Developmental and Comparative Immunology 60 (2016) 108–114

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity

Viktoria A.F. Schneider [a], Albert van Dijk [a], Astrid M. van der Sar [b], Marina D. Kraaij [a], Edwin J.A. Veldhuizen [a], Henk P. Haagsman [a, *]

[a] Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[b] Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 18 November 2015
Received in revised form
21 February 2016
Accepted 22 February 2016
Available online 23 February 2016

Keywords:
Host defense peptide
Chicken cathelicidin-2
Zebrafish embryos
Salmonella enteritidis
Immune stimulation

ABSTRACT

Chicken cathelicidin-2 (CATH-2) is a host defense peptide that exhibits immunomodulatory and antibacterial properties. Here we examined effects of CATH-2 in zebrafish embryos in the absence and presence of infection. Yolk-injection of 0.2–1.5 h post-fertilized (hpf) zebrafish embryos with 2.6 ng/kg CATH-2 increased proliferation of phagocytic cells at 48 hpf by 30%. A lethal infection model was developed to test the prophylactic protective effect of CATH-2 peptide. Embryos (0.2–1.5 hpf) were injected with 2.6 ng/kg CATH-2, challenged with a lethal dose of fluorescently labeled *Salmonella enteritidis* pGMDs3 at 28 hpf and monitored for survival. Prophylactic treatment with CATH-2 was found to delay infection starting at 22 h post-infection (hpi). At 18–20 hpi, significantly lower (2-fold) fluorescence intensity and decreased bacterial loads were detected in peptide-treated embryos. Thus prophylactic administration of low CATH-2 concentrations confer partial protection in zebrafish embryos by boosting the innate immune system.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Host defense peptides (HDP) are key players in the primary defense of the innate immune system and are considered to have potential as new broad-spectrum antimicrobial agents (Hancock and Sahl, 2006). Chicken cathelicidin-2 (CATH-2) is one of the four cathelicidins known in chicken (Goitsuka et al., 2007; van Dijk et al., 2005; Xiao et al., 2006). CATH-2 is a short (26 amino acids), cationic peptide with amphipathic properties that has been shown to exhibit antibacterial activity at micromolar level (van Dijk et al., 2009; Veldhuizen et al., 2013). Besides its microbicidal activity, this peptide is gaining considerable interest due to its immunomodulatory properties. *In vitro* it has been demonstrated that CATH-2 neutralizes LPS and induces human monocyte chemoattractant protein-1 (MCP-1) in PMBCs (van Dijk et al., 2009). As *in vitro* cultures often do not resemble the complex *in vivo* situation, studies in animal models are crucial to investigate the function of HDPs.

Various HDPs have already been tested in *in vivo* models, showing that immunomodulation instead of direct microbial killing activity of a peptide is often essential in order to eliminate an invading pathogen (Hilchie et al., 2013). A clear example of an immunomodulatory mode of action of HDPs *in vivo* was described for the innate defense regulator-1 (IDR-1) peptide. Despite its lack of antimicrobial activity *in vitro*, it can protect mice from bacterial infections. In these studies it was demonstrated that IDR-1 treated mice were more protected from bacterial infections, because the peptide elicited elaborate immune responses in macrophages and monocytes to clear the infection (Scott et al., 2007). Similarly LL-37, the only human cathelicidin, is known to have low antimicrobial activity *in vitro* (MIC values 32–64 μg/ml). However, *in vivo* tests have shown that the bacterial load decreased in mice treated with LL-37, suggesting that triggering the immune system seems to be a key factor to eradicate invading pathogens (Bowdish et al., 2005).

Zebrafish are commonly used as an *in vivo* model, because they have a large number of offspring that develop externally, rapidly and are optically transparent (van der Sar et al., 2004). Furthermore, zebrafish have an innate and adaptive immune system, whereby the latter is not fully functional in zebrafish embryos during the first weeks. (Trede et al., 2004). Their first line of defense

* Corresponding author. Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508 TD, Utrecht, The Netherlands.
E-mail address: h.p.haagsman@uu.nl (H.P. Haagsman).

http://dx.doi.org/10.1016/j.dci.2016.02.023
0145-305X/© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

involves various phagocytic cells, like macrophages and neutrophils, which emerge in the first two days post-fertilization (Crowhurst et al., 2002; Herbomel et al., 1999; Willett et al., 1999). Due to these immunological features, zebrafish embryos are a suitable host model to study the innate immunomodulatory properties of HDPs.

The objective of this study was to investigate the immunomodulatory activity of CATH-2 *in vivo*. Using the zebrafish embryo model we determined the toxicity of the peptide and the effect of CATH-2 on immune cells was explored. Lastly, the prophylactic protective effect of CATH-2 against bacterial infections in zebrafish embryos was determined. Our results showed a protective immunostimulatory effect of CATH-2 in *Salmonella enteritidis* infected zebrafish embryos, due to a peptide-induced increase of phagocytic cells.

