Hepcidin as a Major Component of Renal Antibacterial Defenses against Uropathogenic *Escherichia coli*

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**Abstract**

The iron-regulatory peptide hepcidin exhibits antimicrobial activity. Having previously shown hepcidin expression in the kidney, we addressed its role in urinary tract infection (UTI), which remains largely unknown. Experimental UTI was induced in wild-type (WT) and hepcidin-knockout (Hepc<sup>−/−</sup>) mice using the uropathogenic *Escherichia coli* CFT073 strain. Compared with infected WT mice, infected Hepc<sup>−/−</sup> mice showed a dramatic increase in renal bacterial load. Moreover, bacterial invasion was significantly dampened by the pretreatment of WT mice with hepcidin. Infected Hepc<sup>−/−</sup> mice exhibited decreased iron accumulation in the renal medulla and significant attenuation of the renal inflammatory response. Notably, we demonstrated in vitro bacteriostatic activity of hepcidin against CFT073. Furthermore, CFT073 repressed renal hepcidin, both in vivo and in cultured renal cells, and reduced phosphorylation of SMAD kinase in vivo, suggesting a bacterial strategy to escape the antimicrobial activities of hepcidin. In conclusion, we provide new mechanisms by which hepcidin contributes to renal host defense and suggest that targeting hepcidin offers a strategy to prevent bacterial invasion.


Urinary tract infection (UTI), one of the most common infectious diseases of humans, is mainly caused by uropathogenic *Escherichia coli* (UPEC). When *E. coli*, the intestinal inhabitant, become pathogenic and acquire virulence factors, they reach the urinary tract and affect a wide range of cellular processes. Indeed, the urinary tract is sterile and armed with host defenses such as reduced availability of iron, antimicrobial peptides, the acidic pH of urine, and inflammatory responses mediating the clearance of acute UPEC infection. Iron is particularly deficient in the urine because iron is permanently bound to transferrin in the plasma, which limits its glomerular filtration. This situation may explain why UPEC, such as the prototypical pyelonephritis-associated CFT073 strain, overexpress several iron acquisition systems during UTI including chelate iron siderophores. We have recently shown that renal iron content and the renal iron carriers are under the control of.

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Hepcidin, the major peptide that regulates iron homeostasis.\(^7\) Hepcidin has been discovered in 2000 as an antimicrobial peptide\(^8,9\) and in 2001 as a hyposideremic peptide.\(^10,11\) Hepcidin is mainly produced by hepatocytes in response to increases in serum and tissue iron,\(^10,11\) and inhibits iron efflux from duodenal enterocyte and macrophage stores. Hepcidin binds to ferroportin, the sole iron exporter in mammalian cells, and limits the iron efflux.\(^12,13\) In absorptive enterocytes, we and other authors showed that hepcidin reinforces the control over iron absorption by additionally downregulating the iron importer DMT1 present at the apical plasma membrane.\(^14–17\) Hepcidin is regulated by iron through a complex of integral hemochromatosis proteins, \(i.e.,\) HFE, HJV (hemojuvelin) and transferrin receptor 2. These proteins tightly coordinate signaling through the BMP6/HJV/SMAD pathway.\(^18–20\)

Hepcidin was observed to have additionally an \textit{in vitro} antimicrobial activity and a structure reminiscent of four disulfide defensins.\(^8,9\) Hepcidin synthesis is induced by inflammatory signals such as IL-6, allowing it to play a major role in the anemia associated with chronic diseases and inflammation.\(^21–23\) Hepcidin is also induced in the liver in response to LPS through activin B and Smad1/5/8-signaling\(^24\) and in macrophages through Toll-like receptor 4 (TLR4) signaling.\(^25\) Notably, hepcidin expression was recently identified in several epithelial barriers that are frequently confronted by pathogen infection, including renal distal nephron.\(^7,26–28\) However, whether hepcidin is required for resistance to epithelial barrier infection has received little attention. Here, we provide evidence that hepcidin may represent an effective defense system against UPEC infection and more interestingly that UPEC represses local hepcidin to evade renal host defenses during UTI.

