells were incubated for 24 hours in 37°C. Cells were washed twice with 1X PBS. 200µl freshly prepared media (McCoy's 5A with 10% FBS) containing XTT-menadione was added to each well. XTT-menadione was prepared by adding 2.15µl menadione (Sigma) (172.18g/ml) per ml XTT (Sigma), with 1mg/ml XTT-menadione being dissolved in 1X PBS.

Wells containing only media and XTT-menadione served as background control. Cells were incubated for 3 hours at 37°C with 5% CO_2. Plate was measured at 450 nm and 690nm using the EZ read 400 microplate reader (Biochrom). Where the 690nm measurement (background) was subtracted from the 450nm value. This assay was done in thrice with three natural replicates.

Cytotoxicity for 2 and 10µM CD4-PP for cell line 5637 was completed by Dr. Soumitra Mohanty and Ciska Lindelauf.

Minimal inhibitory concentration (MIC):

Clinical isolates from *E. coli, E. faecalis, E. faecium, K. pneumoniae* and *P. aeruginosa* were grown overnight on blood agar (BA) at 37°C. Single colonies were inoculated in 3% trypticase soy broth (TSB) and incubated at 37°C whilst shaking for 2-4 hours to reach log phase. Inoculum was centrifuged at 2500 rotations per minute (RPM) for 5 minutes and supernatant was discarded. Pellet was resuspended in 3ml 10mM Tris buffer and washed twice.

Concentration of bacteria was measured using a densitometer and adjusted to 0.5 McFarland turbidity. Bacterial concentration was adjusted to 10^6 colony forming units (CFU)/ml by diluting 0.5 McFarland suspension in a 1:150 ratio in 10mM Tris. 50µl 10^6 CFU/ml were added to U-bottomed 96-well plates (polystyrene). Intermediate fold-dilutions starting from 50µM down to 0.78µM of the different AMPs was made by diluting 1mM stocks in 10 mM Tris. 50µl

of each concentration was added to the wells with the bacterial suspension. Making a total of 100µl in each, with final concentrations of AMPs ranging from 25 to 0.39µM.

Wells containing only 10mM Tris and peptide served as blank. Bacterial suspension with AMPs were incubated for 1 hour at 37°C. After 1 hour, 5µl of 20% TSB was added to each well, the plate was then further incubated for another 16 (± 2 hours) at 37°C. MIC was determined by reading the optical density at 598nm using the EZ read 400 microplate reader (Biochrom).

Median MIC assay:

Determination of MIC for each species was based on the above assay, where approx. 10 isolates were used. The median MIC from this assay was used as the lone concentration to investigate the MIC for the rest of the clinical isolates (to make up a total of 20 clinical isolates for each species). The steps were the same as that of the MIC, with only one concentration being used for each species.

Infection assay:

Uroepithelial cell line T24 was seeded with McCoy's 5A medium with 10% FBS and cell line 5637 was seeded using RPMI-1640 with 10% FBS in 24-well plates (Sarstedt), until reaching 80% confluency. All bacterial strains in *Table 1*, excluding *E. faecalis*, was streaked on blood agar and incubated at 37°C overnight.

Three conditions were established and done in duplicate. Uroepithelial cells with only infection served as control. Pre-infection is defined as uroepithelial cells infected with MOI 5 and treated with 2µM CD4-PP at 0 hours. Post-infection is defined as uroepithelial cells infected with MOI 5 and treated with 2µM CD4-PP after 2 hours.

Few bacterial colonises were suspended in 3ml 1X PBS and centrifuged at 1000 RPM for 3 min. Working stock of 10^8 CFU/ml was determined through absorbance at 600nm, which was further diluted to 10^7 CFU/ml using McCoy's 5A (serum and antibiotics free) for T24, and RPMI-1640 (serum and antibiotics free) for 5637. Cells were infected with multiplicity of infection (MOI) 5 of 10^7 CFU/ml bacterial dilution, and pre-infection were treated with 2µM CD4-PP. Cells were incubated for 2 hours at 37°C, 5% CO_2.

Pre-inoculum density (PID) is the CFU present in the 10^7 CFU/ml working stock and allows comparison between how many bacteria was added to the uroepithelial cells at the start versus the end of the experiment. PID was determined by diluting 10^7 stock down to 10^3 CFU/ml, and plating 50µl on blood agar and incubating them at 37°C overnight.

After 2 hours of infection, media was removed, and cells were washed with 500µl 1X PBS thrice. Fresh media was added, both post-and pre-infection was treated with 2µM CD4-PP. Cells were further incubated for 2 hours at 37°C with 5% CO_2. Media was removed, and cells were washed with 1ml 1X PBS once. 200µl of ice-cold 1X PBS with 0.1% Triton X-100 was added, and wells were scraped. Lysate was serially diluted and 50µl was plated on blood agar and incubated overnight at 37°C to verify intracellular count.

Cell line T24 was used for all infections except *E. coli* CFT073, where cell line 5637 was used. This assay was done thrice for all species but *E. coli* CFT073 which was done four times, with two natural replicates for all.

NO production:

Cell line T24 was grown in a 24-well plate until reaching 80% confluency. *E. coli* CFT073 was grown on blood agar overnight at 37°C. A few colonies were suspended in 3 ml 1X PBS,

vortexed and centrifuged for 1000 RPM for 3 minutes. Supernatant was made to 10^7 CFU/ml in 1X PBS by first measuring OD at 600nm and preparing 10^8 CFU/ml.

Four conditions were done, all in triplicate and with McCoy's 5A with 10% FBS. Control contained only media (set one). Set two was treated with 2µM CD4-PP, set three with MOI 5 *E. coli* CFT073, and set four with 2µM CD4-PP and MOI 5 of *E. coli.* Plate was centrifuged for 3 minutes at 1000 RPM and incubated for a total of three hours at 5% CO_2 in 37°C.

Every hour for three hours 150µl of supernatant was mixed with 130µl distilled water and 20µl Greiss reagent (ref. G7921, Invitrogen) in a flat bottomed 96-well plate. One blank was 150µl water, and one was 150µl McCoy's 5A with 10% FBS. Plate was incubated for 30 minutes in the dark at room temperature, and then read at 562 nm using the EZ read 400 microplate reader (Biochrom).

Biofilm:

E. coli CFT073 was grown overnight on Luria Broth (LB) plates without NaCl in 37°C. Few colonies were suspended in 3 ml 1X PBS and centrifuged at 1000 RPM for 3 minutes. Working stock of 10^8 CFU/ml was determined by OD at 600nm, and further diluted to 10^6 CFU/ml in LB without NaCl.

Prevention of initiation of biofilm was set up by diluting CD4-PP from 1mM stock in LB without salt to a quarter of the *E. coli* MIC (see *Table 3*). 50µl 10^6 CFU/ml *E. coli* and 150µl CD4-PP dilution was added in triplicate for each concentration in a flat-bottomed 96 well plate (Costar). Media control was LB without salt only, positive control was with *E. coli.* Plate was incubated for 72 hours at 37°C.

After 72 hours, media was removed, and wells were washed thrice with 225µl 1X PBS, and stained with 225µl 0.03% crystal violet for 10 minutes. Crystal violet (ref. EUH216, ELITech Group) was removed, and wells were washed thrice with 230µl sterile water and left to air dry. The biofilm was dissolved in 225µl of 80% acetone and 20% ethanol and left on a shaker for 10 minutes. Absorbance was read at OD 570 using the EZ read 300 microplate reader (Biochrom). Value from media control was deducted as blank. This assay was done in triplicate with three natural replicates.

Host cell response:

Cell line 5637 was used for measuring gene expression of *CLDN-14*, *OCLN* and *CAMP*. Cell line T24 was used for expression of *CXCL-8.* Uroepithelial cells were grown in a 24-well plate until they reached 80% confluency. Four conditions were established, all done in triplicate, with three natural replicates. Cells grown in only media served as control. Treatment with 10µM CD4-PP, infection with *E. coli* CFT073, or cells both infected with *E. coli* CFT073 and treated with CD4-PP where the three other conditions.

E. coli CFT073 was grown overnight on blood agar in 37°C. Working stock of 10^8 CFU/ml was prepared in 1X PBS, and further diluted to 10^7 CFU/ml in 1X PBS. MOI 5 of 10^7 CFU/ml dilution of *E. coli* CFT073 was added to uroepithelial cells. Simultaneously treated sets were treated with 10µM CD4-PP. Plate was centrifuged at 1000 RPM for 3 minutes, and incubated at 37C, 5% CO_2 for two hours. After incubation, media was removed, cells were washed thrice with 500µl 1X PBS and 350µl RLT lysis buffer (Qiagen) was added.

RNA was extracted according to the RNeasy Midi Kit instructions (Qiagen). RNA quality was measured using nanodrop (ThermoFisher). cDNA was transcribed using 200-300ng RNA using cDNA reverse transcription kit (Applied Biosystems).

Manufacturers protocol for SYBR Green expression assay (Applied Biosciences) was followed to measure the expression of *CLDN-14, OCLN, CAMP* and *CXCL-8* using the Rotor Gene RG-3000 (Applied Biosciences). Comparison in expression was to housekeeping gene *ACTB*. Forward and reverse primers used for each gene can be seen in *Table 2* and was provided by Integrated DNA Technologies.