2. Materials and methods

2.1. Peptide

Synthetic CATH-2 peptides (RFGRFLRKIRRFRPKVTITIQGSARF-NH2) were custom made at CPC Scientific Inc. (Sunnyvale, CA) and were of >95% purity. The peptides were endotoxin-free. Peptide concentrations were made by diluting CATH-2 with injection buffer, a phosphate buffered saline (PBS) containing 1.48 mM NaH_2PO_4, 8.06 mM Na_2HPO_4 and 20 mM NaCl (pH = 7.27). For all experiments CATH-2 was used in its D-form. D-peptides have been shown to have a higher stability *in vivo*, as they are more resistant against proteolytic degradation than peptides in L-form (Molhoek et al., 2011; Wade et al., 1990).

2.2. Zebrafish embryos and injections

For all experiments embryos from the transparent mutant zebrafish line *casper* were used, which are lacking melanocytes and iridophores (White et al., 2008). Zebrafish embryos were harvested from laboratory-breeding animals, which were maintained at 26 °C in aerated 5 L tanks on a 10:14 h light on/light off cycle. Embryos were collected and kept at 31.5 °C until 28 h post-fertilization (hpf). Afterwards embryos were staged at 28 °C for their further development. Depending on the experimental set-up early stage embryos (1–16 cell stage, 0.2–1.5 hpf) or 28 hpf embryos were used for microinjection. Early stage embryos were transferred in agarose trays containing egg water (60 μg/ml instant ocean sea salts) immediately after harvesting the eggs. Zebrafish embryos at 28 hpf, were first mechanically dechorionated and then transferred to agarose trays containing egg water and 0.02% (w/v) buffered 3-aminobenzoic acid (Tricaine; Sigma-Aldrich), to anesthetize the embryos. After positioning early stage and 28 hpf embryos in the agarose trays, the embryos were used for injection purposes. *Danio rerio* (zebrafish) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). The breeding of adult fish was approved by the local animal welfare committee (Animal Experimental licensing Committee, DEC) of the VU University medical center. All protocols adhered to the international guidelines specified by the EU Animal Protection Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5–7 days after fertilization). Because embryos used in this study met these criteria, this specific study was therefore approved by the Animal Experimental Licensing Committee of the VU University medical center (Amsterdam, the Netherlands).

2.3. Toxicity studies of CATH-2

Two different stages (1–16 cell stage [0.2–1.5 hpf] weighed with chorion 2.5 mg and 28 hpf weighed without chorion 0.3 mg) of embryonic development were used for testing the toxic effects of CATH-2. Depending on the developmental stage, embryos were injected in the yolk with various concentrations of the peptide: 6.4 fg (2.6 ng/kg in 0.2–1.5 hpf and 21 ng/kg in 28 hpf embryos), 64.1 fg (25.6 ng/kg in 0.2–1.5 hpf and 210 ng/kg in 28 hpf embryos), 6.41 pg (2.6 μg/kg in 0.2–1.5 hpf and 21 μg/kg in 28 hpf embryos) and 641 pg (256.4 μg/kg in 0.2–1.5 hpf and 2 mg/kg in 28 hpf embryos) or injection buffer. Embryo survival was controlled a few hours after injection and on a daily basis up to five days.

2.4. Whole-mount immunohistochemistry and confocal imaging of peptide treated embryos

To determine the number of phagocytic cells in the embryonic tail, cells were stained with L-plastin antibodies (a kind gift of Prof. P. Martin, Bristol University, UK). L-plastin is a leukocyte specific antibody, and tags both macrophages and neutrophils at 48 hpf (Le Guyader et al., 2008; Redd et al., 2006). To this end 1–16 cell stage (0.2–1.5 hpf) zebrafish embryos were microinjected with 2.6 ng/kg CATH-2 or injection buffer in the yolk. At 48 hpf the embryos were anesthetized and fixed with 4% paraformaldehyde (PFA; EMS) overnight at 4 °C. Embryos were rinsed several times for 10 min in PBTx (PBS with 1% Triton X-100) and permeabilized with a pre-chilled trypsin solution (0.24% in PBS buffer) on ice. After washing the embryos 10 times for 10 min, the embryos were incubated for 3 h with blocking buffer (10% normal goat serum diluted in 1% PBTx) at room temperature (RT). Zebrafish embryos were incubated with anti-L-plastin, diluted 1:500 in antibody buffer [PBTx with 1% (v/v) normal goat serum and 1% (w/v) bovine serum albumin] for 1 h at RT. Subsequently, embryos were extensively washed with PBTx and blocked for 1 h at RT. Finally, embryos were incubated overnight at 4 °C with fluorescently labeled Goat anti-Rabbit antibody (Alexa-Fluor-488, Invitrogen), diluted 1:200 in antibody buffer, and again washed in PBTx. Zebrafish embryos were embedded in 1% low-melting agarose (Sigma-Aldrich) and z-stack analysis was performed of the tail close to the posterior blood island on a Leica SPE-II confocal microscope at the Center for Cell Imaging, University Utrecht. Subsequently, all cells were analyzed with the Image J/Fiji software and green fluorescent phagocytic cells were counted by eye.

