Chapter 4
Antimicrobial Peptides: Maintaining Sterility of the Urinary Tract

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Abstract Due to its close proximity to the gastrointestinal tract, the normally sterile urinary tract is constantly challenged by microbial invasion. To counter this microbial assault, the urinary tract has developed a highly effective antimicrobial "shield" that can rapidly eliminate invading pathogens or prevent their growth. During recent years, considerable advances have been made in our understanding of the immune mechanisms that contribute to urinary tract sterility. Recent evidence indicates that cationic antimicrobial peptides contribute to the innate host defense of the urinary tract. This chapter reviews the published literature on the role(s) of antimicrobial peptides (AMPs) in maintaining urinary tract sterility.

4.1 Introduction

Urinary tract infections (UTI) are one of the most common and serious bacterial infections encountered by physicians (Bachur and Harper 2001; Foxman et al. 2000). Nearly half of all women develop a UTI requiring antimicrobial therapy during their lifetime (Foxman et al. 2000). In 2013, aggregate hospital charges for inpatient UTI management exceeded $640 million US dollars (Spencer et al. 2011a). Specific subpopulations have increased UTI risk, including pregnant women, the elderly, patients with diabetes or multiple sclerosis, patients with acquired immunodeficiency diseases, patients with urologic anomalies, and those having urinary tract intervention such as catheter insertion. Although UTI is not typically associated with significant morbidities, UTI does increase the risk of premature delivery and fetal mortality among pregnant women (Foxman et al. 2000). Long-term complications of UTI include renal insufficiency, renal scarring, hypertension, and chronic kidney disease.

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To date, no treatment strategy has been proven to be effective in the prevention of UTI sequelae. Moreover, antibiotic resistance in uropathogenic bacteria has been increasing in large part due to antibiotic overuse (Spencer et al. 2014a).

4.2 UTI Pathogenesis and Roles for Antimicrobial Peptides in the Host Response

UTI refers to the presence of microbial pathogens within the urinary tract. The site of infection classifies UTI—infection localized to the bladder is referred to as "cystitis" and infection in the kidney is "pyelonephritis." Cystitis typically presents with lower urinary tract symptoms—including dysuria, urgency, and urinary frequency. Pyelonephritis is often associated with more severe or systemic symptoms including fever, back/flank pain, and vomiting. Ascending infection may result in bacteremia and present as the systemic inflammatory response syndrome or overt septic shock (i.e., urosepsis).

Escherichia coli (*E. coli*) is the most frequent bacterial pathogen responsible for UTI—accounting for 85–90 % of cases. Uropathogenic *E. coli* (UPEC) are thought to originate from the fecal flora, spread across the perineum, and enter the bladder through the urethra. Before invading the urothelium, UPEC must overcome several intrinsic characteristics of the urinary tract. Proposed functional mechanisms contributing to urinary tract sterility include barrier formation by uroepithelial cells, unidirectional flow of urine, regular bladder emptying, mucous production, the urinary microbiome, and alterations in the urinary ionic composition (Spencer et al. 2014a; Sobel 1997).

The microbial virulence of UPEC has been linked to many factors (Ragnarsdottir and Svanborg 2012; Mulvey et al. 1998, 2000). The most prominent is type I fimbriae, which are filamentous bacterial appendages that are capped by FimH, a mannose-binding adhesion protein. Type I fimbriae promote tight bacterial binding to a matrix of uroplakin complexes on the surface of superficial bladder epithelial cells (Mulvey et al. 2000). After binding, UPEC invades the bladder uroepithelium where it may establish a state of commensalism or cause a severe, symptomatic infection characterized by a rapid innate host response with cytokine secretion and recruitment of leukocytes to the infection source (Weichhart et al. 2008).

In the kidney, UPEC binds to the apical surfaces of the kidney's collect duct. Within the collecting duct, UPEC specifically targets the intercalated cells (Chassin et al. 2011; Paragas et al. 2014). The signaling pathways activated by UPEC have been investigated in primary cultures of medullary collecting duct cells dissected from the kidneys of LPS-sensitive C3H/HeOuJ mice (Chassin et al. 2011). Signaling pathway analysis demonstrates that UPEC stimulates the expression of pro-inflammatory mediators in the medullary collecting ducts via TLR4-mediated, MyD88-dependent, TRIF-independent NF-kB and MAPK-activated pathways and also via a TLR4-independent, MyD88-independent pathway. The TLR4-independent pathway results from activation of the TNF receptor-associated factor-2 (TRAF2) and apoptosis signal-regulatory kinase 1 (ASK1)-JNK pathway (Chassin et al. 2011; Vandewalle 2008).