Table 2. **Primers used for qPCR:**

Gene	Forward primer	Reverse primer
CLDN-14	5'-TGTACCTGGGCTTCATCTCC-3'	5'-CCTCGCATTCACATTATTTCC-3'
OCLN	5'-CTCCCATCCGAGTTTCAGGT-3'	5'-GCTGTCGCCTAAGGAAAGAG-3'
CAMP	5'-ACCCAGCAGGGCAAATCT-3'	5'-GAAGGACGGGCTGGTGAA-3'
CXCL-8	5'-AAGAGAGCTCTGTCTGGACC-3'	5'-GATATTCTCTTGGCCCTTGG-3'
ACTB	5'-AAGAGAGGCATCCTCACCCT-3'	5'-TACATCGCTGGGGTGTTG-3'

Table 2 shows forward and reversed primer for *CLDN-14, OCLN, CAMP, CXCL-8* and *ACTB* used to measure the host response regarding infection with *E. coli* and treatment with CD4-PP.

Confocal microscopy:

5637 cells were grown on coverslips in RPMI-1640 with 10% FBS in a 24-well plate until they reached 80-90% confluency. Four conditions were established all done in triplicate, with three natural replicates. Cells grown in only media served as control. Treatment with 10µM CD4-PP, infection with *E. coli* CFT073, or cells both infected with *E. coli* CFT073 and treated with CD4-PP were the other three conditions. Protein expression was measured for claudin-14, occludin and LL-37.

Sets which were treated with CD4-PP, was treated with 10µM CD4-PP at 0 hours. Plate was then centrifuged at 1000 RPM for 3 minutes, and the plate was incubated at 5% CO_2 in 37°C. After four hours, MOI 5 of *E. coli* CFT073 10^7 CFU/ml stock was added to infected sets. *E. coli* CFT073 had been grown overnight on blood agar in 37°C. Working stock of 10^8 CFU/ml was prepared in 1X PBS, and further diluted to 10^7 CFU/ml in 1X PBS. Plate was again centrifuged at 1000 RPM for 3 minutes and incubated for another 2 hours at 5% CO_2 in 37°C.

Media was removed, and slides were fixed with 4% PFA (paraformaldehyde, Sigma) for 15 minutes and was then washed with 150µl 1X PBS twice. Cells were permeabilised with 50µl freshly prepared 0.1% Triton-X 100 for 5 minutes, and then blocked with 50µl of 5% bovine serum albumin (BSA) for 30 minutes. Staining with 50µl of primary antibody in 1:200 was diluted in 1:1 1X PBST (1X PBS with 0.1% Tween) and 5% BSA and was left at 4°C overnight. Primary antibody was used against claudin-14 (ref. Ab-19035, abcam), occludin (ref. Sc-133256, Santa Cruz Biotechnology) and LL-37 (ref. Sc-166770, Santa Cruz Biotechnology).

Next day cells were washed thrice with 150µl 1X PBST, and then stained with 1:400 secondary antibody in 1:1 PBST and 5% BSA and incubated in the dark for 1 hour. Claudin-14 was stained with anti-goat Alexa fluor dye 488 (A-21467, Life Technologies Cooperation), occludin and LL-37 with anti-mouse Alexa Fluor dye 594 (ref. A-11032, Invitrogen) and 647 (ref. A-21235, Life Technologies Cooperation) respectively. Cells were washed again thrice with 150µl PBST and stained with 50µl DAPI (ref. D3571, Invitrogen) in a 1:1000 dilution in 1X PBST and incubated for 15 minutes. Cells were washed thrice with 150µl 1X PBST and mounted on glass slides with 3µl Flouromount G (ref. 01001, Southern Biotech), and finally observed under Zeiss LSM 700 confocal microscope with 63X oil immersion objective. Imaging was performed by Dr. Soumitra Mohanty and Dr. John White.

Statistical analysis:
All statistical analysis was calculated using GraphPad Prism 5. Infection assays were calculated by using the one-way ANOVA Dunnett's multiple comparison test. qPCR significance was calculated using an unpaired t-test. The protein expression was calculated using a paired t-test, as these values were normalised to the controls. Outliers were calculated using the Grubb's test.

Results:

CD4-PP shows 10-fold difference in MIC from LL-37:

The toxicity in uroepithelial cells of the novel peptides KR-12 and CD4-PP has never previously been established. Uroepithelial cell line T24 was treated for 24 hours with 2-fold dilutions starting at 100µM for parent peptide LL-37 and cyclic derivative CD4-PP, and 200µM for linear derivative KR-12. After 24 hours, XTT-menadione was added to signal cell viability after treatment. LL-37 and KR-12 cytotoxicity was 25µM and 200µM respectively in uroepithelial cell line T24. Same cell line showed cell viability of approx. 88% when treated with 25µM CD4-PP (*Supplementary, Figure S1*).

To determine the ability of the AMPs to inhibit common uropathogens a MIC assay was set up. Based on the cytotoxicity results, the highest concentration used for all AMPs was 25µM. 20 clinical isolates of *E. coli, K. pneumoniae, E. faecium, E. faecalis* and *P. aeruginosa* were used to establish the median MIC using 2-fold dilutions of the different AMPs starting at 25µM. Bacterial inoculum for each isolate was treated with the different AMPs for a total of 16-18 hours after which the optical density was measured. Information regarding sampling site of these isolates can be found in Supplementary *(Table S1*). The MIC for clinical isolates from the same species with LL-37 and KR-12 was also determined (*Supplementary, Table S2*).

LL-37 showed nearly a 10-fold difference in comparison to CD4-PP, with some clinical isolates having an MIC ≥ 25µM. Treatment with KR-12 showed an MIC over 25µM in most of the clinical isolates. Cyclic peptide CD4-PP showed to in comparison have a more promising MIC.

E. coli, E. faecium and *P. aeruginosa* showed to have a median MIC of 0.39µM CD4-PP. Where 12/20 *E. coli,* 11/20 *P. aeruginosa* and 15/20 *E. faecium* isolates showed to be inhibited at the median MIC. *K. pneumoniae* showed a slightly higher median MIC, where all 20 isolates showed to be inhibited at 1.56µM CD4-PP. *E. faecalis* showed a higher MIC, with only 5/20 being inhibited at 3.125µM CD4-PP, and hence the median MIC for this species is expected to be above 3.125µM. (*Table 3).*

Table 3. **CD4-PP MIC values for clinical strains**

Species	CD4-PP median MIC *(N = 20)*	% of isolates with median MIC (*N = 20)*
Escherichia coli	0.39µM	60% (n = 12)
Enterococcus faecium	0.39µM	75% (n = 15)
Enterococcus faecalis	3.125µM	25% (n = 5)
Pseudomonas aeruginosa	0.39µM	55% (n = 11)
Klebsiella pneumoniae	1.56µM	100% (n = 20)

Table 3 shows median MIC for clinical isolates (*N =20)* of *E. coli, E. faecium, E. faecalis, P. aeruginosa* and *K. pneumoniae.*

Treatment of infected uropathogenic cells shows reduction of bacterial load in infection:

To establish if CD4-PP could inhibit uropathogens *in vitro*, the effect of CD4-PP on intracellular pathogens was determined through infection assays. Uroepithelial cells were treated with 2µM CD4-PP and infected with MOI 5 of susceptible and resistant strains of either *E. coli, E. faecium, K. pneumoniae* or *P. aeruginosa* for a total of 2 or 4 hours (the ATCC or CCUG strains for this can be seen in *Table 1*). After the infection, the uroepithelial cells were lysed and the lysate was serially diluted to compare the inhibition of intracellularly associated bacteria, to untreated control. The 2µM concentration of CD4-PP was chosen as it was above the MIC for these species, and to show whether one concentration would be sufficient to inhibit an array of pathogens. *E. faecalis* had a median MIC above 2µM and was not used for the infection assays.

As previously shown by Chagneau et.al, the UPEC strain CFT073 causes great stress to uroepithelial cell line T24, causing cells to quickly detach and die [47]. For this reason, the CFT073 *E. coli* infection was completed using uroepithelial cell line 5637, which does not show the same behaviour as T24 during infection with this strain. *E. coli* CFT073 were nearly completely inhibited when treated with 2µM CD4-PP at 0 hours infection (*fig 1a).* Giving treatment after 2 hours of infection showed more survival of bacteria, but still significantly less (p-value ≤ 0.01) than control. ESBL producing *E. coli* CCUG 55971 was significantly inhibited at both time points (p-value ≤ 0.001) compared to control (*fig 1b).* Relative survival of *E. coli* CCUG 55971 can be seen to be much lower at treatment at 0 hours compared to 2 hours.