2.5. Bacterial strain, growth condition and transformation

Salmonella enteritidis phage type 13a was transformed with the pGMDs3 (van der Sar et al., 2003). Bacteria were stored in glycerol stocks and grown in LB-broth and LB-plates (Lennox, BD Difco™) containing 100 μg/ml ampicillin (Sigma-Aldrich). Injection stocks with 20% glycerol were made from mid-log phase bacteria in 10 ml ampicillin-LB-broth. Finally, bacteria were washed in Dulbecco's phosphate-buffered saline (DPBS; GIBCO) and diluted 1:100,000. For visualization purposes during injections, bacteria were resuspended 1:1 with 0.17% (v/v) phenol red (Sigma-Aldrich) in PBS (pH 7.4; 136.9 mM NaCl, 2.68 mM KCl, 6.46 mM Na_2HPO_4 and 2.94 mM KH_2PO_4).

2.6. Antibacterial activity of CATH-2

Colony counting assays were used to determine the direct antibacterial activity of CATH-2 against *Salmonella enteritidis* pGMDs3. Bacteria were grown to mid-logarithmic phase and after washing the cells once in ampicillin-LB-broth diluted to

2×10^6 CFU/ml. Mixtures of 25 μl bacteria and 25 μl peptide (0–40 μM) were incubated for 3 h at 37 °C. After serially diluting (50–5000 fold) the mixtures, 100 μl per sample was spread plated on Tryptone Soya Agar (TSA; Oxoid Limited) plates containing 100 μg/ml ampicillin and after 16 h at 37 °C colonies were counted.

2.7. Determination of optimal infection dose

In order to determine the optimal infection dose, 28 hpf embryos were infected via caudal vein injections with various concentrations of *S. enteritidis* pGMDs3, ranging from 1 to more than 500 CFUs (Benard et al., 2012). Groups of 15 embryos were used per bacterial dose. Embryos were maintained after injection at 28 °C and monitored regularly for their rate of survival under the microscope the next day.

2.8. Prophylaxis studies

Immediately after harvesting 1–16 cell stage (0.2–1.5 hpf) zebrafish embryos were microinjected with 1 nl CATH-2 (2.6 ng/kg to 260 μg/kg). On average 28 embryos were used per peptide concentration, injection buffer or uninjected control. Embryos were kept at 31.5 °C overnight and at 28 hpf embryos were systemically infected with *S. enteritidis* pGMDs3 in the caudal vein. After keeping the embryos at 28 °C overnight, embryos were anesthetized and the severity of the *S. enteritidis* pGMDs3 infection was monitored using a Leica MZ16FA Fluorescence Stereo microscope. Fluorescence and bright-field pictures were taken with a Leica DFC420C camera and the fluorescent intensity of the pictures was analyzed with the eLaborant software (www.eLaborant.com). To determine the bacterial load per zebrafish embryo, each embryo was anesthetized and mashed in an Eppendorf tube containing egg water solution. The embryos were further diluted in egg water solution and plated on ampicillin LB plates. Plates were incubated for 16–20 h at 37 °C and colonies were counted the next morning. In addition, rate of survival studies were performed. For this the survival (heart-beating) of *S. enteritidis* challenged embryos, which were pre-injected with buffer or CATH-2, was monitored regularly by eye.

2.9. Graphs and statistical analysis

The graphs in this manuscript were made with the GraphPad Prism 5.0 software. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Dunnett's post hoc test. Significant differences were indicated as * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$). To analyze the survival curves for the prophylaxis studies, a cutoff value of 22 h was used to dichotomize the interval from injection of the first fish in the group till moment of death. A logistic regression analysis was applied with binary variable "death within 22 h" as outcome and experiment as random effect to take the correlation between observations within experiments into account (Bates et al., 2014). Treatment was added to the model as explanatory variable. The Akaike's Information Criterion was used to select the best model. R version 3.1.2 (R Core team, 2014) was used for this analysis.

