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The antimicrobial peptide cathelicidin-BF could be a potential therapeutic for *Salmonella typhimurium* infection

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ABSTRACT

Resistance is increasing to several critical antimicrobials used to treat *Salmonella typhimurium* infection, urging people to search for new antimicrobial agents. In this work, we reported the possibility of a potent antimicrobial peptide cathelicidin-BF found in the venom of the snake *Bungarus fasciatus* in treating *Salmonella typhimurium* infection. We tested its activity in biological fluids and *in vivo* using a mouse model of *Salmonella typhimurium* infection, and examined the effect of cathelicidin-BF on *Salmonella* invasion to epithelial cells. In addition, the biodistribution of cathelicidin-BF was evaluated by using *in vivo* optical imaging. The results revealed that cathelicidin-BF was unstable in gastrointestinal tract, but retained substantially active in murine serum. Cathelicidin-BF attenuated the clinical symptoms of *Salmonella* infected-mice, significantly reduced the number of internalized *Salmonella* and attenuated *Salmonella*-induced decreases in TER in epithelial cells. Our results provide a first indication for the potential of cathelicidin-BF as a novel therapeutic option for salmonellosis.

Salmonella infection is a primary enteric pathogenic disease affecting both human and animals and therefore a major public health concern (Dougan et al. 2011; Maiti et al. 2014). Until recently the most common cause of food poisoning by *Salmonella* species was due to *Salmonella typhimurium* (*S. typhimurium*) (Gordon et al. 2008; Kupz et al. 2014). *S. typhimurium* infection manifest within 48 h after ingestion of contaminated food and include nausea, vomiting, mostly self-limiting, diarrhea. This pathogen is able to colonize the intestinal tract and modulate epithelial tight junction integrity enough to allow the physical movement of PMN across the intestinal monolayer, and penetrate the gut epithelium to ultimately gain access to systemic sites (Galan and Collmer 1999; Gruenheid and Finlay 2003; Kohler et al.

to search for new antimicrobial agents.

Cathelicidins are a family of structurally diverse antimicrobial peptides (AMPs) that exert potent antibacterial activity and acting as multifunctional effector molecules of innate immunity (Bals and Wilson 2003; Hing et al. 2013). Cathelicidin-BF (C-BF) was the first cathelicidin family peptide found in reptiles and has been found to exerting potent antibacterial activity against gram-negative bacteria, especially to *Salmonella.* Minimal inhibitory concentration (MIC) of C-BF to *S. typhimurium* is 4 μg/mL, far more effective than the human cathelicidin peptide LL-37 (MIC = 128 μg/mL) (Liu et al. 2011). C-BF is clearly among the most potent cathelicidins discovered to date. In contrast to most AMPs, C-BF is not toxic to mammalian cells at concentrations well above the MIC against microbes (Wang et al. 2008). These features suggest that C-BF might be used *in vivo* to be effective against *Salmonella* infection. But until now, there is little research on whether C-BF is functional against *Salmonella in vivo*.

The aim of this study was to investigate the activity of C-BF in a more physiological context, such as in simulated gastrointestinal fluids and serum, and examined the effects of C-BF on modulating the *Salmonella* infection *in vivo* mouse models and *in vitro* epithelial cells.

Preparation of peptides

C-BF (KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF) and Fluorescein isothiocyanate-labeled C-BF (FITC-C-BF) was synthesized from GL Biochem (Shanghai, China). Both of them are purified by RP-HPLC

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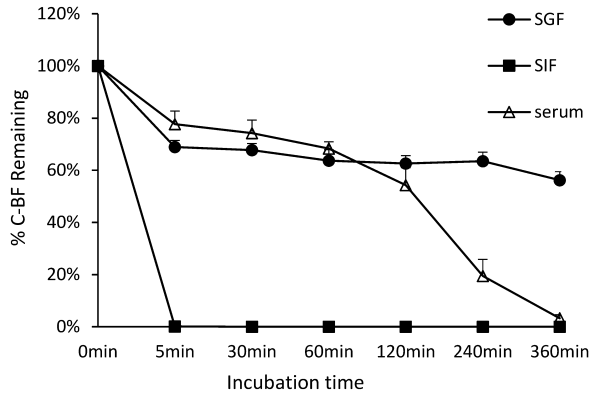
and analyzed by HPLC and mass spectrometry to confirm their purity higher than 95%.