**RESULTS**

**Expression and Localization of Hepcidin in the Kidney**

Quantitative RT-PCR was performed to analyze the relative levels of hepcidin-mRNA in isolated proximal tubules, the cortical and medullary thick ascending limb, and the collecting duct microdissected from wild-type (WT) kidneys. Figure 1A evidenced a large and preferential expression of hepcidin in the distal nephron, as previously described.\(^29\) The hepcidin transcript level was considerably higher in the medullary thick ascending limb but was nearly undetectable in the proximal tubules.

**Decreased Renal Hepcidin Expression in UPEC Infection**

The medullary collecting ducts represent the preferential site of UPEC colonization,\(^30\) we therefore assessed the impact of CFT073 infection on local synthesis of hepcidin. We measured bladder and kidney-associated titers of CFT073 following inoculations of adult CBA/j female mice. CFT073 colony-forming unit was significantly greater in the 48 hours post-infection than 24 hours bladder and kidneys (Figure 1B). In these experiments, there was no bacterial sepsis evaluated by a CFT073 count in the liver homogenates. The mRNA level of lipocalin-2, the siderophore chelator used as a positive control, was greatly increased at 48 hours postinfection while hepcidin-mRNA abundance was significantly decreased at both 24 and 48 hours postinfection (approximately 5-fold decrease, \(P<0.001\)) (Figure 1, C and D). Hepcidin repression was dominant despite a significant local increase of TLR4 and IL-6...
Involvement of Hepcidin in UTI Protection

We then investigated the benefit barriers for UPEC in the absence of hepcidin. Compared with WT mice, Hepc−/− mice exhibited significant iron overload in renal medulla and in the urine (Figure 4A, and Moulouel et al.). Iron excess was mainly sequestered by increased ferritin but the protein levels of both H-ferritin and L-ferritin were significantly reduced in Hepc−/− kidneys following infection, suggesting that in the absence of hepcidin, iron is easily consumed by UPEC (Figure 4B). In WT mice, the basal level of ferritin was quite low, rendering it impossible to be quantified before and after infection. We tested the bacterial load of CFT073 in WT mice previously fed with an iron-rich diet (RD mice). Figure 4C showed that RD mice exhibited high serum ferritin and considerable excretion of iron in urine. However, this urinary iron bioavailability was not advantageous for CFT073 growth (Figure 4D), probably because endogenous hepcidin is already increased in these mice (Figure 4C).

We also explored the expression of lipocalin-2 in Hepc−/− kidneys. Indeed, lipocalin-2 has recently been reported to induce growth arrest of CFT073 by chelation of their siderophores. The expression level of lipocalin-2 was not increased in uninfected Hepc−/− kidneys (Figure 5). However, following UPEC infection, both mRNA and protein levels of lipocalin-2 were induced in WT and Hepc−/− infected mice, and to the same extent, suggesting that lipocalin-2 is not influenced by the lack of hepcidin nor by iron overload in urine and thick ascending limb cells (Figure 5). Thus, for UTI protection, hepcidin and lipocalin-2 are likely to be complementary and have non-redundant functions.
increased in the infected WT mice compared with controls (Table 1). However, for the infected Hepc−/− mice, except IL-6 and IFN-γ that were increased, the expression levels of the other cytokines showed little or no change compared with sham WT mice. Also, TNF-α and IL-1β were induced in sham Hepc−/− animals but they were highly reduced after UTI (Supplemental Table 1). We also investigated macrophage and neutrophil recruitment to the kidney site of infection. In Figure 6A, the positive immunostaining of F4/80 and quantification indicated a similar amount of macrophage infiltrate in the medullary region of both WT and Hepc−/− infected mice. However, myeloperoxidase activity was significantly decreased in Hepc−/− compared with WT infected mice (Figure 6B), confirming the reduced neutrophil influx observed by PAS renal sections staining. Altogether these results suggest that a lack of hepcidin is significantly associated with attenuated inflammatory response to CFT073.