3. Results

3.1. CATH-2 toxicity studies in zebrafish embryos

In order to determine the toxicity of CATH-2, various concentrations of the D-amino acid analog of chicken cathelicidin-2 were yolk-injected in early stage (0.2–1.5 hpf) or 28 hpf embryos. Concentrations ranging from 2.6 ng/kg to 2.6 μg/kg did not affect the survival and development of early stage embryos. However, CATH-2 proved to be lethal to embryos at the highest concentration tested (260 μg/kg). At this dose 50% of early stage embryos died within 7 h after injection (Fig. 1A). Toxicity of CATH-2 was also tested in one-day old embryos (28 hpf). No toxic effects of the different peptide concentrations were observed. Five days post-injection more than 80% of the embryos survived even the highest concentration of 2 mg/kg CATH-2 used (Fig. 1B).

3.2. Phagocytic cell proliferation

To elucidate the immunomodulatory effect of CATH-2 injection in early stage (0.2–1.5 hpf) embryos, immunohistochemistry on phagocytic cells was performed on CATH-2 treated 48-h old embryos (Fig. 2A). Macrophages and neutrophils were stained with the L-plastin antibody and their numbers quantified (Fig. 2B). Interestingly, relative to untreated or buffer injected embryos a 30% increase in phagocytic cells was observed in embryos that received 2.6 ng/kg CATH-2 (Fig. 2C). These results suggest that CATH-2 has an immunostimulatory effect by increasing the amount of phagocytic cells in embryos.

3.3. Embryo infection with S. enteritidis pGMDs3

In order to determine whether the observed increase in phagocytic cells could lead to increased protection against bacterial infections, a *S. enteritidis* pGMDs3 infection model was developed. To this end, embryos (28 hpf) were infected with various doses of *S. enteritidis* pGMDs3 via caudal vein injection. Concentrations exceeding 500 CFUs were lethal to embryos within 17 h post-infection (hpi). Embryos infected with 51–500 CFUs had a very rapid infection progression and were therefore harder to monitor. With this infectious dose approximately 50% of the embryos had died already 17 hpi and at 25 hpi all embryos had succumbed to the infection. When challenged with 10–50 CFUs more than 80% of the embryos survived at 17 hpi, and 23% of these embryos were still alive at 25 hpi. In the lowest concentration tested (1–9 CFUs) 50% of the embryos survived even after 39 hpi, which may indicate self-clearance of the infection (Fig. 3). These results showed that *S. enteritidis* pGMDs3 is lethal for 28 hpf zebrafish embryos and

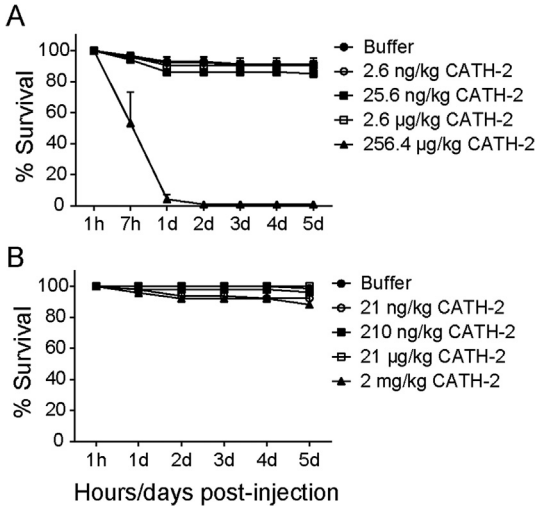

Fig. 1. High concentrations of CATH-2 have toxic effects in early stage embryos. Pools of 20 embryos, at the 1–16 cell stage (0.2–1.5 hpf; A) and 28 hpf (B), were injected in the yolk with various concentrations of CATH-2. Embryos were monitored for survival (and development) 7 h after injection and afterwards daily for five days. All experiments were performed at least in triplicate (data represent the mean ± S.E.M).