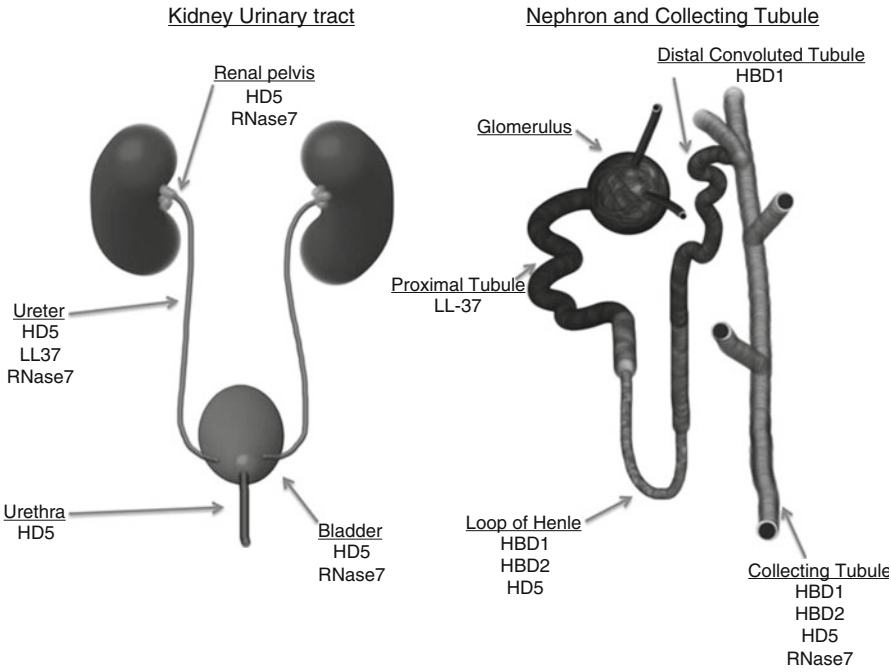

Fig. 4.1 Defined AMP in the kidney and urinary tract. *Left panel*: AMP identified in the human lower and upper urinary tract. *Right panel*: AMPs identified in the nephron and collecting tubule of human kidney (Image reused with permission from Spencer et al. (2014a))

Antimicrobial peptides (AMP) may be induced through TLR-4-mediated and TLR-4-independent pathways. Bacterial attachment may immediately induce uroepithelial AMP production (Spencer et al. 2014a; Zasloff 2007). Alternatively, AMPs may be constitutively produced by the uroepithelium and limit bacterial attachment by direct antimicrobial activity. AMPs may also contribute to urinary tract sterility by depleting vital nutrients required for bacterial growth or serving as chemoattractants for local leukocytes. The following sections outline the published literature on the function(s) of AMPs in the urinary tract. Figure 4.1 shows an overview about identified AMP in the kidney and urinary tract.

4.3 Cathelicidin

The human cathelicidin LL-37 is an amphipathic, α-helical AMP expressed on all epithelial surfaces, myeloid bone marrow cells, and circulating neutrophils. Cathelicidin possesses antimicrobial activity against viruses and Gram-positive and Gram-negative bacteria. Moreover, it acts as a chemoattractant for neutrophils and tissue-derived monocytes by interacting with their fMLP receptors. The optimal chemoattractant

concentrations of cathelicidin are in the micromolar range, considerably higher than observed for traditional chemokines (Zasloff 2007; Yang et al. 2007).

There is limited but convincing evidence that LL-37 plays a role in maintaining urinary tract sterility (Ali et al. 2009). Chromek et al. demonstrate that LL-37 is constitutively expressed in the human upper and lower urinary tract. They detected low levels of cathelicidin in the urine of healthy children (0.2–5.9 ng/mL). Urinary LL-37 levels increased during cystitis and pyelonephritis (0–312.5 ng/mL) (Chromek et al. 2006). The authors concluded that urinary cathelicidin originates from the uroepithelium as there was only a small correlation with urinary leukocytes and myeloperoxidase. They supported this finding through immunostaining localizing cathelicidin production to the renal tubular epithelium in noninfected human renal biopsy specimens. When they exposed these biopsy specimens to UPEC, LL-37 production increased. Additionally, when renal epithelial (A498 and hPTC cells) and uroepithelial cells (J82 and UROtsa cells) were challenged with UPEC, mRNA expression of the LL-37 gene *CAMP* increased within 5 min. This mRNA induction was followed by prolonged LL-37 peptide secretion into the surrounding medium. These data suggest that cathelicidin production originates from the uroepithelium and is designed to facilitate an immediate and sustained response to microbial insult (Chromek et al. 2006; Chromek 2015).

