In vitro and in vivo synergy of cryptdin-2 and ampicillin against Salmonella.

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Running title: cryptdin: an adjunct against Salmonella

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ABSTRACT

In view of the emergence of multidrug-resistant *Salmonella* strains, there is a need for therapeutic alternatives. To reduce the dose of antibiotic required in order to decrease the associated side effects, the present study was aimed at evaluating the synergism, between cryptdin-2 (a Paneth cell antimicrobial peptide) and ampicillin (Amp) against *Salmonella typhimurium*. The synergy was evaluated in terms of fractional bactericidal concentration index, time-kill assay (*in-vitro*), macrophage functions i.e. intracellular killing, lipid peroxidation, superoxide dismutase activity and generation of nitrite (*ex-vivo*) and decrease in colony forming units of *Salmonellae* in livers, spleens and small intestines of infected mice treated with cryptdin-2 and/or amp (*in-vivo*). *In-vitro* synergism was observed between the two agents on the basis of FBC index and time kill assay. When the agents were used in combination, *ex-vivo* studies revealed an enhanced effect on macrophage functions, particularly exhibiting a synergetic effect in terms of SOD levels. *In-vivo* synergy was indicated by a higher log unit decrease in all the target organs of mice treated with the combination. These results point towards the possible use of cryptdin-2 as an adjunct to ampicillin and may help in developing alternate strategies to combat *Salmonella* infections.

**Keywords**: cryptdin-2, *Salmonella typhimurium*, macrophage functions, adjunctive therapy, ampicillin, Paneth cell antimicrobial peptide
INTRODUCTION

Emergence of *Salmonella* strains resistant to first-line antibiotics, as well as showing increased minimum inhibitory concentrations (MICs) of second-generation quinolones is tending to be a serious problem limiting the possibilities for effective treatment of human *Salmonella* infections [15, 33, 34]. Moreover, frequent and lengthy use of antibiotics usually results in alteration of the intestinal commensal flora and leads to chronic toxicity. Recent studies have demonstrated that Gram-negative *S. enterica* serovar Typhimurium has nine functional drug efflux pumps [26]. The expression of this multi drug efflux system has been indicated to decrease cellular drug accumulation by altering cell permeability thereby offering resistance to quinolones and some β-lactams [27]. It provides impetus to the efforts to identify and exploit alternative antibacterial therapies against *Salmonella* infections.

In this context, development of cationic antimicrobial peptides (AMPs) for the treatment of Salmonellosis has recently become a major area of investigation [41]. Among naturally occurring AMPs, defensins form a unique family of cysteine-rich cationic polypeptides with 3–4 disulfide bridges [39]. Mouse enteric alpha-defensins called cryptdins are broad spectrum AMPs due to their ability to kill various bacteria [14, 12, 23, 28], parasites [9] and enveloped viruses [35] *in-vitro*. Recently, we have demonstrated that cryptdin-2 possesses a strong *in-vivo* therapeutic potential against murine salmonellosis without exhibiting any toxicity as indicated by liver and kidney function tests [29]. Additionally, it was found to exhibit very low cytotoxicity towards macrophages even at a concentration twice that of the MBC [29].

It has also been reported that Paneth cell cryptdins are natural pore forming peptides and may be capable of mediating the transport of therapeutic molecules inside the target cell [19]. Hence, cryptdins can be perceived to be one of the promising solutions to the growing problem
of resistance to conventional antibiotics in *Salmonella*, particularly by facilitating the entry of drugs into the cell. The combination therapy of antibiotics and defensins can therefore be used to increase the *in vivo* activity as well as to broaden the antimicrobial spectrum [13]. Earlier, several AMPs have been used in combination with conventional drugs against various bacteria, fungi as well as viruses [10, 16, 21, 31, 42]. However, until now, to the best of our knowledge, no information has been available on the combined activity of cryptdins and conventional anti-
*Salmonella* drugs. The present study was, therefore planned to evaluate the *in-vitro, ex-vivo* and *in-vivo* synergistic effect, if any, of cryptdin-2, a mouse Paneth cell alpha-defensin, in combination with ampicillin, a conventionally used antibiotic against *Salmonella*.