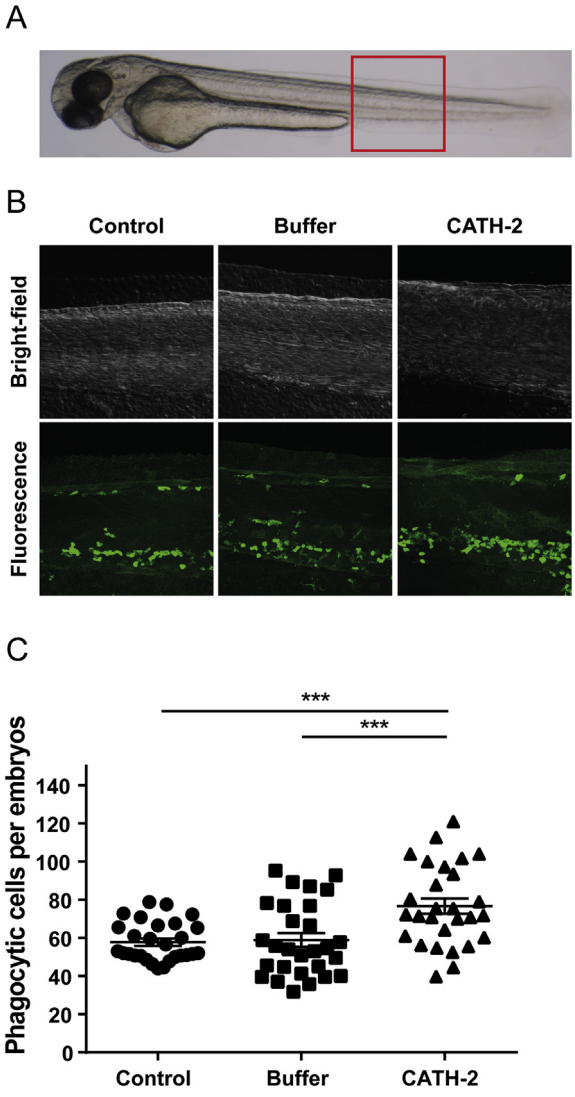

Fig. 2. CATH-2 enhances the number of phagocytic cells in zebrafish embryos. Diagram of a 48 hpf embryo with the area, highlighted in a red box, which was used to measure phagocytic cells (A). Early stage (0.2–1.5 hpf) embryos were treated with CATH-2 and macrophages and neutrophils were visualized in green using a L-plastin antibody (B) and counted (C) (n = 3, in average 9 embryos per group, bar = mean ± S.E.M is depicted). ***$p < 0.001$, one-way ANOVA, multiple comparison.

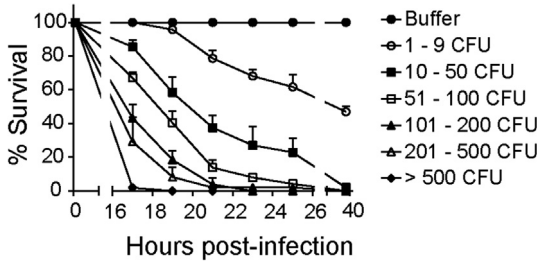

Fig. 3. *S. enteritidis* pGMDs3 is lethal for zebrafish embryos. Groups of 10–15 transparent zebrafish embryos (28 hpf) were systemically infected with various doses of *S. enteritidis* pGMDs3. Embryos were monitored 17 hpi and at regular intervals thereafter. The survival curves represent the mean ± S.E.M of three independent experiments per inoculum class.

10–100 CFUs seems to be the appropriate bacterial dose to monitor the infection in zebrafish embryos one day after infection.

3.4. Prophylactic effect of CATH-2 on S. enteritidis infection

The proposed prophylactic effect of CATH-2 in zebrafish embryos was tested by injecting various concentrations of CATH-2 into the yolk of early stage (0.2–1.5 hpf) embryos and to subsequently systemically challenge these embryos with *S. enteritidis* pGMDs3 at 28 hpf. To determine the effect of CATH-2 in the early phase of infection, the bacterial infection in the embryos was analyzed by confocal microscopy. The fluorescent signal of *S. enteritidis* pGMDs3 in the embryos was monitored 18 to 20 hpi. Embryos, which were treated with CATH-2, had a lower fluorescent intensity compared to the control groups (Fig. 4A and Fig. S1). The measured fluorescent intensity level of CATH-2 injected embryos was significantly (two-fold) lower than observed for untreated and buffer injected embryos. The median values of the pixel count demonstrated even a two-log-reduction in CATH-2 pre-injected embryos compared to the control groups (Fig. 4B).

Additional plating of embryos at 20–22 hpi on agar media revealed that prophylactic administration of 2.6 ng/kg CATH-2 into the yolk resulted in a significantly two-fold reduction of bacterial survival in embryos. Median values confirmed these findings, as again a two-fold lower CFU count was observed in peptide pre-injected embryos (Fig. 4C).

Next the rate of survival of *S. enteritidis* infected embryos, which were pretreated with CATH-2, was monitored. Although prophylactic treatment with CATH-2 did not prevent mortality, a significant delay in the progression of infection could be observed 22 hpi for embryos treated with 2.6 ng/kg CATH-2 (Fig. 5). Quantification of the effect using logistic regression showed that the odds on death within 22 h for untreated embryos was 1.9 times larger (95% CI: 1.1–3.2) than the odds for the buffer pre-injected group. For 2.6 ng/kg, 25.6 ng/kg and 2.6 μg/kg, odds were 0.5 (95% CI: 0.3–0.9), 0.7 (95% CI: 0.3–1.5) and 0.7 (95% CI: 0.4–1.3) relative to the buffer pretreated embryos respectively. Fig. 5 is a result from six pooled independent experiments, only in one experiment the buffer injected embryos, showed a higher survival compared to the peptide injected embryos at 110 hpi.