In the same study, Chromek et al. evaluated the biological relevance of cathelicidin in vivo using a mouse model of pyelonephritis. Immunofluorescent staining suggests that Cramp, the murine homologue of human cathelicidin, is upregulated during the acute stages of pyelonephritis in the renal tubular epithelium. During more advanced stages of infection, cathelicidin is also released from leukocytes, indicating that urinary cathelicidin originates from both the urothelium and leukocytes. Deletion of the *Cramp* gene leads to increased bacterial attachment to bladder uroepithelium compared to wild-type mice. Cathelicidin-deficient mice also experienced a higher rate of ascending infection compared to wild-type animals with increased renal bacterial burden. Thus, cathelicidin appears to participate in epithelial antimicrobial defense, recruitment of immune cells, and neutrophil killing of pathogens. During UTI, epithelial cells rapidly increase production of cathelicidin to protect the urinary tract from bacterial invasion, and the "second wave" of cathelicidin comes from leukocytes (Chromek et al. 2006).

4.4 Defensins

Defensins, characterized by a 15–20 amino acid sequence including six cysteine residues, are one of the most studied families of AMPs. Defensins typically have broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, viruses, fungi, and protozoa (Lehrer et al. 1993). Along with their direct antimicrobial properties, defensins play a role in cell-mediated immunity as chemoattractants for immature dendritic cells (Zasloff 2007). Defensins are initially synthesized as

pre-pro-proteins and undergo processing to become mature, biologically active peptides. In humans, defensins are classified into one of two families depending on their disulfide-bridging pattern—the α-defensins or the β-defensins. The clusters of genes encoding the α-defensin subfamily and the majority of members of the β-defensin subfamily are located on chromosome 8p22 and 8p23 (Liu et al. 1997).

4.5 α-Defensins

The α-defensins HNP1–HNP4 are primarily found in neutrophils where they provide non-oxidative microbicidal activity. HNPs encounter pathogens after they are secreted onto the cell's surface via degranulation or after a pathogen undergoes phagocytosis and the phagocytic vacuole fuses with the neutrophilic granule (Ganz 2003). Regarding the urinary tract, Ihi et al. demonstrated that urinary levels of HNP1–HNP3 significantly increased in the setting of UPEC and *Enterococcus faecalis* UTI (6.5 ± 1.1 pg/μL to 29 ± 5.7 pg/μL) (Ihi et al. 1997). Similarly, Tikhonov et al. demonstrated that urinary HNP1 increased eightfold in patients with chronic pyelonephritis compared to control patients and patients with glomerulonephritis. Urinary HNP1 levels correlated with urinary IL-8 levels as well as leukocyte count, suggesting that urinary HNP1 may reflect neutrophil recruitment to the infection site (Tikhonov et al. 1997). No microbiological information has been published in regard to the HNPs and UTI.

The expression and function of epithelial human defensin HD5 have been reported mostly in the small intestine where it is secreted by Paneth cells into the intestinal crypts. HD5 has also been described in the male and female reproductive tracts, with evidence suggesting that it is inducible and important in eradicating infection (Porter et al. 2005; Quayle et al. 1998). Recombinant mature HD5 has been shown to have bactericidal activity against uropathogenic bacteria, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and UPEC (Wang et al. 2010).

In the urinary tract, HD5 has been localized to the uroepithelium of the kidney, ureter, and bladder (Spencer et al. 2012). Schwaderer et al. demonstrate that HD5 gene and protein expression were significantly greater in human kidney biopsy specimens from patients with pyelonephritis compared to noninfected controls. Their data also show that secreted HD5 was not routinely detected in culture-negative human urine samples; however, HD5 levels significantly increased in urine samples infected with UPEC (300–670 ng HD5/mg urine creatinine). Although urinary HD5 did not reach concentrations likely to be directly antimicrobial against common uropathogens, the mucosal surface concentrations may be higher (Spencer et al. 2012). Urinary HD5 levels are higher in patients who have undergone ileal neobladder reconstruction and ileal conduit urinary diversion (Porter et al. 1998; Townes et al. 2011).