Preparation of biological fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in the United States Pharmacopoeia (Anonymous 1995). SGF consists of 3.2 mg/mL pepsin (Sigma P7012) in 0.03 M NaCl at pH 1.2. SIF consists of 10 mg/mL of pancreatin (Sigma P7545) in 0.05 M KH_2PO_4, pH 7.5. They are prepared when needed. Mouse serum was prepared by centrifuging the coagulated healthy mouse blood at 3000 rpm for 15 min to get supernatant, and stored at −20 °C.

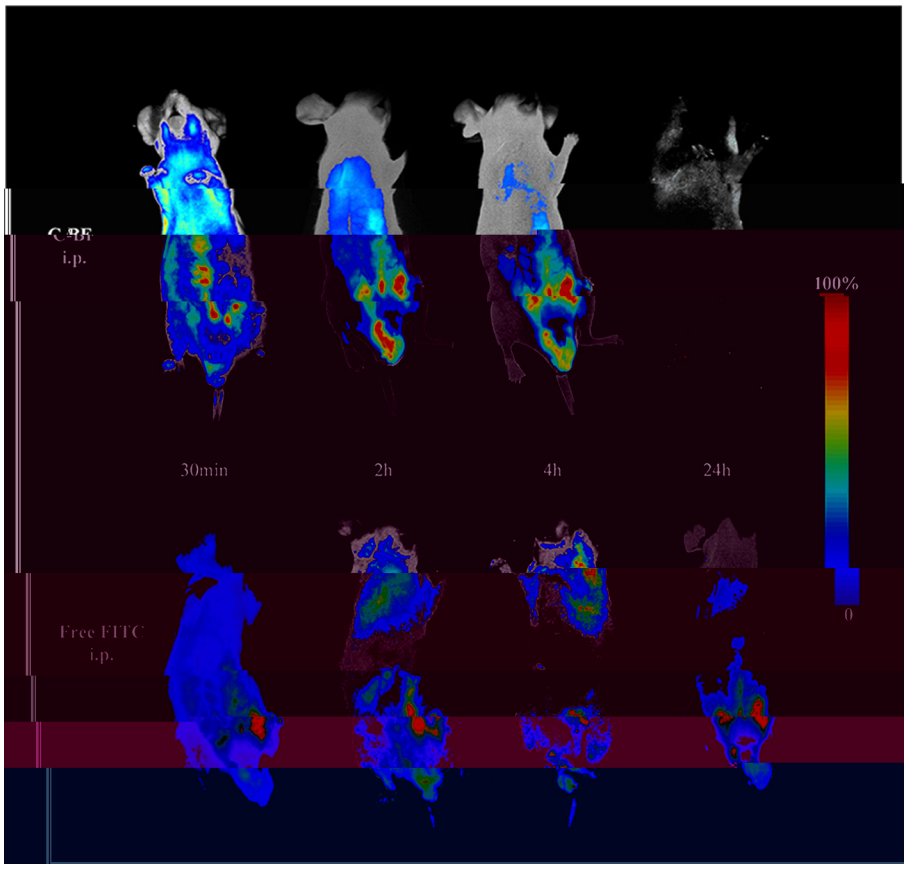
Peptide stability in biological fluids

To test the peptide stability in biological fluids, RP-HPLC analysis was performed with Water-600 series HPLC system using a Zorbax SB C18