***Fig 1*. Infection assay for *E. coli* CFT073 and CCUG 55971**

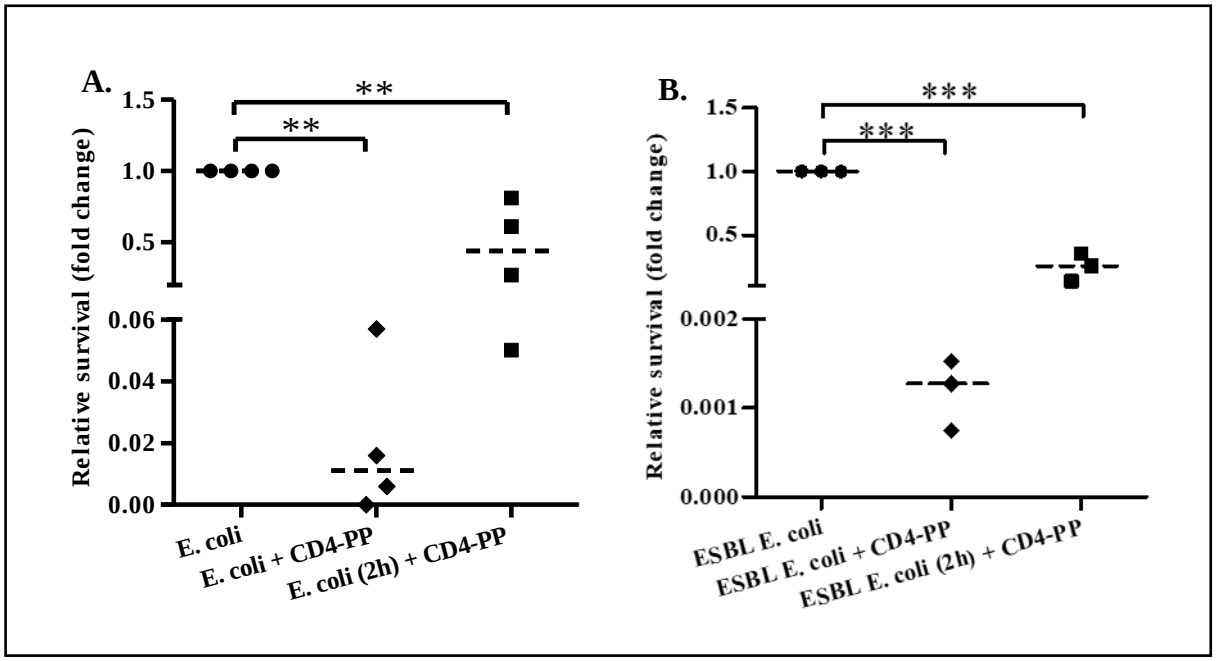

Fig 1. Results from infection assays, shows relative survival compared to the control infection, to treatment with 2µM CD4-PP at 2 and 4 hours. Survival assay for A) *E. coli* CFT073 was conducted in cell line 5637, p-value ≤ 0.01 for treatment at 0 and 2 hours. B) *E. coli* CCUG 55971 was conducted in cell line T24, p-value ≤ 0.001 for treatment at 0 and 2 hours. Statistics were calculated using the ANOVA Dunnett's multiple comparison test.

K. pneumoniae ATCC 25955 and MDR *K. pneumoniae* CCUG 58547 were inhibited at both time points (*fig 2a-b).* With treatment of CD4-PP at 0 and 2 hours significantly reducing the relative survival (p-value ≤ 0.001). For both strains treatment at 0 hours show less survival of bacteria compared to treatment at 2 hours.

***Fig 2*. Infection assay for *K. pneumoniae* ATCC 25955 and CCUG 58547**

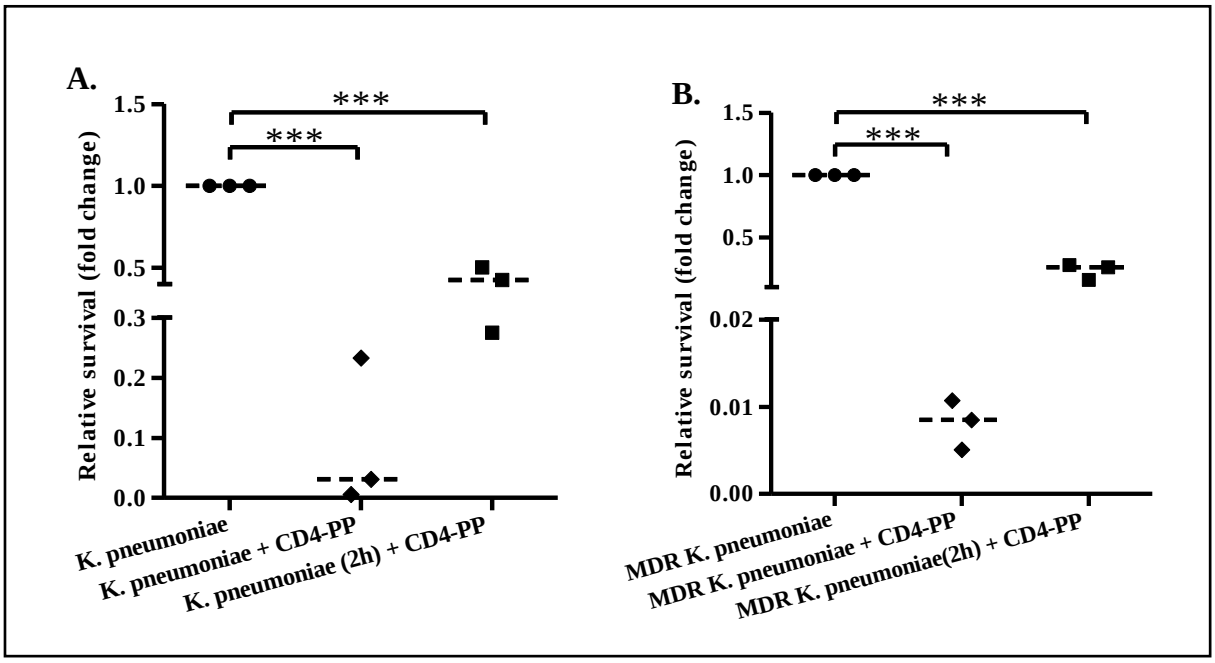

Fig 2. Results from infection assays, shows relative survival compared to control infection, to treatment with 2µM CD4-PP at 2 and 4 hours. Survival assay for both was done in cell line 5637. A) Shows *K. pneumoniae* ATCC 25955, p-value ≤ 0.001 for treatment at 0 and 2 hours. B) *K. pneumoniae* CCUG 58547, p-value ≤ 0.001 for treatment at 0 and 2 hours. Statistics were calculated using the ANOVA Dunnett's multiple comparison test.

P. aeruginosa ATCC 27853 was inhibited when treated at 0 hours (p-value ≤ 0.5) (*fig 3a).* But treatment after 2 hours showed no difference compared to control. *P. aeruginosa* CCUG 51971 was not inhibited at either time points (*fig 3b).*

***Fig 3*. Infection assay for *P. aeruginosa* ATCC 27853 and CCUG 51971**

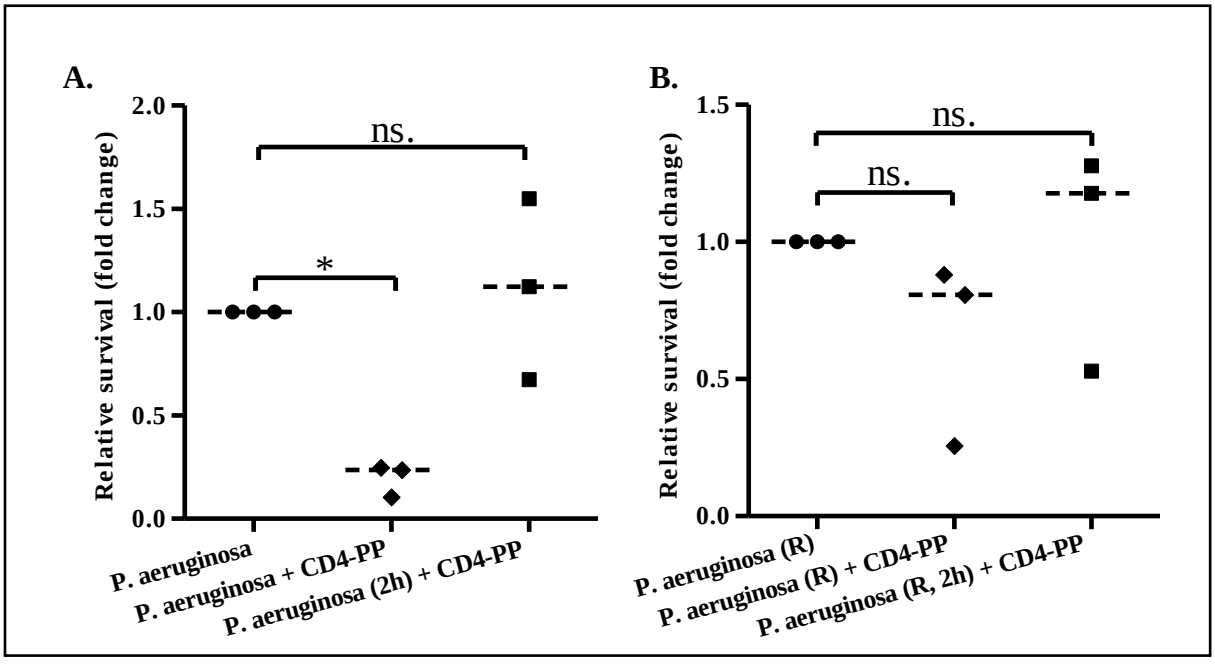

Fig 3. Results from infection assays, shows relative survival compared to control infection, to treatment with 2µM CD4-PP at 2 and 4 hours. Survival assay for both was done in cell line 5637. A) Shows *P. aeruginosa* ATCC 27853, p-value ≤ 0.05 for treatment at 0 hours, ns at 2 hours. B) *P. aeruginosa* CCUG 51971, p-value was ns at treatment at both 0 and 2 hours. Statistics were calculated using the ANOVA Dunnett's multiple comparison test.