4. Discussion

In this study we demonstrated for the first time the *in vivo* activity of CATH-2 peptide. Our data showed that prophylactic CATH-2 administration in the yolk of zebrafish embryos led to a partial protection against lethal bacterial infections. Zebrafish are an established *in vivo* model, which has been extensively used to study host-pathogen interactions and to model various diseases (Meijer et al., 2014; van Leeuwen et al., 2014). Furthermore, zebrafish embryos have been used to determine the toxicity and mode of action of various biochemical and biological compounds (Berghmans et al., 2008; Fleming A., 2013; Ordas et al., 2015; Rubinstein, 2006). This model has, however, to the best of our knowledge, not yet been used to study the effect of HDPs on infection *in vivo*. To date only one study reports on the use of HDPs in zebrafish embryos to test cytotoxicity of such peptides. In this study magainin-2, pleurocidin and pleurocidin derived peptides were tested for their cytotoxic effects towards 4, 28 and 52 hpf zebrafish embryos, when added to the water (Morash et al., 2011). Such compound screening on zebrafish embryos has indeed mainly been performed by adding the compound to the water (Goldsmith, 2004; Ordas et al., 2015; Ridges et al., 2012; Zon and Peterson, 2005). This administration route has the disadvantage that compound uptake through the skin can be limited and is dependent on