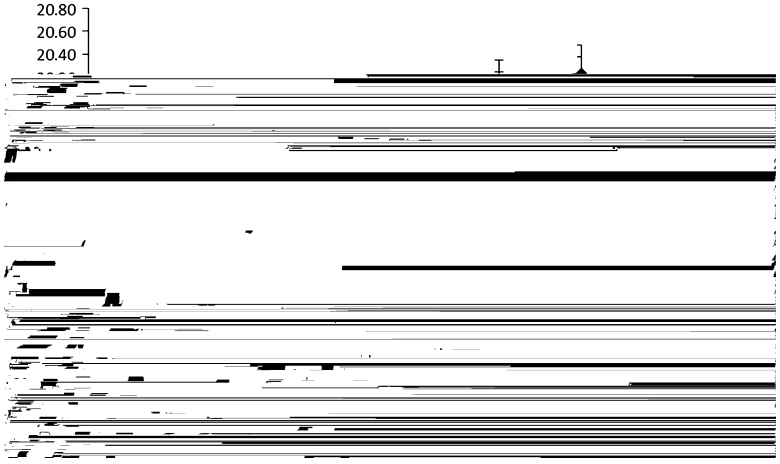


.1. Degradation of C-BF in biological fluids. The peptides were incubated with the SGF or SIF or serum for different time periods and the remaining peptide amounts were determined by RP-HPLC.

(Millicell-ERS; Millipore, Bedford, MA)(Schierack et al. 2006). The cells were randomly divided into four groups for the measurement of TER. The groups consisted of the following: the control group (treated with sterile saline), the *Salmonella* group (treated with 2×10^6 CFU/mL *Salmonella* for 3 h), and the C-BF + *Salmonella* group (treated with 2 μg/mL C-BF for 1 h, washed out before exposure to *Salmonella*), and the C-BF group (treated with 2 μg/mL



. 3. *In vivo* spectral fluorescence imaging in nu/nu mice. The animal was placed in lying position.

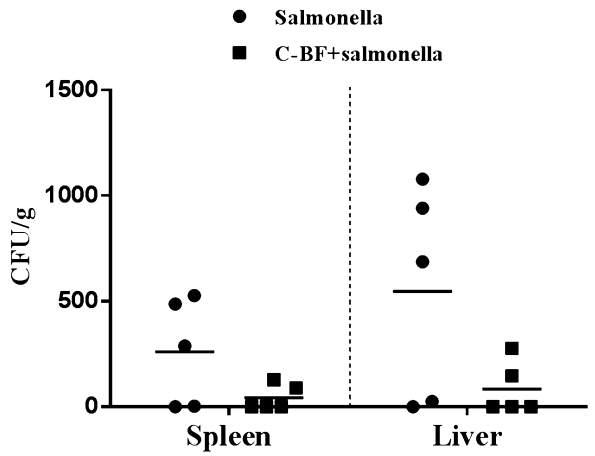


. 4. Body weight of mice in each group ($n = 10$). Data show means and SEM.

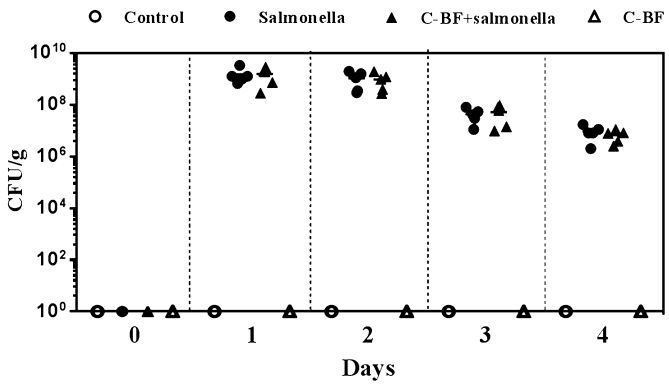
treatment on the colonization of spleen and liver by *Salmonella*. Bacteria translocation to both spleen and liver was significantly reduced ($P < 0.05$) by C-BF + *Salmonella* group compared with the *Salmonella* group (Fig. 5). The amount of *Salmonella* shed in the feces was also monitored daily. But there was no significant difference between the *Salmonella* group and C-BF + *Salmonella* group (Fig. 6).