CD4-PP showed to inhibit *E. faecium* CCUG 33829 and CCUG 36804 significantly (p-value ≤ 0.001), nearly completely clearing the infection in T24 in both time points (*fig 4a-b)*.

***Fig 4*. Infection assay for *E. faecium* CCUG 33829 and CCUG 36804**

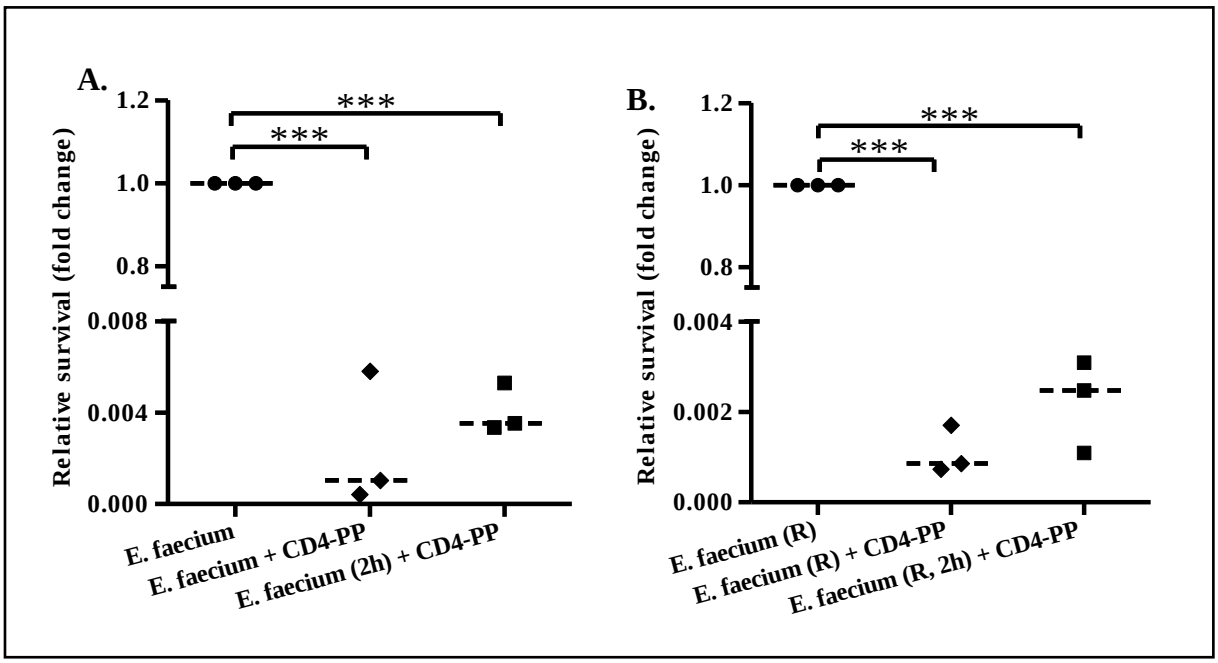

Fig 4. Results from infection assays, shows relative survival compared to control infection, to treatment with 2µM CD4-PP at 2 and 4 hours. Survival assay for both was done in cell line 5637. A) Shows *E. faecium* CCUG 33829, p-value ≤ 0.001 for treatment at 0 and 2 hours. B) *E. faecium* CCUG 36804, p-value ≤ 0.001 for treatment at 0 and 2 hours. Statistics were calculated using the ANOVA Dunnett's multiple comparison test.

Treatment with 2µM CD4-PP showed to inhibit infection *in vitro* for both strains tested for *E. coli, K. pneumoniae* and *E. faecium. P. aeruginosa* ATCC 27853 was only inhibited when treatment was given at 0 hours, but not at 2 hours. *P. aeruginosa* CCUG 51971 was not shown to be inhibited independent of if CD4-PP treatment occurred at the same time as infection, or after 2 hours.

CD4-PP upregulates *CLDN-14, OCLN* and *CAMP:*

After seeing the effect CD4-PP had in the infection assays, the immunomodulatory effect of CD4-PP treatment during infection in uroepithelial cell lines was investigated. Tight junction proteins are known to be downregulated during infection and the parent peptide LL-37 is known to be upregulated during infection. As such the gene expression of *CLDN-14* and *OCLN* encoding tight junction proteins claudin-14 and occludin, and *CAMP* encoding LL-37 was measured using standard qPCR protocol. Cell line 5637 was infected with MOI 5 of *E. coli* CFT073 and treated with 10µM CD4-PP. The CD4-PP concentration was chosen as it showed a significant immunomodulatory effect. This allowed for two comparisons; comparing the gene expression between uninfected 5637 cells to CD4-PP treated cells, and infected 5637 cells to infected cells treated with CD4-PP.

Treatment of 5637 uroepithelial cell line with 10µM CD4-PP showed upregulation of tight junction genes *CLDN-14 (fig. 5a)* and *OCLN (fig. 5b)*, compared to control, and to *E. coli* CFT073 infected cells (*fig 5d-e)*. Gene encoding LL-37, *CAMP* was upregulated in a similar fashion to *CLDN-14* and *OCLN*, with a higher expression of *CAMP* when CD4-PP was present (*fig 5c & 5f).*

***Fig 5*. mRNA expression of *CLDN-14, OCLN* and *CAMP* in cell line 5637**

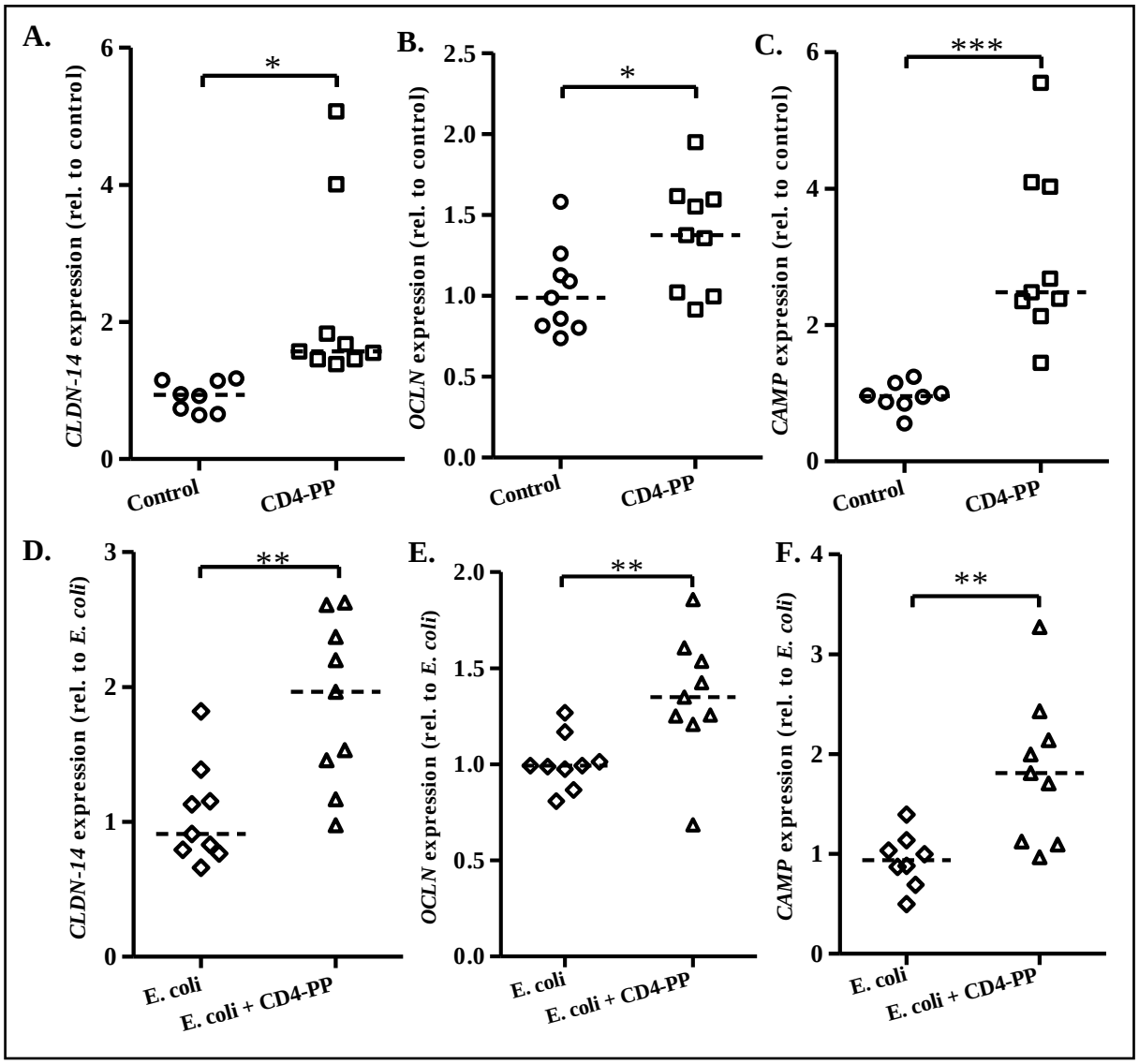

Fig 5. Shows mRNA expression of tight junction genes claudin-14, occludin and the antimicrobial peptide LL-37. Cell line 5637 was used for all three genes. A-C show expression during 10µM CD4-PP treatment relative to untreated control: A) *CLDN-14*, $p \leq 0.05$. B) *OCLN*, $p \leq 0.05$ C) *CAMP* $p \leq 0.001$. D-F show expression during infection with MOI 5 *E. coli* CFT073 and 10µM CD4-PP treatment relative to *E. coli* infected control: D) *CLDN-14*, $p \leq 0.01$. E) *OCLN*, $p \leq 0.01$. F) *CAMP*, $p \leq 0.01$. Statistics were calculated using an unpaired t-test.