**Hepcidin Triggers Urinary Acidic pH**

In the collecting duct, the apical H+/K+-ATPase pumps largely mediate acid secretion. Moreover, Schwarz et al.²⁸ reported that the Atp4a pump is regulated by hepcidin via the autocrine/paracrine pathway in gastric parietal cells. Therefore, we attempted to evaluate the mRNA expression of both Atp4a and Atp12a pumps in Hepc−/− kidneys. Compared with WT mice, the level of the two pumps was significantly lower in the Hepc−/− mice with a pronounced effect on the Atp4a transcript (Figure 7B). The degree of reduction of both pump transcripts was correlated with a significant increase of the pH value of the urine (Figure 7C). To investigate further whether hepcidin modulates the urinary pH, we treated Hepc−/− mice intravenously with the hepcidin peptide and measured the pH of urine daily. Figure 6D showed that treated Hepc−/− mice exhibited significant recovery of acidic pH by d 2 of hepcidin injection. A normal range of pH value was attained as soon as d 3 (6.39±0.09 versus 7.00±0.06 at d 0; *P=0.001). Because CFT073 inhibited hepcidin synthesis in the kidney, we analyzed the expression of Atp4a and Atp12a pumps in WT mice at 48 hours postinfection and found that their transcripts were reduced (Figure 7, A and B). The mRNA levels of NHE3 and BSC1 that are sensitive to the body acid-base balance were not affected (Supplemental Figure 4).³²⁻³⁵

**Hepcidin as an Antimicrobial Peptide Active Against CFT073**

We finally analyzed hepcidin antibacterial activity on UPEC in vitro. We first treated CFT073 that were cultured in healthy adult male sterile urine containing increasing concentrations of hepcidin peptide, and found that the growth kinetics of CFT073 were significantly reduced in a dose-dependent fashion (Figure 8A). At the end of the treatment we analyzed UPEC survival on lysogeny broth (LB) agar plates. Figure 8B showed that treated CFT073 started to grow again, suggestive of a bacteriostatic action of hepcidin. We then determined the structural architecture of CFT073 by electron microscopy analysis, and observed that within 2 hours of incubation with 200 µg/ml of hepcidin, about 41% of CFT073 (compared with 3.5% in controls) exhibited disruption of their envelopes with obvious cell lysis (Figure 8C), indicating that high concentrations of hepcidin may also act as bactericidal CFT073.

**Mechanism by which CFT073 Targets Hapcidin in the Kidneys**

We examined both BMP6/SMAD and IL-6/Stat3 pathways in the kidneys of WT and Hepc−/− mice at 48 hours postinfection. Consistent with the induction of IL-6 in our experiments, Stat3 phosphorylation was increased in infected mice
compared with the control group but there was no difference in the extent of Stat3 induction between the two groups (Figure 9A). However, the level of SMAD3 phosphorylation we reduced in WT mice at 48 hours postinfection (Figure 9B). This SMAD inactivation was more obvious in Hepc−/− mice in which the steady-state level of phosphorylated SMAD kinase was already high. Thus, CFT073 may decrease renal hepcidin expression by acting on the BMP6/SMAD pathway.

We then infected both medullary collecting duct mIMCD-3 cells and hepatocyte HepG2 cells with formaldehyde (PFA)-fixed CFT073 and examined hepcidin mRNA expression at different multiplicities of infection (MOIs; number of bacteria per number of monolayer cells) and different incubation times. Figure 10A shows that an MOI of 5:1 was sufficient to reduce by 4-fold the mRNA level of hepcidin in mIMCD-3 cells compared with controls. Full suppression was observed by 2 hours postinfection, persisting up to 24 hours postinfection (Figure 10B). Reduced SMAD phosphorylation was also confirmed in inoculated mIMCD-3 cells (Figure 10C). In contrast, we found that 4 hours postinfection with LPS led to a significant increase in hepcidin mRNA in mIMCD-3 cells (Figure 10D). Furthermore, lipocalin-2 was significantly induced in mIMCD-3 cells, and hepcidin mRNA reduced in HepG2 cells, following CFT073 incubation (Supplemental Figure 5).

**DISCUSSION**

In the present study, we provide evidence that hepcidin is an effective mediator of renal defense systems against UPEC infection. Indeed, a total lack of hepcidin was found to alter renal host barriers, including urinary iron restriction, acidic...
pH of urine, renal inflammatory response and antimicrobial activity. Consequently, CFT073 UPEC infection and growth were favored within the urinary tract. Our data also suggest that renal hepcidin may be one of the targets that UPEC triggers to become more pathogenic and evade host defenses during UTI.