**MATERIALS AND METHODS**

**Bacterial strain and growth medium**

*Salmonella typhimurium* NCTC74, procured from Central Research Institute, Kasauli, India was used in the present study. This strain was maintained on Mac Conkey agar medium and has been used in earlier studies both as a virulent as well as a reference strain [29]. Overnight culture was harvested by centrifugation (3783 × g, 10 min), washed once with 10 mM sodium phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS to a final concentration of approx $1 	imes 10^7$ cells/mL.

**Animals**

BALB/c mice (18–22 g) of either sex (4–5 weeks old) obtained from Central Animal House, Panjab University, Chandigarh, India were housed under standard conditions with free access to feed and water ad libitum. Throughout the study, the guidelines of the Institutional Animal Ethics Committee, Panjab University, Chandigarh (India) were followed.
Synthetic cryptdin-2 and ampicillin

Chemically synthesized peptide with an amino acid sequence LRDLVCYCRTRGCKRERMNGTCRKGHLMYTLCCR, identical to the sequence of mouse Paneth cell cryptdin-2 with disulphide linkages between Cys<sub>1</sub>-Cys<sub>6</sub>, Cys<sub>2</sub>-Cys<sub>4</sub>, Cys<sub>3</sub>-Cys<sub>5</sub>, was obtained from Taurus Scientific, USA. It was suspended in 0.01% acetic acid, stored as a stock solution of 100 mg/L at -20°C and was used within 3 weeks. Ampicillin (Amp) powder was procured from Sigma Aldrich, USA.

In-vitro activity of cryptdin-2 and Amp against Salmonella

Minimum bactericidal concentrations (MBCs)

The anti-Salmonella activity of cryptdin-2 and Amp was monitored by broth-dilution technique. In brief, cells were grown individually in the presence of different concentrations of cryptdin-2 (5-20μg/ml) and Amp (0.5–16μg/ml) in specially designed flat-bottom tubes containing 3ml of nutrient broth (peptone 5.0g/L, NaCl 5.0g/L, beef extract 1.5g/L, yeast extract 1.5g/L, pH 7.4±0.2) followed by the addition of an inoculum of 10<sup>7</sup> cells of S. typhimurium. Growth was monitored during the mid-log phase by measuring the optical density at 620 nm. In addition, cell cultures from the mid-log phase were diluted appropriately in PBS and plated on MacConkey Agar medium. After 16-18h, the number of colony-forming units (cfu) was enumerated, and the data obtained was used for calculating the MBCs of cryptdin-2 and Amp. The MBC was defined as the concentration at which there was greater than 99% inhibition of growth.

Fractional bactericidal concentrations (FBCs)

Checkerboard test was performed in 96-well microtiter trays using an 8 x 8 well configuration. Two-fold serial dilutions of cryptdin-2 and Amp were prepared with concentrations ranging from...
0 to 2 MBC. 10 µl of each cryptdin-2 dilution was added to the wells of a 96-well plate in a vertical orientation and 10 µl of each Amp dilution was added in a horizontal orientation so that the plate contained various concentrations of combinations of the two agents. Then each well was supplemented with 80µl (10⁷ cfu/ml) of *S. typhimurium* and the plate was incubated at 37°C.

Wells not containing any antibacterial agent were used as the positive growth controls. FBC was calculated by dividing the MBC of the combination of cryptdin-2 and Amp by the MBC of cryptdin-2 or Amp alone. The FBC index, obtained by adding both FBCs, was interpreted as indicating a synergistic effect when it was ≤0.5, as additive or indifferent when it was >0.5 and ≤2.0, and as antagonistic when it was >2.0 [42].