CD4-PP downregulates CXCL-8 during infection:

AMPs are a part of the innate immune system, and hence the effect of the synthetic CD4-PP on chemokine IL-8 expression could prove interesting. To measure the expression of *CXCL-8*, the gene encoding IL-8, in response to CD4-PP treatment, cell line T24 was infected with MOI 5 of *E. coli* CFT073 and treated with 10µM CD4-PP, establishing the same conditions as described above. Gene expression was measured using standard qPCR protocol. Treatment with CD4-PP without infection of *E. coli* CFT073 showed to significantly upregulate *CXCL-8* (p-value ≤ 0.01) (*fig 6a).* But expression was significantly reduced if the cells were infected with *E. coli* CFT073 and treated with CD4-PP (p-value ≤ 0.05) (*fig. 6b).*

***Fig 6*. mRNA expression of *CXCL-8* in cell line T24**

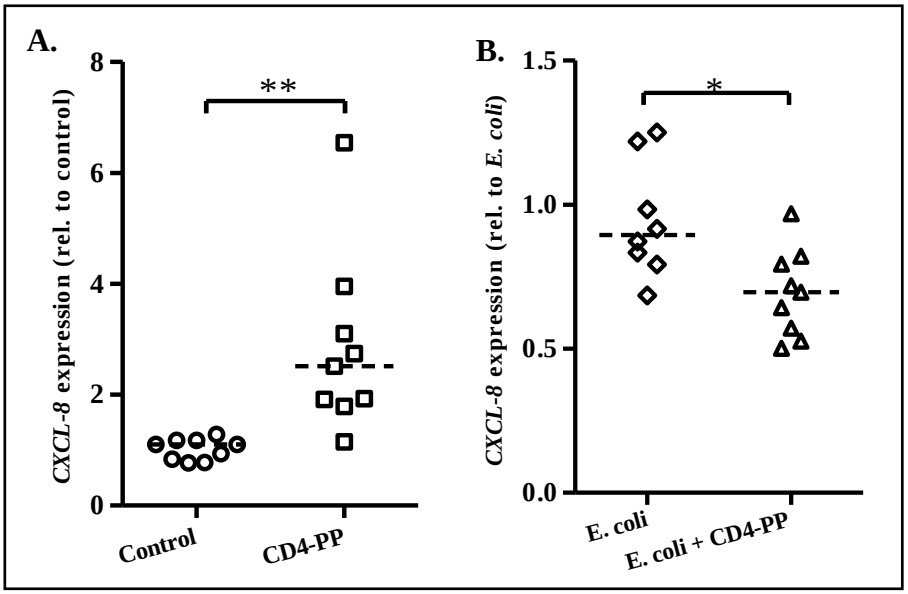

Fig 6. Shows mRNA expression of chemokine *CXCL-8* in cell line T24. Infection was with *E. coli* CFT073, and treatment with 10µM CD4PP. A) *CXCL-8* expression during treatment with CD4PP, $p \leq 0.01$. *CXCL-8* expression during infection and treatment, $p \leq 0.05$. Statistics was calculated using an unpaired t-test.

Immunofluorescence show protein upregulation during CD4-PP treatment:

Gene expression of *CLDN-14, OCLN* and *CAMP* were all shown to be upregulated during CD4-PP treatment in both uninfected and infected 5637 cells. To determine if the same change was seen for the protein expression of claudin-14, occludin and LL-37, confocal microscopy was done using 5637 cells. Conditions remained the same as for gene expression, where treatment was with 10µM CD4-PP and infection with MOI 5 of *E. coli* CFT073. Cells treated with CD4-PP was treated for a total of 6 hours, and cells infected with *E. coli* CFT073 was infected for 2 hours. Afterwards cells were stained using Alexa Fluor secondary antibodies and mounted on glass slides and was observed in the confocal microscope Zeiss LSM 700 using 63X immersion oil. Antibodies bound to target proteins show immunofluorescence, images from confocal microscopy can be seen in *Figure 7*.

Intensity of immunofluorescence for claudin-14 (green) seemed stronger during CD4-PP treatment in both uninfected and infected cells when compared to untreated cells (*7a, d, g & j*).

Occludin intensity (red) was slightly stronger during treatment of uninfected cells compared to control (*7b & e*). No difference was seen in occludin intensity between infected, and CD4-PP treated infected cells (*7h & k*). LL-37 (magenta) showed no clear difference in intensity between control and CD4-PP treated cells (*7c & f*). Slightly stronger intensity can be seen in CD4-PP treated infected cells compared to infected control (*7i & l*).

Fig. 7: **Immunofluorescence of Claudin-14, Occludin and LL-37 in cell line 5637:**

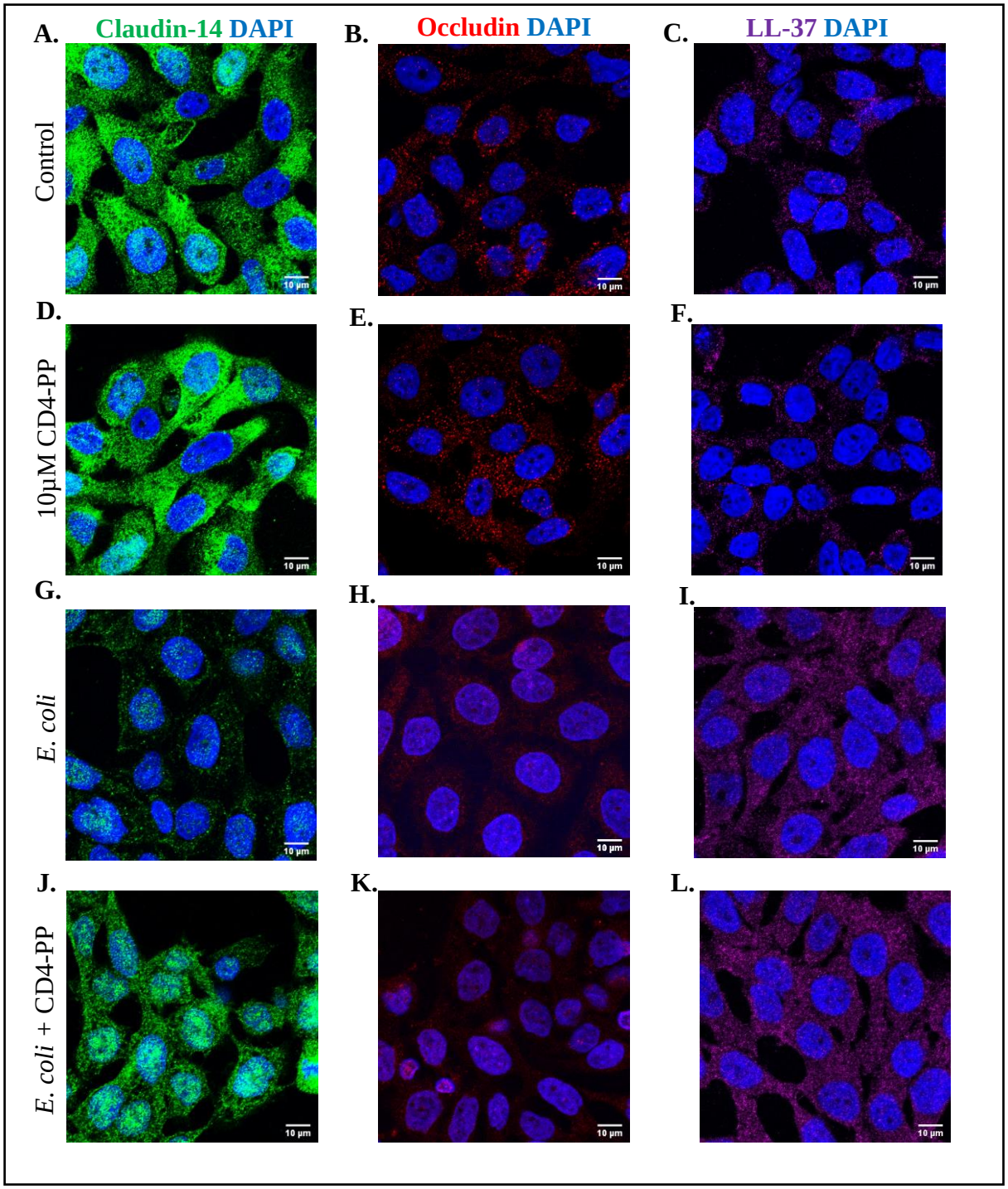

Fig. 7 shows protein expression of claudin-14, occludin and LL-37 in 5637 cells. Green fluorescence indicates protein expression of claudin-14, red show occludin, and magenta show LL-37. A-C are untreated and uninfected control (5637 cells). D-F are 5637 cells treated with 10µM CD4-PP. G-I are 5637 cells infected with MOI 5 *E. coli* CFT073. J-L are 5637 cells infected with MOI 5 *E. coli* CFT073 and treated with 10µM CD4-PP.