**Renal Inhibition of Hepcidin by UPEC**

Numerous studies have recently reported direct links between local synthesis of hepcidin and pathogen infection in several epithelial barriers, although no study has been performed in the kidney so far. For example, hepcidin produced by the gastric parietal cells and by the retina was reported to be upregulated following *Helicobacter pylori* and *Staphylococcus aureus* infections, respectively.²⁸,³⁶ Our study revealed that renal hepcidin is also regulated on UTI but in an opposite way. Indeed, renal hepcidin was unexpectedly downregulated 24 hours postinfection, whereas hepatic hepcidin increased, despite a delayed response (at 48 hours postinfection). Furthermore, as both hepatic and renal hepcidin transcripts were repressed when renal mIMCD-3 and hepatic HEPG2 cells were infected with CFT073, we assumed that a direct contact of host cells with UPEC is required to repress hepcidin. Bacterial LPS was shown to induce hepcidin mRNA in mIMCD-3 cells, thereby excluding this endotoxin from the repression mechanism of hepcidin and suggesting the involvement of an unknown bacterial compound during this process. Repression of hepcidin has previously been described in a mouse model of gastric mucosal infection with *Helicobacter felis.*³⁷ The authors attributed this effect to the reduced number of parietal cells that synthesize hepcidin. However, in our UTI model, renal epithelium remained intact up to 48 hours postinfection. Moreover, because the decline of hepcidin was detected earlier following infection, we believe that UPEC may directly modulate hepcidin expression and that discrepant effects of pathogens on hepcidin must result either from the nature of the pathogenic strain or from the host environment. Indeed, UPEC differs from *Helicobacter* by the high degree of virulence.³⁸ And consistent with the influence of the host environment are the results from Gaddy *et al.*³⁹ showing in gerbils that a high-salt diet led to reverse from induction to repression the effect of *H. pylori* on hepcidin expression. This observation may also explain our results with UPEC UTI because the kidneys are the exclusive desalting sites with high osmolarity of urine and of the renal medulla counterpart.

Another notable finding in our study is the UPEC-mediated signaling pathway leading to hepcidin repression. We observed that SMAD1/5/8 phosphorylation, the primary BMP6-induced pathway that controls hepcidin expression in the liver, was preferentially inhibited by UPEC in both WT and Hepc−/− infected kidneys. The IL-6-induced Stat3 phosphorylation was not affected by the UTI infection. Because renal hepcidin was repressed in this model, we concluded that UPEC might block BMP signaling as well as inflammatory IL-6 and LPS/TLR4 signaling by targeting the SMAD pathway. Our results support evidence showing that IL-6 and LPS-dependent activin B pathways may tightly overlap with the BMP/SMAD

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**Table 1. Expression profile of inflammatory cytokines in WT and Hepc−/− kidneys prior to and after CFT073 infection**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Sham (ng/ml)</th>
<th>WT 48 h (ng/ml)</th>
<th>P Value</th>
<th>Hepc−/− 48 h (ng/ml)</th>
<th>Hepc−/− versus WT in 48 h P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>15.9±0.7</td>
<td>70.5±15.8</td>
<td>0.01</td>
<td>87.1±41.4</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>51±3.1</td>
<td>124.8±13.2</td>
<td>0.001</td>
<td>105.2±15.3</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.3±0.3</td>
<td>4.7±0.02</td>
<td>0.02</td>
<td>1.9±0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>272±12.3</td>
<td>696±40.2</td>
<td>0.001</td>
<td>356±51.2</td>
<td>0.002</td>
</tr>
<tr>
<td>CXCL2</td>
<td>12.6±1.6</td>
<td>61.5±14.2</td>
<td>0.001</td>
<td>10.5±1.1</td>
<td>0.02</td>
</tr>
<tr>
<td>CCL5</td>
<td>58.1±9.6</td>
<td>304.6±50.8</td>
<td>0.001</td>
<td>50±12.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*The results are the mean±SEM. NS indicates nonsignificant.*
with hepcidin was sufficient to prevent UTI. Indeed, we found that hepcidin conserved bacteriostatic and bactericidal actions reminiscent of the mechanism by which defensins exert their antibacterial activity.8,9 Finally, we observed that hepcidin increased the acidic pH of urine by acting on the expression of the Atp4a and Atp12a pumps. Thus, as in gastric parietal cells, an autocrine/paracrine regulation of Atp4a and Atp12a by hepcidin may occur in renal tubular cells.