**Time-kill Assay**

To determine the bactericidal action of cryptdin-2 and Amp, separately and in combination, *S. typhimurium* was exposed to one of the antimicrobial agents or to both simultaneously and the viable count was monitored. Cryptdin-2 (19µg/ml) and Amp (4.0 µg/ml), alone and in combination {Cryptdin-2 (5.0µg/ml) + Amp (0.5µg/ml)} were added to nutrient broth (peptone 5.0g/L, NaCl 5.0g/L, beef extract 1.5g/L, yeast extract 1.5g/L, pH 7.4±0.2) containing 10⁷ cfu of *S. typhimurium* and incubated at 37°C. 100µl aliquots were withdrawn at 0, 30, 60, 90, 120 minutes and spread plated on MacConkey agar plates. The plates were then incubated at 37°C for 24 hours for enumeration of cfu.

**Ex-vivo effect of cryptdin-2 and Amp**

Murine peritoneal macrophages were isolated by the method as described by us earlier [29]. Briefly, peritoneal macrophages were isolated by intraperitoneal elicitation of mice with 1ml of 3% thioglycollate media. Two days later, mouse peritoneal cavity fluids were flushed with 8-10
ml chilled RPMI-1640 containing 10% FCS, centrifuged and macrophages were resuspended in cold RPMI-1640. Cell viability was checked by trypan blue exclusion method.

**Intracellular killing of *S. typhimurium***

Mouse peritoneal macrophages were infected with *S. typhimurium* at a multiplicity of infection (MOI) of 1:100 for 90 min. Infected macrophages were washed three times in RPMI-1640 and treated with cryptdin-2 (19µg/ml) and Amp (4µg/ml), separately and in combination at reduced concentrations {Cryptdin-2 (5.0µg/ml) + Amp (0.5µg/ml)}. After every 30, 60 and 90 min of treatment period, treated and untreated macrophages were pelleted (2000rpm, 10min) and lysed with 500µl of 0.25% TritonX-100. Lysates were serially diluted and plated on MacConkey agar medium. After an incubation of 24h at 37°C, cfu were counted. Mean percentage intracellular killing was calculated as described earlier [29]. Values were expressed as mean ± SD of three independent experiments. Statistical analysis was done by Student’s *t* test and *p*<0.05 was considered statistically significant.

**Interaction of macrophages with *S. typhimurium*** – Being an intracellular pathogen, *Salmonella*-macrophage interactions play a central role in the pathogenesis. *S. typhimurium* encounters oxidative stress within the macrophage milieu, mounted in the form of respiratory burst. The respiratory burst that is concomitant with phagocytosis produces a number of toxic byproducts. Therefore, to assess the effect on the extent of lipid peroxidation, superoxide dismutase activity and to measure the nitric oxide generation, murine peritoneal macrophages were interacted with *Salmonella* at an MOI of 1:100 in the presence and absence of cryptdin (19µg/ml) and Amp (4µg/ml) alone and in combination at reduced concentrations {Cryptdin-2 (5.0µg/ml) + Amp (0.5µg/ml)} in a six-well tissue culture plate at 37°C in CO₂ incubator for 18 hrs. After incubation, lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-
100, 1 mM PMSF) was added (1:1 ratio) to all the wells and the plate was further incubated at 4°C for 20 min. Reaction mixtures from each well were centrifuged (2000 rpm, 15 min) and supernatants thus obtained were used to study the following.

**Extent of lipid peroxidation (LPO)**

Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids (PUFAs). The peroxidation of PUFA is a feature of many types of cell injuries in which free radical intermediates are produced in excess. Lipid peroxidation can cause changes in membrane fluidity and permeability and increase the rate of protein degradation, which eventually leads to cell lysis. A measure of the LPO products such as malondialdehyde (MDA) is an indication of the extent of damage. Quantitative measurement of lipid peroxidation in the culture supernatants of macrophages was performed as described by Wills [40]. The results were expressed as nanomoles of MDA per milligram of protein, using the molar extinction coefficient of chromophore (1.56×10⁵ M⁻¹ cm⁻¹). Protein content of the samples was estimated by the method as described by Lowry et al. [20].