4.6 β-Defensins

The human β-defensins, encoded by over 28 genes, are widely expressed in human epithelia and certain family members exhibit antimicrobial activity toward Gram-positive and Gram-negative bacteria (Ali et al. 2009; Schutte et al. 2002). Of the β-defensins, human β-defensin-1 (HBD1, encoded by the *DEFB1* gene) and human β-defensin-2 (HBD2, encoded by *DEFB4*) have been described in the human urinary tract. *DEFB1* mRNA is constitutively expressed by the epithelial lining of the kidney's loop of Henle, the distal tubule, and the collecting duct (Valore et al. 1998). HBD1 is translated as a 68 amino acid pro-peptide and undergoes variable amino-terminal processing to a 36–47 amino acid (Valore et al. 1998; Zucht et al. 1998). Mature HBD1 is constitutively detected in sterile urine (10–100 μg/L) and levels increase up to threefold in patients with UTI (Valore et al. 1998; Hiratsuka et al. 2000). Although these urinary levels of HBD1 are insufficient to kill invading bacteria, HBD1 may provide a fast-acting antimicrobial coating of urothelium and prevent infection by inhibiting bacterial attachment to the urothelium (Valore et al. 1998).

Defb1 encoding mouse BD1 (mBD1) has been considered orthologous to HBD1 based on conserved gene structure, expression pattern, and antimicrobial activity, though the amino acid sequences of mBD1 and HBD1 are only 51 % identical (Huttner et al. 1997; Bals et al. 1998; Morrison et al. 1998). *Defb1* is transcribed within lower urinary tract and the kidney's distal tubules and collecting ducts (Bals et al. 1998; Becknell et al. 2013). The mature mBD1 peptide exhibits salt-sensitive antimicrobial activity toward Gram-positive and Gram-negative bacteria (Bals et al. 1998; Morrison et al. 1998). Mice lacking both copies of *Defb1* (*Defb1*$^{-/-}$) exhibit increased incidence of spontaneous bacteriuria compared to *Defb1*$^{+/-}$ and wild-type controls, with *Staphylococcus* species predominating among urine isolates (Morrison et al. 2002). Wild-type mice undergoing transurethral inoculation of UPEC exhibit significant reduction in bladder *Defb1* transcript levels within 2 h of infection that persists up to 2 days. These results suggest that successful UPEC colonization of the urinary tract is achieved through local inhibition of Defb1 expression. However, when *Defb1*$^{-/-}$ animals were challenged with UPEC, no difference in upper or lower tract bacterial burden was observed relative to wild-type controls (Becknell et al. 2013).

Additional *Defb* family members are detectable at the mRNA level in the murine urinary tract. *Defb3* (encoding mBD3) and *Defb14* (encoding mBD14) mRNA are enriched in mouse bladder, compared to ureter and kidney (Becknell et al. 2013). Both mBD3 and mBD14 exhibit bactericidal activity toward UPEC in the low-micromolar range. Other *Defb* transcripts, such as *Defb2*, *Defb28*, *Defb29*, and *Defb42*, are enriched or exclusively expressed in murine ureter and kidney, compared to bladder (Becknell et al. 2013). The lack of available antibodies has limited the study of the levels and distribution of these mBD peptides in the urinary tract. The establishment of mouse strains with deletions in one or more of *Defb* family members will facilitate studies that determine their biological relevance within the urinary tract (Zhou et al. 2013; Navid et al. 2012).

4.7 Hepcidin

Hepcidin, also known as liver-expressed antimicrobial peptide-1 (LEAP-1), is produced in the liver and excreted in the urine (urinary concentrations range from 10 to 30 μg/L). It is translated in the liver as an 84 amino acid pre-pro-peptide. After processing and excretion through the kidneys, a 25 amino acid peptide (hepc-25) is the predominant form in the urine (but shorter urinary peptides are also detected). Hepcidin does not show sequence similarity to any of the other described AMPs but structurally resembles the defensin family given the four disulfide bridges in its tertiary structure (Park et al. 2001; Krause et al. 2000).