We provided new insights into the regulatory networks of the antimicrobial peptide hepcidin to prevent UPEC pathogenesis in the kidneys. Hepcidin appears to be a bridge between innate and adaptive immunity in this organ. The targeting of renal endogenous hepcidin by UPEC appears to be beneficial for these pathogens, especially at the initial stages of invasion. Our data may lead to the identification of new therapeutic strategies that target hepcidin or specific pathways regulating this peptide to enhance host defense or prevent maladaptive inflammatory responses leading to kidney injury in a variety of infectious and non-infectious conditions.

CONCISE METHODS

Animals

Seven-week-old CBA/J female mice were purchased from the Janvier-Europe laboratory and acclimated in our animal facility for 1 wk. Hepcidin-deficient C57BL/6 mice (Hepc−/−) were generated by Sophie Vaulont (Institut Cochin, Paris, France). All experimental procedures involving animals were performed in compliance with the French and European regulations on animal welfare and public health service recommendations.

Bacterial Strains

The E. coli strain used was the uropathogenic CFT073 (O6 :K2 :H1) isolated from a urosepsis episode and belongs to the B2 phylogenetic group.

Ascending UTI Model

The mouse model of ascending unobstructed UTI was used as described in Labat et al.47 Briefly, female mice were anesthetized using a xylazine/ketamine mixture before inoculation into the bladder of 50 µl of 0.9% NaCl containing 10⁶ bacteria. The urethral catheter used was immediately removed after inoculation. Sham-infected mice were infused with 50 µl of 0.9% NaCl. Animals were killed 24 hours or 48 hours later. The kidneys, bladder and liver were aseptically removed and frozen at −80°C for further study. For bacterial counts,
Cytokine and Chemokine Assays
Screening for cytokines and chemokines in renal extract was performed using MILLIPLEX MAP Mouse Cytokine (EMD Millipore, Saint-Quentin-en-Yvelines, France) according to the manufacturer’s instructions. For renal extracts, the kidneys were homogenized in a lysis buffer (50 mM HEPES pH 7.0, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 1X protease inhibitor cocktail ((EDTA Complete; Thermo Fisher Scientific, Paris France), and 10 μL/mL phosphatase inhibitor cocktail 2 (Sigma-Aldrich, Saint-Quentin Fallavier, France)). The homogenate was centrifuged at 100g at 4°C for 30 s, and the supernatants were transferred to a fresh tube. Ten per cent NP-40 was added to a final concentration of 0.05%, and the tubes were mixed by gentle inversion before an additional centrifugation at 1000g for 7 min. This final supernatant was used for cytokine and chemokine measurements. Eight-point standard curves were generated for each cytokine using the Luminex bead technology.

Myeloperoxidase Activity
Myeloperoxidase activity was assessed in kidney tissues by the spectrophotometric assay based on myeloperoxidase-catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) by H2O2. Samples were homogenized in a potassium phosphate buffer (50 mM, pH 6.0) and then centrifuged at 10,000g for 15 min at 4°C. The pellets were resuspended again in an equivalent volume of phosphate buffer (50 mmol/L, pH 6). Protein concentration was determined using the Bradford assay. Each sample of 100 μg of protein was resuspended in hexadecyl trimethylammonium bromide (HTAB) buffer (0.5% w/v, in 50 mM potassium phosphate buffer, pH 6.0), sonicated at 4°C, and centrifuged again at 10,000g for 15 min at 4°C. Fifty microliters of the supernatants were combined with 150 μL H2O2 (3 mM diluted in citrate), and 150 μL TMB solution (3 mM). In this assay, TMB was oxidized to a blue product that absorbs at 655 nm. The enzyme activity was measured continuously by monitoring the absorbance changes at 655 nm with a spectrophotometer.

Histologic Examination
The kidneys were fixed in 4% formaldehyde, embedded in paraffin and sections of 4 μm were then stained with Perls’ Prussian blue, H&E and picrosirius red. Images were acquired using a ScanScope digital scanner (Aperio, TRIBVN, France). Morphologic assessments were conducted by two independent renal pathologists who were uninformed about the treatments and mouse phenotypes.