**Estimation of nitrite concentration**

Nitric oxide is an important signalling molecule regulating diverse range of physiological processes. Nitrite levels were measured by slight modification of the method of Green et al. [11] as described by us earlier [5] and served as an indicator of nitric oxide production. 100µl aliquots of sample were mixed with 400µl of distilled water and 500µl of Griess reagent. The reaction mixture was incubated at room temperature for 10 min (in dark) and optical density was measured at 546 nm. Nitrite was quantified using standard graph of sodium nitrite. Nitrite levels
in the cell-free supernatants of infected macrophages (treated with the agents) were compared
with those of control (uninfected macrophages) and infected macrophages (untreated).

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Estimation of superoxide dismutase (SOD) activity

Host cells are protected from oxygen-derived radical injury by naturally occurring free-radical
scavengers and antioxidant pathways, including superoxide dismutase. However, the antioxidant
defense mechanism fails either due to overproduction of free radicals or decreased activities of
scavenging enzymes, or both, causing lipid peroxidation. Therefore, SOD activity was assayed
according to the method of Kono [17]. The reaction was initiated by the addition of 0.5ml of
hydroxylamine hydrochloride to the reaction mixture containing 2 ml of nitroblue tetrazolium
(NBT) and 0.1ml of culture supernatant. Change in absorbance was measured spectro-
photometrically at 560nm. SOD activity expressed as units of SOD per milligram of protein
where one unit of activity is defined as the amount of SOD required to inhibit the rate of
reduction by NBT by 50%.

In-vivo effect of cryptdin-2 in conjunction with Amp against murine salmonellosis

Mice were infected with $10^7$ cfu of *Salmonella typhimurium* orally. Seven days after the
challenge, establishment of *Salmonella* infection was confirmed by the bacterial translocation in
the intestines, livers and spleens of the infected mice. Seven days post-infection, mice were
randomized in eleven groups with five mice in each group and treated with cryptdin-2 and
Ampicillin. Cryptdin-2 was injected subcutaneously (sc) at a dose of 5µg/mouse while Amp was
administered sc at 16, 32 and 64mg/kg body weight, individually and in combination. In addition
to these doses, lower concentrations of both the agents in combination were also tested.
Group I: Mice in this group were injected subcutaneously (sc) with 0.1ml sterile saline which served as the control (infected) group. Group II: mice were administered 16mg/kg body weight of ampicillin, sc in this group. Group III: mice in this group were administered Amp (32mg/kg body weight, sc). Group IV: mice in this group received 64mg/kg body weight of Amp (sc). Group V: mice were sc administered cryptdin-2 at a single dose of 5µg/mouse. Group VI: mice were co-administered cryptdin-2 (5µg/mouse, sc) + Amp (16mg/kg body weight, sc). Group VII: in this group, mice were co-administered cryptdin-2 (5µg/mouse, sc) + Amp (32mg/kg body weight, sc). Group VIII: mice were co-administered cryptdin-2 (5µg/mouse, sc) + Amp (64mg/kg body weight, sc) in this group. Group IX: mice were co-administered cryptdin-2 (2.5µg/mouse, sc) + Amp (8mg/kg body weight, sc). Group X: mice in this group were co-administered cryptdin-2 (2.5µg/mouse, sc) + Amp (16mg/kg body weight, sc). Group XI: mice were co-administered cryptdin-2 (2.5µg/mouse, sc) + Amp (32mg/kg body weight, sc). 48h post therapy, mice were sacrificed and their livers, small intestines and spleens were removed aseptically. Tissues were weighed and 10% homogenates were prepared in PBS. Serial 10-fold dilutions of each homogenate were plated on MacConkey agar medium for enumeration of cfu in different groups.