Hepcidin has broad-spectrum antimicrobial activity against *E. coli* (ML-35), *S. epidermidis*, *S. aureus*, *C. albicans*, and group B *Streptococcus* (Park et al. 2001). Hepcidin also plays an important role in iron homeostasis. Genetically modified mice, engineered to overexpress hepcidin, die shortly after birth secondary to severe iron deficiency. These two findings indicate that hepcidin may participate in the innate defense of the urinary tract by direct antimicrobial activity and/or reduction of available iron—which is an essential nutrient for uropathogens (Park et al. 2001; Weinstein et al. 2002).

4.8 Lactoferrin and Lipocalin

Lactoferrin inhibits bacterial growth through free iron chelation or the effects of lactoferricin, a bactericidal protein generated by the proteolytic cleavage of lactoferrin (Bellamy et al. 1992). Lactoferrin is detectable in human urine; however, these levels are relatively low (14–145 ng/mL). In the human kidney, lactoferrin is expressed in the cells lining the distal collecting ducts of the medulla (Abrink et al. 2000). Lipocalin 2 (LCN2) is a member of the large lipocalin protein family, which has a wide range of biological functions. Several reports indicate that lipocalin 2 (LCN2) mediates a bacteriostatic effect by sequestering iron (Berger et al. 2006; Flo et al. 2004; Holmes et al. 2005). Specifically, LCN2 binds the secreted siderophore enterochelin (Ent), which UPEC and other pathogens release into the extracellular milieu to acquire essential iron. Upon complexing with Ent:Fe^{3+}, LCN2 reroutes Ent:Fe^{3+} for degradation and prevents iron transfer to bacteria. Without iron, uropathogens are unable to grow, facilitating immune clearance (Paragas et al. 2014). Thus, *Lcn2*-deficient mice demonstrate increased susceptibility to bacterial infections when challenged with intraperitoneal *E. coli* (Berger et al. 2006).

Recently, Paragas et al. observed a significant induction of Lcn2 in the urine of mice with UTI compared with uninfected animals. The degree of Lcn2 upregulation correlated with the number of infecting bacteria in the urine and reduction of bacterial load with antibiotics resulted in decreased Lcn2 production. A similar correlation between bacterial load and LCN2 was also seen in the clinical setting—with urinary levels increasing tenfold in patients with UTI (30–300 ng/mL) compared to

healthy controls (7.4–36.3 ng/mL). These results provide clinical support for LCN2 production in immune defense of the urinary tract (Paragas et al. 2014). Additionally, using an *Lcn2* global knockout mouse, Paragas et al. demonstrate that after transurethral UPEC inoculation, *Lcn2*$^{-/-}$ mice have higher bladder bacterial burden than their littermates and take longer to clear the infection. Both wild-type and *Lcn2*$^{-/-}$ mice were comparably infected with an Ent mutant UPEC strain, indicating that the antimicrobial actions of Lcn2 on uropathogens require iron acquisition (Paragas et al. 2014).

Using an Lcn2-*Luc2*-mCherry reporter mouse, this same research group demonstrates that Lcn2 is constitutively produced by the thick ascending limbs of the loop of Henle and the α-intercalated cells of the renal collecting tubule (Paragas et al. 2011, 2014). During experimental UTI, GFP-labeled UPEC bound the kidney's α-intercalated cells and induced Lcn2 expression in a TLR-4- and NF-κB-sensitive fashion (Paragas et al. 2014; van Adelsberg et al. 1994; Xie et al. 2008).

4.9 Ribonuclease A Superfamily

Four lineages of the ribonuclease (RNase) A superfamily encode proteins associated with host defense: (1) angiogenins, (2) eosinophil RNases, (3) RNase 6, and (4) RNase 7 and RNase 8 (Rosenberg 2008; Simanski et al. 2010; Becknell et al. 2015). To date, RNase 6 and RNase 7 have been evaluated in the urinary tract and during UTI. Both RNases 6 and 7 have potent antimicrobial activity against common Gram-positive and Gram-negative uropathogens (Becknell et al. 2015; Spencer et al. 2011b, 2013). The mechanisms accounting for the antimicrobial properties of these RNase A proteins are not completely understood (Boix and Nogues 2007). The bactericidal activity of RNase 7, for example, has been linked to its capacity to permeate and disrupt the bacterial cell membrane, which is an action independent of its ribonuclease activity (Huang et al. 2007; Harder and Schroder 2002). Moreover, RNase 7 is highly cationic (isoelectric point 10.7), and the cationic charge is necessary for antibacterial activity. Finally, distinct regions of the RNase 7 peptide appear to be responsible for its antimicrobial activity against various pathogens (Huang et al. 2007; Wang et al. 2013).