Immunohistochemistry
Antigen retrieval from kidney sections was performed in citrate buffer at pH 6 (Vector Laboratories, Burlingame, CA) in a steamer.
Endogenous peroxidase activity was blocked with 3% H2O2, and non-specific protein binding was blocked with serum-free protein blocking solution (RTU Kit; Vector Laboratories). Kidney sections were first incubated overnight at 4°C with primary anti-F4/80 monoclonal antibody (AbD Serotec, a Bio-Rad company, Marnes-la-Coquette, France) applied at 1:50 dilution and incubated. The signal was revealed by a secondary antibody already labeled with horseradish peroxidase-conjugated streptavidin (Dako, Les Ulis, France), and visualized using Nova Red substrate kit (Vector Laboratories). The sections were then stained with hematoxylin.

Quantitative RT-PCR
Total RNA was isolated using the SV Total RNA isolation system (Promega, Charbonnières-les-Bains, France), and subsequent cDNA synthesis was performed with SuperScript II reverse transcription kit (Invitrogen, Life Technologies, Saint Aubin, France) according to the manufacturer's instructions. Real-time PCR was performed in duplicate in a Light Cycler II LC 480 (Roche Diagnostics, Meylan, France) using the SYBR Green PCR mix (Roche Diagnostics, Meylan, France). The primers used were as shown in Table 2.

To validate experiments examining the mRNA expression of hepcidin, liver RNA was systemically used as a positive control, and RNA from kidneys of hepcidin knockout mice (Hepc2/2, in which hepcidin is fully absent) was used as a negative control.

Protein Extraction and Western Blotting
Both tissues and cell pellets (approximately 40 mg) were harvested in a lysis buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.6, 1% Triton, 0.1% SDS and 26.6 mg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) containing a 1× protease inhibitor cocktail (EDTA Complete; Thermo Fisher Scientific). The homogenate was incubated in the same buffer for 60 min at 4°C. After centrifugation at 16,000 g for 15 min, the supernatant containing total proteins was transferred to a fresh tube and stored at –80°C until used. Protein concentrations were determined using the Bradford protein assay with bovine albumin standards.

Proteins were separated by electrophoresis on 8% or 10% SDS-polyacrylamide gels depending on the molecular weight of the target proteins. Primary antibodies were used at a 1:6000 dilution for anti-FPN (a kind gift from Dr. D. Haile, San Antonio, TX), 1:1000 for anti-TFR1 (Zymed, Invitrogen, Life Technologies, Saint Aubin, France), 1:1000 for anti-H-ferritin or anti-L-ferritin (a kind gift from Paolo Arosio, Brescia, Italy), 1:1000 for anti-P-SMAD1/5/8 (EMD Millipore, Saint-Quentin-en-Yvelines, France), 1:2000 for anti-P-stat3 (Cell Signaling-Ozyme; Cell Signaling Technology, Danvers, Massachusetts, USA).

Massachusetts), 1:1500 for anti-Lcn2 (R&D Systems), 1:2000 for anti-ZO-1 (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 1:10,000 for anti-b-actin mouse monoclonal antibody (Sigma-Aldrich Fine Chemicals), which was used as an additional control to check for equal loading and transfer onto the nitrocellulose membranes.

Immunoreactive bands were revealed by horseradish peroxidase-conjugated secondary antibodies using Amersham enhanced chemiluminescence.

**Bacterial Growth Measurement**

E. coli CFT073 was cultured in 4 ml of LB medium for 16 hours at 37°C. The E. coli were then centrifuged at 2500g for 10 min, washed with 20 ml of isotonic saline, recentrifuged and washed twice with 10 ml of sterile urine (see below). Cultures were diluted to obtain a starting concentration of 5×10⁵ bacteria/ml. The growth experiments in the absence or presence of increasing concentrations of hepcidin (0, 5, 10, 25, 50, 200, 400 µg/ml) were assayed using 96-well plates, and OD600 was measured at intervals of 5 min over 24 hours with a Tecan Infinite M200 plate reader. Each experiment was performed in triplicate and repeated in three different cultures.