**STATISTICAL ANALYSIS**

Data were expressed as mean ± standard deviation of three to five independent experiments. Statistical analysis was done by Student’s unpaired \( t \) test and one way analysis of variance (ANOVA) followed by pair wise comparison procedures (Tukey test) using Jandel Sigma Stat Statistical Software, version 2.0. In all cases, statistical significance was defined as p value of atleast <0.05.
RESULTS

**In-vitro activity of cryptdin-2 and Amp against Salmonella**

**Minimum bactericidal concentrations**

Cryptdin-2 and Amp decreased the cfu of *Salmonella typhimurium* in-vitro in a concentration-dependent manner. When *Salmonella typhimurium* cells were incubated with 0.5, 1, 1.5, 2, 4, 8 and 16 µg/ml of Amp, no visible growth was observed at 4µg/ml (10.77 micromoles/L) concentration of Amp indicating this concentration as the MBC of Amp against *S. typhimurium*. Similarly, MBC of cryptdin-2 against *Salmonella* was evaluated to be 19µg/ml (4.47 micromoles/L) as reported earlier [29].

**Fractional bactericidal concentrations**

The combination of cryptdin-2 and Amp was found to be highly effective in-vitro as evidenced by the reduced MBCs of 5µg/ml and 0.5µg/ml for cryptdin-2 and Amp respectively. The FBC index was calculated to be 0.388 which indicated *in vitro* synergy between the two agents.

**Time-Kill Assay**

After 120 minutes, cryptdin-2 (19µg/ml) and Amp (4µg/ml) separately gave a significant decrease (p<0.01) of 4.11 and 3.26 log units respectively, as compared to control (120min). However, when used in combination, cryptdin-2 (5µg/ml) + Amp (0.5µg/ml), a decrease of 5.32 log units was observed after 120min (p<0.05) indicating an *in vitro* synergetic effect (Fig.1).

**Activity of cryptdin-2 and amp against intracellular Salmonella**

The mean intracellular killing in untreated macrophages was found to be 17.1±3.4%, 27.6±3.67% and 43.4±5.2% at 30, 60 and 90 minutes, respectively. On the other hand, the mean percentage intracellular killing in presence of cryptdin-2 (19µg/ml) alone at 30, 60 and 90 minutes was 31.5±4.98% (p<0.05), 46±5.16% (p<0.05) and 61.8±8.57% respectively as
compared to the untreated ones. Similarly, when infected macrophages were treated with Amp
(4µg/ml) alone, the mean intracellular killing was 30.2±4.23% (p<0.05), 40.7±3.95% (p<0.05)
and 59.2±6.17% at 30, 60 and 90 minutes respectively. A higher intracellular killing was
observed when infected macrophages were treated with cryptdin-2 (5µg/ml) in conjunction with
Amp (0.5µg/ml) at lower doses indicating the ex-vivo synergy. In this case, the killing was found
to be 43.4±5.5% (p<0.05), 57.8±6.09% (p<0.01) and 82.8±10.05% (p<0.001) at 30, 60 and 90
minutes, respectively. These results indicate that both cryptdin-2 and Amp might act in
conjunction with macrophage antibacterial effectors leading to an enhanced killing of
intracellular Salmonellae.

Estimation of lipid peroxidation levels

Significant decrease in the levels of MDA was observed when macrophages were interacted with
*S. typhimurium* in the presence of cryptdin-2 (p<0.01) and Amp (p<0.05) alone as compared to
macrophages interacted with *Salmonella* in absence of any antimicrobial agent. However, a
much higher reduction in MDA levels was observed (p<0.01) when macrophages were interacted
in the presence of both cryptdin-2 and Amp (Fig.2).

Estimation of nitrite levels

Significant decrease (p<0.05) in the levels of nitrite produced by macrophages was observed in
the presence of cryptdin-2 as compared to the macrophages interacted with *Salmonella* in the
absence of any antimicrobial agent. Similarly, nitrite levels of infected macrophages were
significantly reduced in the presence of Amp also but to a lesser extent (p<0.05) (Fig.3).
However, a more pronounced effect (p<0.05) was observed when macrophages were interacted with *Salmonella* in the presence of both cryptdin-2 and Amp (Fig.3).