Densitometry of protein expression:

Confirmation of protein expression was done by densitometry analysis of immunofluorescence. Claudin-14 and occludin were both upregulated (p-value ≤ 0.05) during CD4-PP treatment in uninfected cells (*fig. 8a-b).* Claudin-14 was also upregulated (p-value ≤ 0.01) during CD4-PP treatment in *E. coli* infected cells (*fig. 8d).* Occludin showed no significant upregulation during the same conditions (*fig. 8e).*

LL-37 was not significantly upregulated during CD4-PP treatment in uninfected cells (*fig. 8c).* But was significantly upregulated (p-value ≤ 0.05) during CD4-PP treatment in *E. coli* infected 5637 cells (*fig. 8f).*

***Fig. 8*. Protein expression of claudin-14, occludin and LL-37 in cell line 5637**

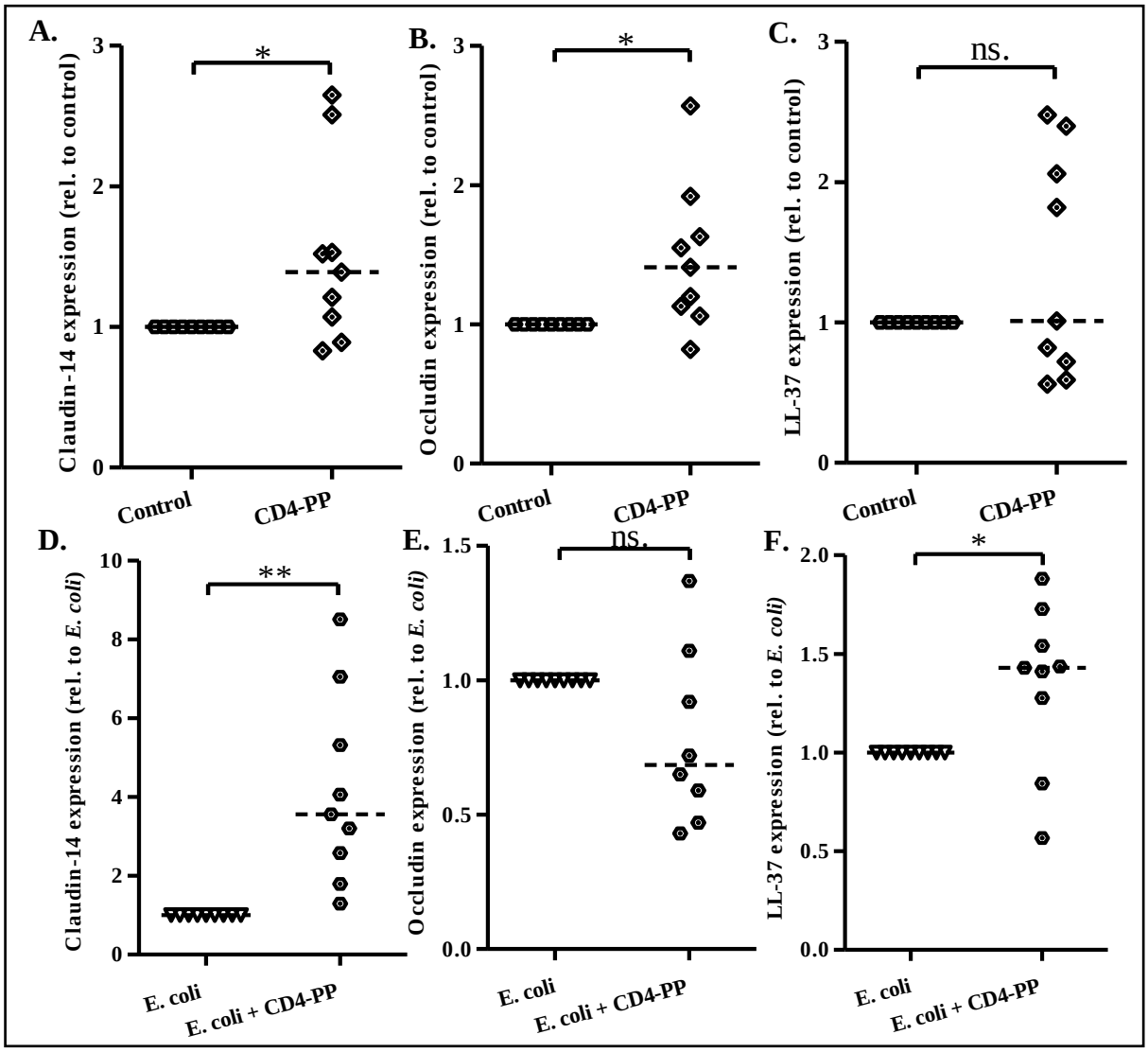

Fig. 8 shows the protein expression of claudin-14, occludin and LL-37 in cell line 5637. A-C show expression during 10µM CD4-PP treatment relative to control: A) Claudin-14, p ≤ 0.05. B) Occludin, p ≤ 0.05. C) LL-37, ns = non-significant. D-F show expression during MOI 5 *E. coli* infection with 10µM CD4-PP treatment relative to *E. coli* infected control: D) Claudin-14, p-value ≤ 0.01 E) Occludin, ns = non-significant. F) LL-37, p ≤ 0.05. Statistics were calculated using a paired t-test.

Discussion:

CD4-PP showed to successfully inhibit both Gram negative and positive species during infection of uroepithelial cells *in vitro,* demonstrating MIC values nearly 10-times lower than the parent peptide LL-37 for the same species. Treatment of CD4-PP in uroepithelial cells also indicated upregulation of genes and proteins involved in prevention of infection. Here it is shown for the first time that CD4-PP is active against uropathogens and has desirable traits which are important for novel treatments. Demonstrating that CD4-PP is active against a broad-spectrum of bacteria is pertinent, as commonly treatment against UTIs fail due to resistance or is prolonged for extensive periods due to recurrent infections [26].

Development of novel treatment options for infectious diseases is desperately needed to battle the ongoing spread of antibiotic resistance. UTIs affect millions of individuals annually, and the ability to treat them are becoming scarce. Currently, the annual cost of treating community acquired UTIs in the United States alone cost $1.6 billion [48]. This is associated with higher morbidity as well as a high socioeconomic burden, both on the patient and the society. The societal cost of UTIs alone in the United States is estimated to be $3.5 billion per year [5]. As prolonged, or recurrent infection will directly be associated with loss of work, increasing expenses in medicine and healthcare and a significant decrease in the patient's quality of life [49]. Synthetic derivatives of known antimicrobial peptides have previously been discussed as good starting points for new antimicrobials [50].

One concentration inhibits uropathogenic infection *in vitro:*

LL-37 has several times been described as an AMP which can inhibit a wide range of bacteria [51]. Here it is shown that its synthetic derivative CD4-PP, possess the same characteristics, but with greater potency. Both peptides induce cell cytotoxicity at 25µM in human uroepithelial cells, with CD4-PP having reduced MIC values for all species compared to LL-37. Based on the results for the MICs, one concentration was determined which was slightly higher than the MICs for all but *E. faecalis.* As *E. faecalis* showed to have a higher MIC than all the other clinical isolates.

The concentration chosen was 2µM, which is greatly below the cytotoxic concentration of 25µM. Infecting uroepithelial cells with MOI 5 of *E. coli, K. pneumoniae* and *E. faecium* and treating with CD4-PP showed to significantly reduce the intracellular, or membrane associated bacteria. This was independent of if the cells were treated simultaneously as they were infected, or if they were treated 2 hours after. An interesting observation is that the peptide does not discriminate in its action depending on if the bacteria carry resistance to other antibiotics. This further means there is no intrinsic resistance present against CD4-PP in these pathogens. Here it is shown for the first time that multidrug resistant urinary pathogens are susceptible to CD4-PP.

It is important to note that for all strains, there was a lower relative survival when CD4-PP treatment was given at the same time as cells were infected. This is not how a natural infection occurs, as in a clinical setting antibiotic will not be given until the diagnosis is confirmed. Uroepithelial cells were also treated with CD4-PP after two hours of infection, this *in vitro* set-up can reflect a simplified model for UTIs. It is an effective way to determine how active the peptide is against intracellular, or membrane-associated uropathogens during a UTI.

P. aeruginosa was the only bacterial species which showed not to be inhibited *in vitro.* Except for when CD4-PP was given at the same time as the cells were infected with *P. aeruginosa* ATCC 27853. *P. aeruginosa* is known for being a difficult pathogen to treat, with multiple intrinsic resistance mechanisms [28-29]. The outer membrane of *P. aeruginosa* has low permeability due to outer membrane porins (OMPs) such as OprF and OprH [52]. Treatment

with the parent peptide LL-37 has also shown to promote virulence factors as well as upregulate genes involved with quorum sensing in *P. aeruginosa* [53]. More detailed studies into why CD4-PP has a decreased activity against *P. aeruginosa* during infection needs to be explored.