C-BF ameliorates Salmonella-induced alterations to the architecture of intestine

To analyze *S. typhimurium*-induced intestinal pathology, we observed the alterations of gut structure by



. 5. Bacterial counts evaluated 4 days post-infection in the liver and spleen. The bars indicate the median bacterial load.



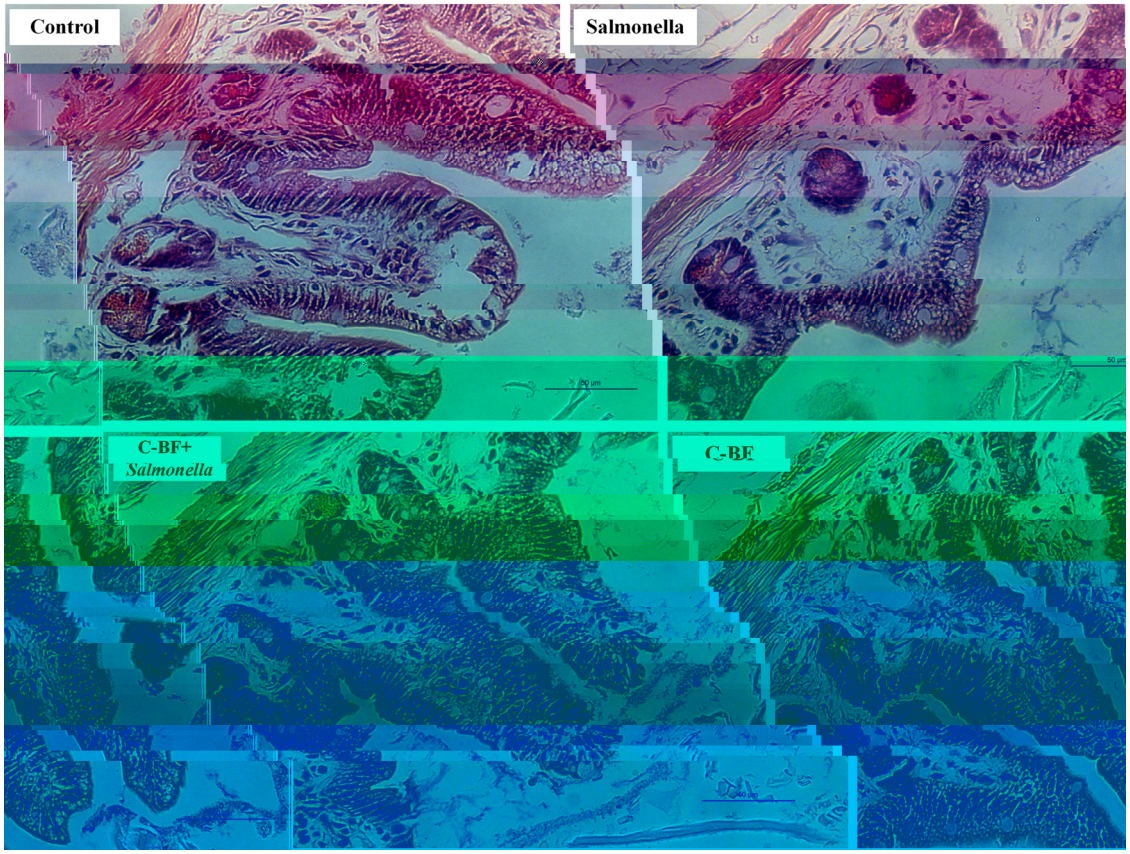
. 6. Fecal shedding of *S. typhimurium* in mice after inoculation.