**Estimation of superoxide (SOD) levels**

Interaction of macrophages with *Salmonella* induced a significant decrease in the SOD activity in the culture supernatant of macrophages (p<0.01). No significant change in SOD activity was observed in the presence of cryptdin-2 and Amp alone as compared to normally interacted macrophages in the absence of any antimicrobial agent. However, supplementation of the reaction mixture with both cryptdin-2 and Amp significantly restored the SOD activity (p<0.05) (Fig.4).

**Therapeutic potential and synergetic efficacy of cryptdin-2 and Amp against experimental salmonellosis.**

Therapeutic efficacy of cryptdin-2 and Amp alone, as well as in conjunction, was investigated in terms of reduction in the number of *Salmonellae* in different target organs of mice infected with *Salmonella typhimurium* after 48h of chemotherapy. In the livers of treated mice, the log unit decrease in bacterial loads was 1.1, 2.2, and 3.23 for 16, 32 and 64/kg body weight Amp respectively while a 1.96 log unit decrease was observed after treatment with cryptdin-2. Co-administration of 5µg of cryptdin-2 with 16, 32, 64 mg/kg body weight Amp gave a higher log unit decrease of 3, 3.79 and 4.52 respectively (Fig. 5A). In spleens of mice, log unit decrease in bacterial loads was observed to be 1.86, 2.98, 3.57 and 2.59 after treatment with 16, 32, 64mg/kg body weight Amp and cryptdin-2 (5µg), respectively. Here also, the adjunct therapy of 5µg cryptdin-2 with 16, 32, 64 mg/kg body weight Amp was found to be more effective as a higher
log unit decrease of 3.4, 3.61 and 3.98 respectively was observed (Fig.5B). In small intestines also, cryptdin-2 (5µg/mouse) alone gave a decrease of 1.67 log units in bacterial loads as compared to untreated controls. There was 1.43, 1.92 and 3.25 log unit decrease in the number of Salmonellae in small intestines of the mice treated with 16, 32 and 64mg/kg body weight Amp, respectively (Fig.5C). However, when 5µg of cryptdin-2 was used in combination with 16, 32 and 64mg/kg body weight Amp, the log unit decrease in intestinal bacterial loads was found to be 2.47, 3.62 and 4.58 units respectively. Interestingly, when the mice were treated with respective half of each Amp dose in conjunction with half the dose of cryptdin-2 (2.5µg/mouse) of cryptdin-2, the log unit decrease in the number of salmonellae in all the target organs was observed to be comparable to the decrease observed when both the agents were used together at twofold higher concentrations.

**DISCUSSION**

An alternative to overcome the problem of emerging drug resistance in Salmonella strains is to avoid overuse and misuse of antibiotics [1, 24]. In this regard, the use of cationic antimicrobial peptides in conjunction with conventional antibiotics might prove useful as this would reduce the dose of antibiotics required to treat the disease thereby decreasing the associated side effects. In the light of these facts, the present study aimed at evaluating the synergetic effect of ampicillin in conjunction with cryptdin-2 against Salmonella typhimurium.

The in vitro synergy is evidenced from the FBC index and time-kill assay as a higher antibacterial effect was exhibited when the two agents were used in combination at the concentrations much lower than their individual MBCs against Salmonella. It can be attributed to an increased permeability of Salmonella cell membrane by cryptdin-2 thereby leading to an
increased diffusion of Amp into the intracellular targets. These observations are consistent with previous studies pertaining to the synergetic actions of $\alpha$ helical peptide p18, reproductive tract $\beta$-defensins, HNP-1, magainin, cathelicidins and several other novel cationic peptides with commonly used antibiotics against *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacter cloacae* [7, 16, 32, 36, 37].

*Salmonella* has been reported to establish a niche within the macrophages where it replicates and causes a systemic disease. The dynamic interplay between host responses and bacterial virulence mechanisms determines the outcome of the infection. At this stage, antibacterial agents may directly or indirectly modulate the natural phagocyte-bacterium interactions [18]. Keeping this in view, the *ex-vivo* effect of cryptdin-2 and amp on macrophage functions, in terms of intracellular killing, extent of lipid peroxidation, superoxide dismutase activity and generation of reactive nitrogen intermediates (RNIs) was evaluated.