E. coli CFT073 cause stress in CD4-PP untreated uroepithelial cells:
Another discovery was that infection of cell line T24 with *E. coli* CFT073, the uropathogenic strain, showed to kill the uroepithelial cells in the absence of CD4-PP. An explanation for why the T24 cells detached and died is the virulence factors possessed by this strain [47]. For example, the toxin colibactin inhibits DNA in human cells and can cause such stress that cells detach and die [54]. For this reason, cell line 5637 was used for the CFT073 infection assay, as these cells showed to be less stressed by infection. *E. coli* CCUG 55971 did not show to kill T24 cells. This can be explained by the presence of fewer virulence factors, or less potent virulence factors in this ESBL producing strain, where the fitness cost of having resistance has been a reduction in virulence factors [55]. This interesting finding could be further explored as a separate study, focusing on the virulence factors expressed by these bacteria, and the impact they have on uroepithelial cells.

All strains used during the infection assays showed to some degree to induce stress in the uroepithelial cell lines, but during treatment with CD4-PP more uroepithelial cells appeared to be adherent. It is to be expected that epithelial cells lose their structure and integrity during infection. This allows pathogens to invade deeper into the epithelium and can during a UTI cause uroepithelial cells to exfoliate [56]. Hence maintaining the integrity of the uroepithelium aids in preventing further invasion of pathogens.

CD4-PP impact on NO and biofilm production needs improvement:
Nitric oxide (NO) production was measured in the absence and presence of *E. coli* CFT073 infection in uroepithelial T24 cells during CD4-PP treatment (*Supplementary, Figure S3).* No detectable difference in free nitrate concentration was seen between cells with and without CD4-PP treatment, or between infected cells with and without treatment. Reactive nitrogen and oxygen species are generally produced by phagocytic cells to combat pathogens [57]. Nitrate, which is an oxidation product of NO, has been shown to be upregulated in urine during UTIs [58]. Free nitrate concentration did not differ between untreated and treated T24 cells, indicating that CD4-PP alone does not induce NO production. Important to note is that the free nitrate was measured in T24 cells after infection, and as mentioned previously, *E. coli* CFT073 causes these cells to quickly detach and die, which could impact this experiment.

Production of biofilm is common during CAUTIs [19]. Prevention of initiation of formation of biofilm was not shown to be effective for sub-inhibitory CD4-PP (*Supplementary, Figure S4*). To successfully inhibit biofilm formation and production, higher concentrations of CD4-PP is most likely required. These concentrations should preferably be higher than the tested species MIC value. Mature biofilm may not be preventable using CD4-PP alone, as the peptide could be too small to penetrate it.

CD4-PP helps in preventing invasion of pathogens:
The effect and impact of a new antimicrobial cannot solely be determined by the effect it has on the pathogen. But also, how the host responds to treatment. As mentioned earlier, the cytotoxicity concentration was determined to be 25µM. To see how the host cells responds to treatment, a higher concentration than used for both MIC and infection assays was used to see exactly how CD4-PP affect host cell gene and protein expression.

Urinary pathogens infecting uroepithelial cells trigger a downregulation of tight junction proteins [59]. These protein structures work on maintaining the epithelial integrity, keeping the cells attached, and by such prevent the entry and invasion of pathogens into deeper tissues [60]. Therefore, it would be beneficial if new treatment could help upregulate both the gene and protein expression of tight junctions. Claudin-14 and occludin are two well-known tight junction proteins, encoded for by *CLDN-14* and *OCLN*, respectively. It was here seen that the gene expression for both was upregulated in 5637 cells treated with CD4-PP, independent of if the cells were infected with *E. coli* CFT073 or not.

Protein expression of claudin-14 showed upregulation during CD4-PP treatment in both uninfected and infected cells. Occludin was only upregulated during treatment of uninfected cells. This can indicate that CD4-PP helps to promote the expression of tight junction genes, as well as the protein expression of claudin-14, and by such aids in preventing invasion of pathogens. The upregulation at the protein level can be seen clearly in the immunofluorescence staining (*fig. 7*), where the intensity for claudin-14 can visibly be seen as stronger for when cells were treated with 10µM CD4-PP. Occludin expression in the same figure is more difficult to see, but as densitometry showed that its expression was only upregulated in uninfected cells treated with CD4-PP, further research into the occludin expression during treatment of infected cells could prove interesting.

LL-37 and innate immunity is regulated in response to CD4-PP treatment:

It has previously been shown that LL-37 induces several different immune modulatory events and is upregulated immediately after *E. coli* infection in uroepithelial cells [38]. *CAMP* which encodes LL-37 will later be downregulated because of negative feedback. In the current study it was shown that CD4-PP significantly upregulates *CAMP* expression during CD4-PP treatment in both uninfected and infected 5637 cell. Production of LL-37 has shown to enhance and induce IL-1β production of cytokines and chemokines, and as a result mount a specific immune response [61]. Upregulation of *CAMP* allows us to assume that the cells are ready to produce LL-37 if an infection occurs. The protein expression of LL-37 was shown to be significantly upregulated during infection only, with LL-37 not being upregulated during treatment of uninfected cells (*fig. 8c & f*), which confirms this hypothesis.

Lastly, the level of *CXCL-8,* the gene encoding the chemokine IL-8 was measured in uroepithelial cell line T24 (*fig. 6).* IL-8 works as a chemical signal, attracting neutrophils to the site of the infection [62]. There was an upregulation of *CXCL-8* when comparing untreated to CD4-PP treated cells. *CXCL-8* expression was reduced in CD4-PP treated cells which were infected with *E. coli* CFT073. The expression of *CXCL-8* occurs quite rapidly, and to fully understand how CD4-PP impacts this gene expression further investigation is needed. Determining the expression of IL-8 during the same conditions could shed more light on how this chemokine is produced during CD4-PP treatment.

Future aspects and applications:

There are further intriguing aspects which could be added to the research concerning CD4-PP than the ones presented in this study. Such as the mode of action. LL-37 is known to create toroid pores on bacterial surfaces, but the way in which CD4-PP invade bacteria is not established [41-42]. As CD4-PP is an AMP, it can strongly be assumed to work by pore formation on the bacterial surface. Albeit it might work in a different fashion than from LL-37. Understanding the mode of action for CD4-PP could lead to investigation into dual treatment, using an antibiotic to reach synergy, and could potentially allow for inhibition of pathogens such as *P. aeruginosa.*

Another crucial aspect which should also be explored is the possibility for development of resistance against CD4-PP, as it has been shown that bacteria can inactivate AMPs such as LL-37 or in unique cases develop resistance [63]. There has not yet been any synthetic derivative of an AMP that has passed clinical trials [36]. The most likely utilisation of CD4-PP in treatment of UTIs would come from coating catheters to prevent biofilm formation and subsequent CAUTI. Prevention of CAUTIs by using antimicrobial urinary catheters coated with antimicrobial compound is currently being utilised [20]. Hence, future research concerning the effectiveness of CD4-PP as coating on catheters to prevent CAUTIs could be an interesting next step for this synthetic AMP in clinical treatment.

Conclusion:

Here it has been shown for the first time, that CD4-PP, the cyclised synthetic derivative of LL-37 effectively inhibits clinical uropathogens, and in some cases at concentrations nearly 10-fold lower than the parent peptide. CD4-PP has been used effectively to inhibit infection by the most common urinary pathogens *in vitro* and was further proven to be effective against bacteria with extensive resistance against several classes of antibiotics. The peptide also actively upregulates important host factors in uroepithelial cell lines, such as tight junction proteins, which promote the structure of the uroepithelium and prevent exfoliation. As well as upregulating the parent peptide LL-37 during treatment in infected cells.

Prevention of biofilm formation needs to be further analysed using stronger concentrations, and the mode of action is still to be confirmed. There is more to be learnt from CD4-PP, but this study has shown that it has every possibility to become a novel treatment option for UTIs.

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Supplementary:

***Table S1*. Information regarding collection site for clinical isolates**

Species	Blood	Deep airways	Deep secretion	Nasopharynx	Puncture	Urine	Wound
E. coli	3			1	1	14	1
E. faecalis	5					15	
E. faecium	5				1	14	
K. pneumoniae	2		3			13	2
P. aeruginosa		8					12

Table S1 shows the collection site of the clinical isolates used for minimal inhibitory concentration. All samples were collected at Karolinska University Hospital, Stockholm, Sweden.