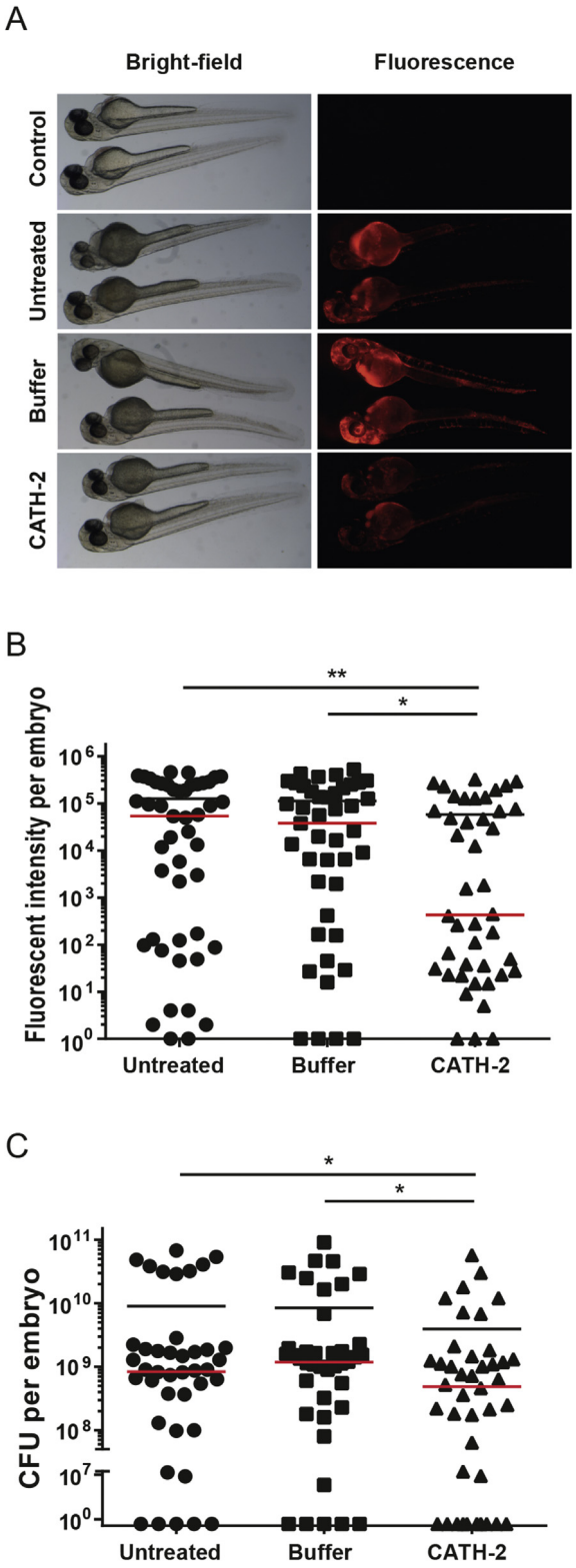

Fig. 4. CATH-2 decreases the bacterial load in embryos. Groups of 10 embryos received either 2.6 ng/kg CATH-2 or buffer by yolk injection at 1–16 cell stage (0.2–1.5 hpf) followed by challenge with *S. enteritidis* pGMDs3 at 28 hpf. Fluorescence intensity was monitored at 18–20 hpi. Bright-field and fluorescent images of two representative embryos per condition are shown (A). Images were further analyzed based on their red fluorescent pixel counts (B) (n = 4, 10 embryos per group, mean is depicted). CFUs of the *S. enteritidis* pGMDs3 infected embryos were determined 20–22 hpi (n = 4, 10 embryos per group, mean and median are depicted black and red bar, respectively) (C). $^{*}p < 0.05$, $^{**}p < 0.01$. A log-transformation was applied on the CFU and pixel data to obtain normally distributed data. Statistical analysis was performed using one-way ANOVA, multiple comparison.

the biophysical characteristics of the compound. The yolk-injection of compounds, as performed in the current study, circumvents this problem of inadequate uptake efficacies.

Toxic effects of micro-injection of CATH-2 were analyzed at two different developmental stages of the zebrafish (1–16 cell stage and 28 hpf). Only a relatively high concentration of CATH-2 (260 μg/kg) was toxic for early stage embryos (Fig. 1A). At the early point of development embryos consist only of a few cells (1–16 cells). In contrast at 28 hpf embryos are more developed, at that time they have a beating heart, blood circulation, pigmentation of their eyes and a straight tail (Benard et al., 2012; Kimmel et al., 1995). These later stage embryos were less susceptible to high peptide concentrations, exhibiting only low toxic effects with 2 mg/kg CATH-2 (Fig. 1B).

To investigate the immunostimulatory properties of CATH-2, the peptide was injected in early stage zebrafish embryos. Immunohistochemistry with L-plastin antibody was performed to detect macrophages and neutrophils in 48 hpf embryos upon pre-injection with CATH-2 at early cell stage. Injection of the peptide led to a significant 30% increase of these phagocytic cells in CATH-2 injected embryos compared to the control group (Fig. 2C).

To study the prophylactic activity of HDPs *in vivo*, a novel approach was developed by combining peptide micro-injections in early stage zebrafish embryos and challenging the same embryos at 28 hpf by caudal blood vein injection. These experiments showed a prophylactic potential of CATH-2 against bacterial infection. Both CFU and pixel counts demonstrated a significant two-fold decrease in CATH-2 pretreated embryos compared to the untreated control groups (Fig. 4). In addition, the survival rate of the embryos was monitored. At 22 h post-infection a significant delay in the progression of infection could be observed in 2.6 ng/kg of CATH-2 zebrafish embryos (Fig. 5). Colony counting assays using *S. enteritidis* pGMDs3 have shown that CATH-2 has a minimal inhibitory concentration (MIC) value of 10 μM (Fig. S2). Due to the low peptide concentrations used for the zebrafish experiments a direct killing effect of CATH-2 *in vivo* is unlikely. Taken together these findings suggest that a peptide-induced increase in phagocytic cells may contribute to the prophylactic effect of CATH-2 in the infections experiments.

Interestingly, 2.6 ng/kg seemed to be the optimum concentration during this study as higher peptide concentrations led to a lower survival rate. At a dose of 2.6 ng/kg D-CATH-2 recruitment of phagocytic cells occurs. Chemotaxis has been reported to be highly concentration dependent, i.e. immune cells will react to chemotactic ligands within a limited concentration range (De et al., 2000). It may well be that the higher concentrations tested are beyond this range. Several other explanations can be offered for the observation that higher peptide concentrations (25.6 ng/kg and 2.6 μg/kg) have less prophylactic effects. A high peptide concentration may result in specific detrimental effects on immune cells during infection. Another possibility is that at higher peptide concentrations the anti-inflammatory properties of CATH-2 prevail and cause disabling of the immature immune system.

During these studies also the L-form of CATH-2 was tested. Toxicity experiments with the L-form of CATH-2 showed similar results at all concentrations described as with the D-CATH-2. Only with the highest concentration of L-CATH-2 (260 μg/kg) toxic effects were observed in early stage embryos already 7 h post-injection (data not shown). In addition, the rate of survival of L-CATH-2 pre-injected embryos upon bacterial challenge was monitored, showing that the protective effect of L-CATH-2 against infection indicates the same tendency but was less strong compared to D-CATH-2 pre-injected embryos (data not shown). These findings support our hypothesis that the D-form of the peptide is more stable *in vivo* than the L-form.