C-BF prevented Salmonella invasion to epithelial cells

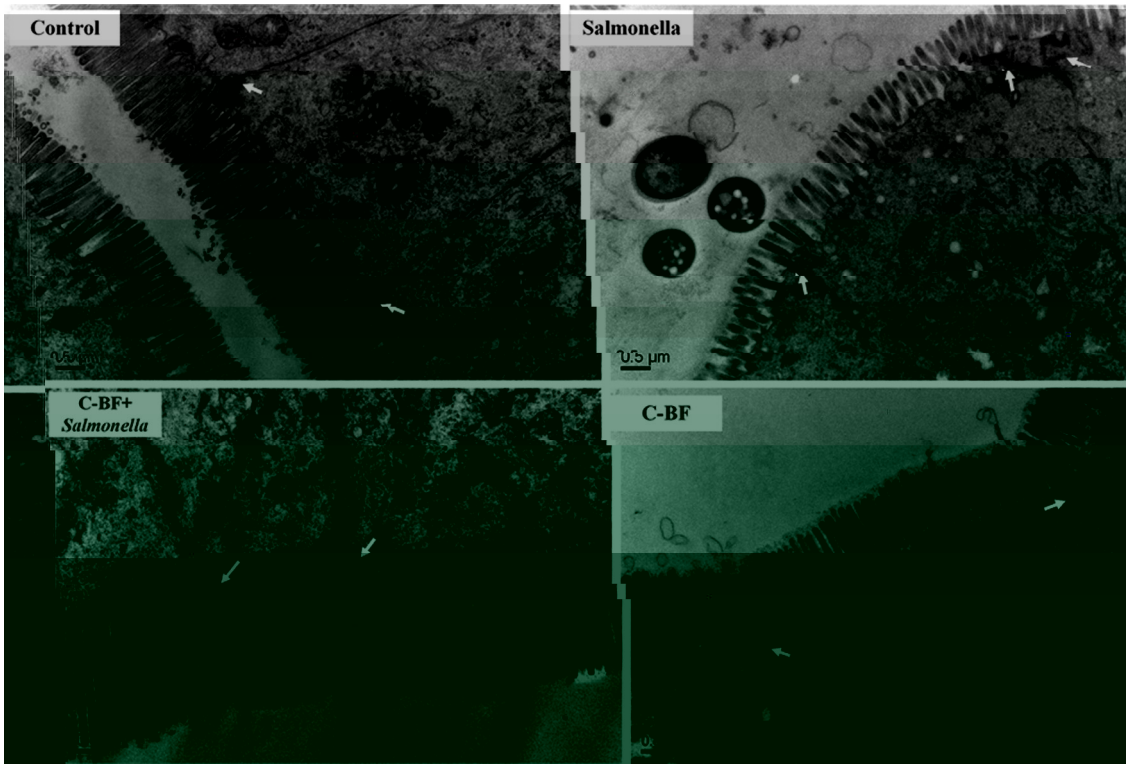
Following the results of mice infection that C-BF ameliorates the architecture of intestine and reduced the bacteria load in liver and spleen, we speculated that C-BF can somehow enhance the gut barrier function to prevent bacteria invasion, so we next examined the effect of C-BF in preventing *Salmonella* invasion using an epithelial cell line IPEC-J2. Because reduced *Salmonella invasion* in the presence of C-BF might be due to antibacterial effects of C-BF, We performed the experiment in two settings in which cells were treated with C-BF for 1 h and then washed out before bacteria addition or kept in the medium during the infection period. The data in Fig. 9 showed that both washed out or consistent C-BF, at concentrations of 2 μg/mL, could significantly reduce the number of internalized *Salmonella*. When concentrations of C-BF up to 10 μg/mL, constant C-BF had strong killing activity against *Salmonella*, while washed out C-BF exhibited equivalent effect as concentrations of 2 μg/mL.