Sterile urine was collected over 24 hours from three healthy adult male volunteers with no history of UTIs or antibiotic use during the previous 6 months. The urine samples were pooled, filtered (0.22 µm pore size) and used within 48 hours.

**Cell Culture and Infection**

The mIMCD-3 cell line derived from a mouse inner medullary collecting duct (ATCC CRL-2123) was cultured in DMEM:F-12 medium supplemented by 10% FBS (ATCC with LGC Standard Co., Molsheim, France). HEPG2 cells derived from human hepatocellular carcinoma (ATCC CRL-2123) were cultured in DMEM with 10% FBS. For cell infection, the CFT073 bacterial strain was grown overnight in a LB medium and then centrifuged and resuspended in 2% formaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) and incubated for 20 min at 37°C. Fixed bacteria were centrifuged again, washed twice and resuspended in sterile PBS. They were then added to confluent

![Figure 10.](image)

**Table 2. Primers sequences**

<table>
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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>Mouse Hepc1</td>
<td>CGATACCAATGCAGAAGAGAAGG</td>
<td>TTTCGCAACAGATACCACTGGG</td>
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<tr>
<td>Mouse Atp4a</td>
<td>AGCACCAGGCACCATGGGGAAG</td>
<td>CACCAGGGCCAGACCCATGTT</td>
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<td>Mouse Atp12a</td>
<td>GCCACGGGCCACCAGAAAGAT</td>
<td>CTGGGGCTGTAGGATGACC</td>
</tr>
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<td>Mouse Tlr4</td>
<td>ACCAGGGAGCTTGAATCCCTGCA</td>
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<td>Mouse Ngal</td>
<td>CTGAATGGGTGGTGGGTGGTTG</td>
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<td>Mouse BSC1</td>
<td>CGGTCCCCAAAGATTGAA</td>
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<td>Mouse Aq2</td>
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<td>Mouse GAPDH</td>
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<td>Human HEPC1</td>
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<tr>
<td>Mouse Aq2</td>
<td>CGGCCATCTCCATGAGATT</td>
<td>GGAAGAAGCTCAGCTGACCC</td>
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mIMCD-3 and HEPG2 cells at a concentration of 1, 5, or 10 bacteria per cell. Infected cell lines were harvested after 2, 4, 6, and 24 hours after inoculation. Cells treated with PBS or LPS (LPS-EK Ultrapure; Invivogen, France) were used as controls. Fixed bacteria showed no further growth on LB agar plates.

**Electronic Microscopy Analysis**

E. coli CFT073 bacteria were cultured in 4 ml of Luria-Bertani medium for 16 hours at 37°C. They were then centrifuged at 2500g for 10 min and washed twice with 20 ml of isotonic saline. Cultures were then incubated with or without hepcidin (200 μg/ml). After 1 hour, the cell suspensions were centrifuged at 10,000g for 15 min, washed with PBS, resuspended in fixative (3% glutaraldehyde and 4% PFA in 0.1 M cacodylate buffer at pH 7.2) for 24 hours at 4°C, postfixed in osmium tetroxide, dehydrated with ethanol, and embedded in Epon. Ultrathin sections stained with lead citrate were examined on a Jeol 1010 electron microscope. Images were captured by a digital imaging system.

**Chemical Synthesis of Mouse Hepcidin Analog**

The mouse hepcidin 1 analog was manually synthesized as described. A linear precursor with protected side chains was assembled manually by solid-phase peptide synthesis on Wang resin load low, preloaded with a threonine substituted at 0.27 mmol·g⁻¹ using Fmoc chemistry. After deprotection of Fmoc groups, using 20% piperidine/DMF (v/v), coupling was performed using 1:1:1:2 amino acid/PBOP/HOBt/DIEA in 5 ml of DMF (30 min) for all amino acids, except Fmoc-Cys(Tig)-OH, Fmoc-Cys(Acm)-OH, and Fmoc-Cys(Bu)-OH, for which collidine was used instead of DIEA. The resin bound peptide was cleaved from resin support and the side-chain protecting groups were removed by treatment with tri-fluoro-acetic acid. After cleavage from the resin, the peptide was dissolved in a mixture of DMSO/H₂O (1/2, v/v) and air oxidized to allow the Cys-S7-Cys23 disulphide bond formation. The oxidized peptide was extensively diluted in water (10 v/v) and lyophilized to dryness and kept at −20°C as a white powder until use. The resulting peptide was analyzed by mass spectrometry and its biologic activity was systematically checked for its ability to promote ferroportin degradation on J774 macrophages.