In the urinary tract, our research group has demonstrated that RNase 6 and RNase 7 expression differ. Using human biopsy samples and a murine model of experimental UTI, Becknell et al. demonstrate that RNase 6 expression localized to resident urinary tract leukocytes or infiltrating granulocytes and macrophages in mouse cystitis bladders, mouse pyelonephritis kidneys, and human pyelonephritis kidneys. RNase 6 was not routinely detected in noninfected human or mouse kidney and bladder tissues. With UPEC infection, RNase 6 peptide production markedly increased, likely representing the recruitment of RNase 6 expression leukocytes to the infected kidney (Becknell et al. 2015).

In contrast, RNase 7 is an epithelial-derived AMP that is constitutively produced by the uroepithelium of the lower urinary tract and the α- and β-intercalated cells of

the renal collecting tubule. When urinary RNase 7 is neutralized in human urine specimens in vitro, urinary bacterial growth increases (Spencer et al. 2011b, 2014b). During UTI, urine from children with UPEC infection contained about twice the concentration of RNase 7 as the urine of noninfected controls. Similarly, human kidney biopsies from patients with pyelonephritis demonstrate that tissue concentrations of RNase 7 protein are about twice what was measured in normal controls. What distinguishes the expression of RNase 7 within the urinary tract from the other previously described AMPs is the considerably greater amount secreted. Whereas median urinary concentrations of cathelicidin and HBD1 are 1.6×10^{-5} μM and 2.5×10^{-5} μM, respectively, RNase 7 is present at around a 1000-fold greater concentrations (Spencer et al. 2011b, 2013; Zasloff 2013). These results suggest that RNase 7 provides a front-line antimicrobial shield that permits uropathogenic organisms from invading the urothelium (Zasloff 2013). If this barrier is breached during microbial assault, other defenses like RNase 6 or other AMPs are activated to combat infection.

4.10 Tamm-Horsfall Protein (THP)

THP or uromodulin is the most abundant protein in human urine, and mounting evidence points to its role in maintaining urinary tract sterility. Mice deficient in Thp (*Thp*$^{-/-}$) exhibit delayed clearance of UPEC and higher UPEC recovery from urine and bladders following experimental UTI (Bates et al. 2004). This was confirmed independently by Mo and colleagues, who further demonstrated that this protective effect of Thp existed specifically toward type 1-fimbriated UPEC (Mo et al. 2004). This may be explained by the observation that type 1-fimbriated UPEC interacts with THP's mannose residues and relies on these structures for attachment to uroplakin plaques on bladder urothelium (Pak et al. 2001). While THP does not possess bactericidal activity in vitro, it is capable of inhibiting bacterial attachment to cultured kidney epithelial cells (Hawthorn et al. 1991).

4.11 AMPs as Therapeutics

The therapeutic potential of AMPs to prevent or treat UTI has been given added incentive as antibiotic resistance toward uropathogenic bacteria has been increasing. The use of AMPs does require some degree of caution as AMPs are powerful biomolecules that can elicit native cell death if applied in high concentration (Ali et al. 2009). The most straightforward method of AMP UTI therapy is direct application to the source of infection (i.e., through bladder washes) or oral therapy. The use of oral lactoferrin B effectively decreased infection and inflammation in the mouse urinary tract after UPEC infection (Haversen et al. 2000). An alternative possibility for using AMPs therapeutically would be to induce or augment their natural production using

homeopathic therapies like specific nutrients and vitamins. Treatment of human bladder cell lines with 25-hydroxyvitamin D_3 and 1,25-hydroxyvitamin D_3 boosted cathelicidin production and antibacterial activity against UPEC. Moreover, bladder biopsy specimens from postmenopausal women treated with 25-hydroxyvitamin D_3 rapidly show increased *CAMP* expression in response to UPEC infection compared to biopsy specimens from women who did not receive vitamin D supplementation (Hertting et al. 2010). Since urothelium and intercalated cells have emerged as the chief parenchymal sources of AMPs, future efforts to induce AMP production should focus on these cell populations. As some of the basic issues that control urinary tract AMP expression and function are clarified, strategies and possible agents for preventing and treating UTI can be developed to limit antibiotic overuse.

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