C-BF attenuated Salmonella-induced decreases in TER in epithelial cells

IPEC-J2 cells were used to assess barrier function in response to *Salmonella* infection in the absence or presence of C-BF. As shown in Fig. 10, administered with C-BF alone did not alter the TER of polarized IPEC-J2 cells. *Salmonella* infection resulted in a rapid decrease in TER in the IPEC-J2 cell monolayer 90 min post-infection, but



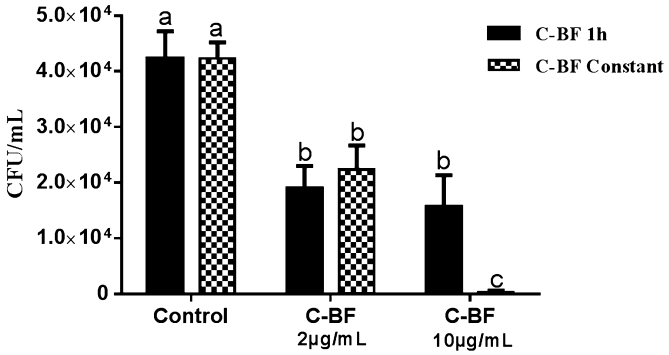
. . Hematoxylin and eosin (H&E) staining of distal ileum tissues.



. . Electron micrograph images of the colonic tissues. Arrows point to the tight junctions of intestine epithelium.

TER recovered in cells treated with C-BF which had much slower decreases.

A major limitation of antimicrobial peptides is their low stability toward proteases, this severely limits the practical application of many peptides as drugs or feed additives (Andres and Dimarcq 2004; Marr et al. 2006). Our study reveals that antimicrobial peptide C-BF exerts antibacterial activity in murine serum, but not stable in simulated intestinal fluids. The small intestine is the most degradative environment for the widest range of peptides and proteins, it contains luminal secreted proteases and membrane-bound peptidases. It has been reported that small peptides (less than 6-aa) have high stability in gastrointestinal fluids. This may be due to their lack of specific cleavage motifs (Smart et al. 2014). C-BF is a 30-aa peptide with amphipathic α-helical conformation (Wang et al. 2008), there is plenty of hydrolysis site for peptidase. The reason why C-BF remains active in serum needs to be further explored. These results implicate C-BF is better administrated by intraperitoneal injection other than gavage. Next we observed the biodistribution of FITC-C-BF using *in vivo* imaging system. FITC-C-BF's MIC against *Salmonella* is equivalent to C-BF, and



. . Invasion of *S. typhimurium* in IPEC-J2 Cells.

mice orally infected with *S. typhimurium* and allowed pathogen growth in the gut lumen and developed mucosal inflammation in colon and cecum (Stecher et al. 2010). So we use the streptomycin mouse model for *Salmonella*, in this model, infection with *S. typhimurium* leads to massive pathogen growth in large intestine and pronounced acute mucosal inflammation within 6–8 h (Barthel et al. 2003; Suar et al. 2006; Kaiser et al. 2012). The current study has shown that C-BF attenuated the symptoms of *Salmonella* infection and reduced the bacterial loads in the spleen and liver, this may due to the antibacterial activity of C-BF against *Salmonella.* At the same time, C-BF also ameliorates *Salmonella*-induced detrimental alterations to the architecture of intestine, indicate C-BF may have multiple roles *in vivo*.

The results of *in vitro* epithelial cells model reveal that C-BF can reduce *Salmonella* invasion and attenuate *Salmonella*-induced decreases in TER in epithelial cells even without the contact with the *Salmonella*. Therefore, we speculated that C-BF can protect host from *Salmonella* infection by affecting certain functions of epithelial cells. Our study contradicts a prior finding that infection with *S. typhimurium* did not influence TER (McCormick et al. 1993). There are key differences between our report and this earlier work that could account for such differences. First, the cell lines and *Salmonella* species used in these two studies are different. Second, they only measure the TER within 120 min, whereas we record it up to 180 min, and the TER of *Salmonella*-infected cells begins decreasing markedly in 120 min.

Our results indicating that C-BF has potentials to play a role in modulating *Salmonella* infection, as well as ameliorating intestinal barrier functions, suggesting that C-BF is an excellent therapeutic candidate for *S. typhimurium* infection in human and farm animals.

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