Figure S1. **Cytotoxicity of cell line T24 for LL-37, KR-12 and CD4-PP, and cell line 5637 for CD4-PP**

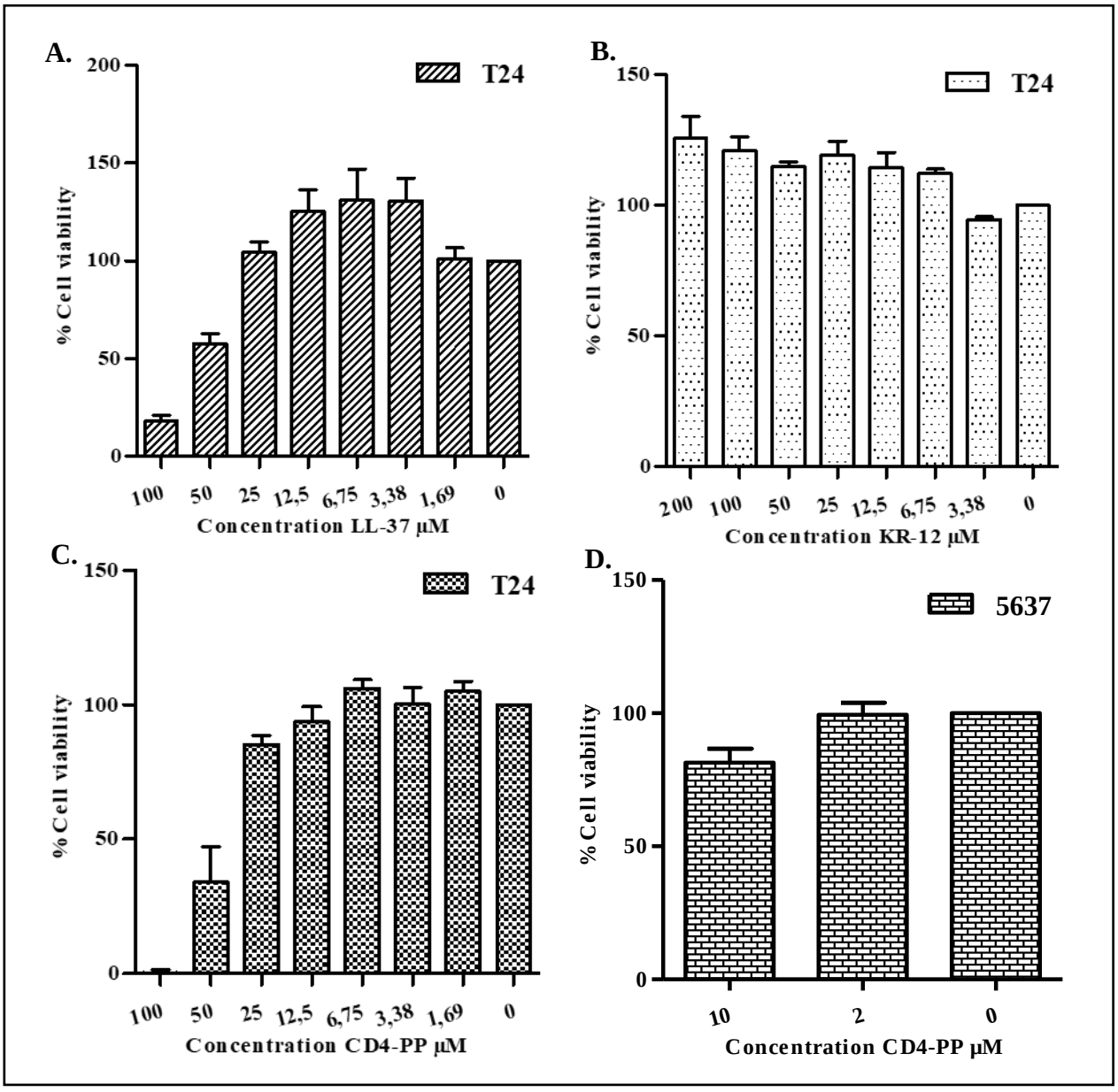

Fig. S1. shows the cytotoxicity of uroepithelial cell line T24 when treated with 2-fold dilutions of CD4-PP, LL-37 and KR-12. Cytotoxicity was observed above 25µM for CD4-PP and LL-37, and above 200µM for KR-12. As well as the cytotoxicity of uroepithelial cell line 5637 for 2 and 10µM CD4-PP, concentrations used for infection assay as well as gene and protein expression.

***Table S2*. Median MIC values for LL-37 and KR-12 using clinical isolates**:

Species	LL-37 median MIC	% of isolates with LL-37 median MIC	KR-12 median MIC	% of isolates with KR-12 median MIC
Escherichia coli N=18	3,125µM	83,3%	6,25µM	66,6%
Enterococcus faecium N = 8	3,125µM	87,5%	>25µM	100%
Enterococcus faecalis N = 11	≥25 µM	63,6%	≥25 µM	90,1%
Pseudomonas aeruginosa N = 11	3,125µM	81,8%	6,25µM	54,5%
Klebsiella pneumoniae N = 14	3,125-6,25µM	100%	≥25µM	42,8%

Table S2 shows the median MIC values for clinical isolates collected and provided by the Department of Clinical Microbiology at the Karolinska University Hospital, Stockholm.

***Figure S3.* Free nitrate concentration by T24 cells:**

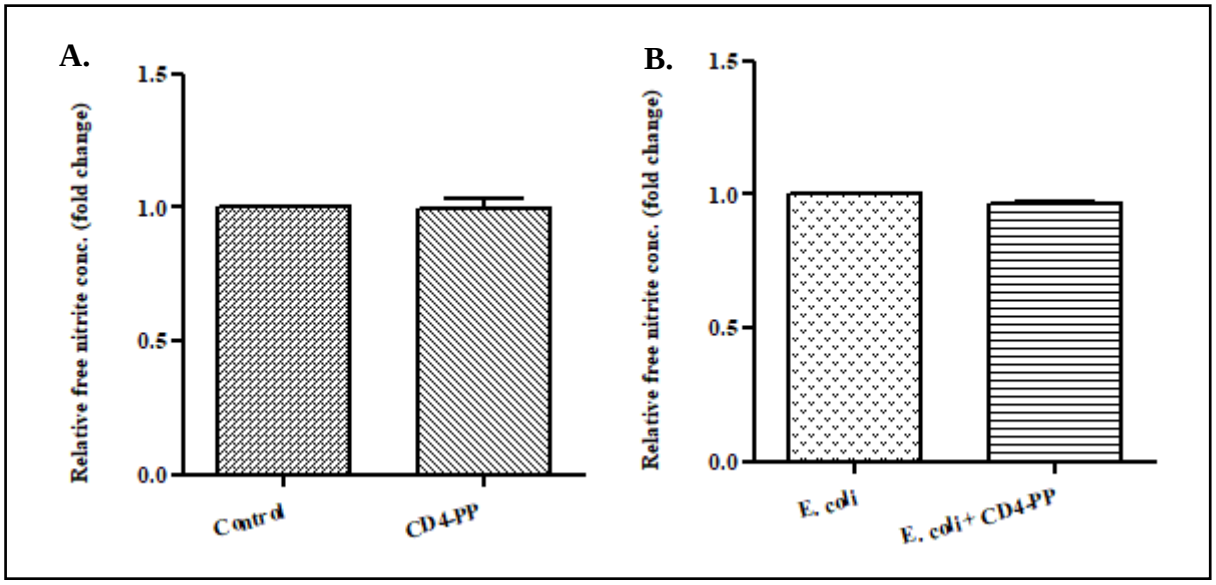

Figure S3 shows the production of free nitrate in T24 cells. A) Shows the free nitrate concentration of T24 cells treated with 2µM CD4-PP relative to untreated control. B) Shows the free nitrate concentration of T24 cells infected with *E. coli* CFT073 and treated with 2µM CD4-PP relative to cells only infected with *E. coli* CFT073. Assay was done in triplicate twice as a trend of no significance was established after two sets.

Figure S4: **Prevention of initiation of biofilm for *E. coli* CFT073**

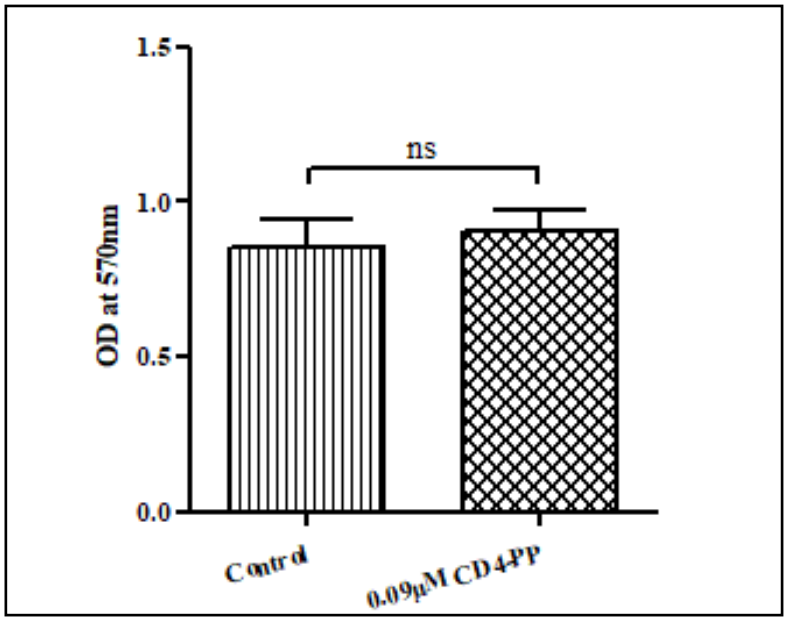

Figure S4 show the prevention of initiation of *E. coli* CFT073 biofilm when treating with a fourth of the MIC = 0.09µM CD4-PP. No significance was detected between control biofilm and biofilm treated with CD4-PP. Prevention of biofilm formation was done in triplicate, thrice.