Treatment of infected macrophages with cryptdin-2 and Amp individually, exhibited a higher killing of intracellular Salmonellae as compared to the untreated macrophages. Interestingly, the killing effect was found to be more pronounced when both the agents were used in combination to treat intracellular Salmonellae. These results are in concordance with the earlier reports wherein it has been proposed that ampicillin kills intracellular Salmonellae within the mouse macrophages owing to its internalization by cells through pinocytosis [18]. The fact that alpha-defensins may enhance phagocytosis by interacting directly with the pathogen, may account for the higher killing observed when macrophages were treated with cryptdin-2 [29]. In support of our findings, defensins have been reported to enable macrophages to inhibit intracellular proliferation of *Listeria* [2].
There is evidence that *Salmonella* infection results in excessive production of reactive oxygen species thereby leading to lipid peroxidation and finally to tissue damage [25]. Interaction of macrophages with *Salmonella* in the presence of cryptdin-2 in conjunction with amp decreased the extent of LPO and restored the SOD levels. In particular, the SOD activity appeared to be enhanced synergistically in the presence of the combination. It is indicated that these agents might have decreased LPO by scavenging the free radicals and by upregulating the anti-oxidant activity, thus counteracting the oxidative stress. In agreement to our findings, it has been reported that antibacterial agents including defensins may directly scavenge the oxidants produced as a result of phagocyte-bacterium interaction [18]. It may also be possible that cryptdin-2 might be contributing to the inhibition of quorum sensing (in terms of bacterial density due to direct killing) thereby rendering the number of surviving *Salmonellae* insufficient enough to activate macrophages.

Nitric oxide (NO) is an important signalling molecule that acts in many tissues to regulate a diverse range of physiological processes [25]. However, excessive amounts of NO are potentially toxic and have been implicated in numerous pathological situations and chronic inflammation [3]. Reactive nitrogen intermediates (RNI) such as nitrites are known to be the end products of oxidative metabolism of labile nitric oxide and their quantification is regarded as an indicator of NO generation [5]. A significant increase in the levels of nitrite by macrophages when infected with *Salmonella* might combine with superoxide anions to form peroxynitrites, which further stimulates the production of pro-inflammatory cytokines such as TNF-α contributing to tissue injury. However, when macrophages were infected with *Salmonella* in presence of cryptdin-2 alone or in combination with ampicillin, significant decrease in the nitrite levels was observed. It might be possible that these agents neutralize the *Salmonella*- induced ...
release of nitric oxide in macrophages, as has been reported earlier [43]. Additionally, lower levels of nitrite as observed in the macrophages treated with cryptdin-2 might be attributed to the anti-inflammatory activity of cryptdin as has been reported for various other AMPs [12].