**Statistical Analysis**

For statistical analyses of quantitative variables, Student’s t test was performed. A finding of P<0.05 was considered significant.

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**DISCLOSURES**

None.

**REFERENCES**


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Figure S1: TLR4 and IL6 mRNA expressions in the kidney
The kidneys of Sham and 48-hour-infected WT mice were harvested, and the mRNA levels of both TLR4 and IL-6 were quantified by RT-qPCR. Data were normalized by GAPDH-mRNA levels. The results are the mean ± s.e.m. of at least six individual mice in each group. *P < 0.05, ***P < 0.0001.

Supplementary S2:

Figure S2:
CBA/J WT mice were infected with $10^9$ CFUs of CFT073, and bacterial counts were performed on bladder and kidneys at 24 and 48 h postinfection. (A) Hepcidin mRNA quantification in WT liver at 24 and 48 hours postinfection. (B) WT serum hepcidin measured by LC-MS/MS at 24 and 48 hours post-infection. (Sham) means uninfected mice. The results are the mean ± s.e.m. of at least six individual mice in each group. *P < 0.05, ***P < 0.0001. NS indicates non-significant.
Figure S3:
Histologic examination
The kidneys were fixed in 4% formaldehyde, embedded in paraffin and sections of 4-µm were then stained with picro–sirius red (PSR). Images were acquired using a ScanScope digital scanner (Aperio, TRIBVN, France). Morphologic assessments were conducted by two independent renal pathologists who were uninformed about the treatments and mice phenotypes.
Figure S4: Renal cell damage evaluation
The kidneys of Sham and 48-hour-infected WT mice were harvested for the following studies. (A) The mRNA levels of Na+/H+ exchanger 3 (NHE3), bumetanide-sensitive Na+/K+/2Cl-cotransporter 1 (BSC1) and the water channel aquaporin-2 (AQ2) were quantified by RT-qPCR. Data were normalized by GAPDH-mRNA levels. (B) Protein levels of tight junction ZO-1 were quantified by Western blot. The left panel shows a representative western blot, and the right panel shows the quantification. Data were normalized by β-actin protein level. The results are the mean ± s.e.m. of at least six individual mice in each group. NS indicates non-significant.
Figure S5: Effect of CFT073 on lipocalin-2 and hepcidin expressions in cultured renal mIMCD-3 and hepatic HepG2 cells respectively.
mIMCD-3 and HepG2 cell lines were infected with PFA-fixed CFT073, and the mRNA level of lipocalin-2 was explored at different MOIs (MOI, multiplicity of infection, number of bacteria per number of monolayer cells) and different incubation times. (A) shows the effect of different MOIs on lipocalin-2 mRNA abundance in mIMCD-3 cells. (B) shows the time-course effect using an MOI of 5:1. (C) Hepcidin-mRNA repression in HepG2 cells in response to different MOIs of CFT073. All mRNA quantifications were determined by RT-qPCR. Data were normalized by GAPDH-mRNA levels. The results are the mean ± s.e.m. of at least three independent cultures. *P < 0.05, **P <0.03. NS indicates non-significant.
Table S1

Expression profile of inflammatory cytokines in WT and Hepc-/- kidneys prior CFT073 infection (Sham). The results are the mean ± s.e.m. NS indicates non-significant.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT Sham (ng/ml)</th>
<th>Hepc-/- Sham (ng/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>15.9 ± 0.7</td>
<td>7.5 ± 4.4</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>51 ± 3.1</td>
<td>65.5 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.3 ± 0.3</td>
<td>5.3 ± 1.1</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-1α</td>
<td>272 ± 12.3</td>
<td>417 ± 19.3</td>
<td>0.002</td>
</tr>
<tr>
<td>CXCL2</td>
<td>12.6 ± 1.6</td>
<td>27.4 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>CCL5</td>
<td>58.1 ± 9.6</td>
<td>141.3 ± 18.8</td>
<td>NS</td>
</tr>
</tbody>
</table>