The results of in vivo studies also indicated synergism between Amp and cryptdin-2 as higher clearance of Salmonellae in all target organs was observed when both the agents were used in combination as compared to the bactericidal effect observed when the agents were used alone at the same doses. These results are in concordance with earlier reports suggesting the synergetic effect of antimicrobial peptides with β-lactam antibiotics [16, 36]. The most interesting finding of our study was that the addition of cryptdin-2 can not only potentiate the effectiveness of ampicillin but also reduces the therapeutic dosage of both the agents to half (from 5µg to 2.5µg per mouse of cryptdin-2 and from 16, 32, 64 mg/kg to 8, 16, 32mg/kg body wt. of Amp) while maintaining the therapeutic efficacy, compared with drugs alone, against experimental salmonellosis. In the present study, significantly low concentration of cryptdin 2 (5µg per mouse) was found to clear Salmonella in vivo relative to that required for in vitro anti-Salmonella activity (19µg/ml) in concordance with our earlier report [29]. The therapeutic efficacy of cryptdin-2 might be attributed to the property of antimicrobial-immunomodulatory duality exhibited by host defense peptides. Earlier also, various AMPs have been shown to possess a broad range of immunomodulatory properties including chemotraction of immune cells, induction of chemokines and resolution of infections [14, 12, 38]. Thus, it can be hypothesized that cryptdin-2 may act as an antibacterial as well as immunostimulatory molecule in vivo in Salmonella-infected mice, where it is recognized as an effector molecule of innate immunity. The implication of the present study is that cryptdin-2 can be used as a possible adjunct to ampicillin for superior treatment of salmonellosis. These observations seem to be of
significance as reducing the therapeutic concentration of the antibiotics may be a valuable strategy to avoid the development of emerging antibiotic resistance. In addition to the findings of the present study, cryptdin-2 has also been observed to possess antibacterial activity against other Gram negative organisms such as *Y. enterocoliica* (21µg/ml, MBC) and *E. coli* (10µg/ml, MBC) as well as against *S. aureus* (12µg/ml, MBC) and *E. histolytica* (4µg/ml, minimum amoebicidal concentration) (data communicated). In the light of these data, the peptide seems to be a promising broad-spectrum antimicrobial agent.

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**CONFLICT OF INTEREST STATEMENT**

The authors declare that there are no conflicts of interest.


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Figure Legends

**Figure 1:** Log$_{10}$ cfu of *Salmonella typhimurium* NCTC 74 at various time intervals in presence of Ampicillin- 4µg/ml (■), cryptdin-2: 19 µg/ml (▲) alone, and in combination i.e Amp(0.5µg/ml) + Cry (5ug/ml) (●). Values are expressed as mean ± S.D. of three independent experiments. *p<0.01 vs. log$_{10}$ cfu of *Salmonella* after 120 mins in the absence of any antibacterial agent (control ♦); *p<0.05 vs. log$_{10}$ cfu of *Salmonella* after 120 mins in the presence of Amp (4µg/ml) (■).

**Figure 2:** Effect on MDA levels of macrophages (MΦ) when interacted with *S. typhimurium* in presence of Amp and Cryptdin (Cry) alone, and in combination. Values are expressed as mean± S.D. of four independent experiments. Statistical analysis was done using 1-Way ANOVA. *p<0.01 vs. uninteracted macrophages (baseline); *p<0.05 vs. macrophages interacted with *Salmonella*; †p<0.05 vs. macrophages interacted with *Salmonella* in presence of Amp.

**Figure 3:** Effect on the nitrite levels of macrophages (MΦ) when interacted with *S. typhimurium* in the presence of Amp and Cryptdin (Cry) alone, or in combination. Values are expressed as mean ± S.D. of four independent experiments. Statistical analysis was done using 1-Way ANOVA. *p<0.05 vs. uninteracted macrophages (baseline); *p<0.05 vs. macrophages interacted with *Salmonella*.

**Figure 4:** Effect on the activity of superoxide dismutase of macrophages (MΦ) when interacted with *S. typhimurium* in presence of Amp and Cryptdin (Cry) alone, and in
combination. Values are expressed as mean ± S.D. of four independent experiments. Statistical analysis was done using 1-Way ANOVA. *p<0.05 vs. uninteracted macrophages (baseline); #p<0.05 vs. macrophages interacted with Salmonella.

Figure 5: Log_{10} cfu of S. typhimurium in (A) livers, (B) spleens and (C) small intestines of infected mice after 48h of therapy with cryptdin-2 (Cry) and ampicillin (Amp) separately and in combination. Values are expressed as mean ± S.D. of two independent experiments. Statistical analysis was done using 1-Way ANOVA. *p<0.05 vs. log_{10} cfu of S. typhimurium in untreated mice (control); #p<0.05 vs. log_{10} cfu of S. typhimurium in mice treated with Amp (16mg/kg body weight); †p<0.05 vs. log_{10} cfu of S. typhimurium in mice treated with cryptdin (5µg/mouse).