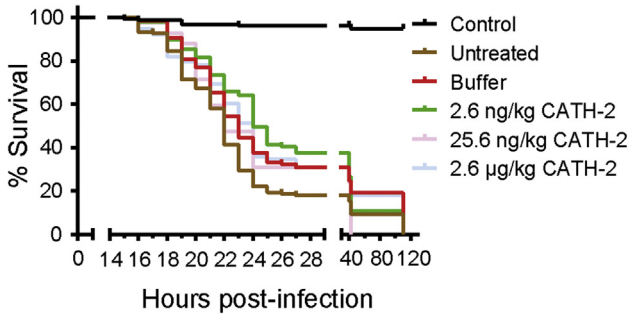

Fig. 5. CATH-2 delays the infection in zebrafish embryos in their early phase of infection. Embryos were injected with various concentrations of CATH-2 or buffer at 1–16 cell stage (0.2–1.5 hpf) and were challenged with 10–100 CFUs of *S. enteritidis* pGMDs3 at 28 hpf. Embryos were monitored for survival at 16 hpi and at regular intervals thereafter. Kaplan Meyer survival analysis was based on the pooled data of six independent experiments (n = 6, on average 28 embryos per group).

Several studies in other animal models have described comparable immunomodulatory properties of HDPs *in vivo* (Bommineni et al., 2014; Elahi et al., 2006; Lisanby et al., 2008). One such peptide is human neutrophil peptide-1 (HNP-1), which has antibacterial activity and was proven to be effective in mice where it demonstrated high activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* infections. Low doses (0.4 ng) of HNP-1 were sufficient to rescue mice from bacterial infections and this activity was related to the presence of neutrophils since no effect was observed for neutrophil depleted mice (Welling et al., 1998). Similarly, *in vivo* studies were performed on various innate defense regulator (IDR) peptides, which are derived from natural antimicrobial peptides but lack antimicrobial activity. These peptides show immunomodulatory properties by recruiting monocytes, macrophages or neutrophils during infection (Nijnik et al., 2010; Scott et al., 2007). These studies including our current findings all point towards an important role for HDPs in innate immunity related to numbers and activity of neutrophils or macrophages and possibly other innate immune cells.

In conclusion, microinjection in zebrafish embryos was used for the first time as a method to study *in vivo* immunostimulatory and prophylactic effects of a HDP. At low doses CATH-2 was found to stimulate the immune system of zebrafish embryos by enhancing the proliferation of phagocytic cells. This increased amount of phagocytic cells is thought to be crucial in lowering bacterial survival and postponing the infection in *S. enteritidis* infected zebrafish embryos.

Acknowledgement

The authors thank Gunny van den Brink-van Stempvoort, Theo Verboom, Wim Schouten and Lisanne van Leeuwen (Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam) for technical assistance and Prof. P. Martin (Bristol University, UK) for providing the L-plastin antibody used in these experiments. Furthermore we thank Hans Vernooij (Department of Farm Animal Health, Utrecht University) for helping with the statistical analysis of the survival data.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.02.023.

References

Bates, D., Maechler, M., Bolker, B., Walker, S., 2014. lme4: Linear Mixed-effects Models Using Eigen and S4. The Comprehensive R Archive Network, 1.1-7.
Benard, E.L., van der Sar, A.M., Ellett, F., Lieschke, G.J., Spaink, H.P., Meijer, A.H., 2012. Infection of zebrafish embryos with intracellular bacterial pathogens. J. Vis. Exp. 61 http://dx.doi.org/10.3791/3781 pii: 3781.
Berghmans, S., Butler, P., Goldsmith, P., Waldron, G., Gardner, I., Golder, Z., Richards, F.M., Kimber, G., Roach, A., Alderton, W., Fleming, A., 2008. Zebrafish based assays for the assessment of cardiac, visual and gut function - potential safety screens for early drug discovery. J. Pharmacol. Toxicol. Methods 58, 59–68.
Bommineni, Y.R., Pham, G.H., Sunkara, L.T., Achanta, M., Zhang, G., 2014. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. Mol. Immunol. 59, 55–63.
Bowdish, D.M., Davidson, D.J., Lau, Y.E., Lee, K., Scott, M.G., Hancock, R.E., 2005. Impact of LL-37 on anti-infective immunity. J. Leukoc. Biol. 77, 451–459.
Crowhurst, M.O., Layton, J.E., Lieschke, G.J., 2002. Developmental biology of zebrafish myeloid cells. Int. J. Dev. Biol. 46, 483–492.
De, Y., Chen, Q., Schmidt, A.P., Anderson, G.M., Wang, J.M., Wooters, J., Oppenheim, J.J., Chertov, O., 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J. Exp. Med. 192, 1069–1074.
Elahi, S., Buchanan, R.M., Attah-Poku, S., Townsend, H.G., Babiuk, L.A., Gerdts, V., 2006. The host defense peptide beta-defensin 1 confers protection against *Bordetella pertussis* in newborn piglets. Infect. Immun. 74, 2338–2352.
Fleming, A., A, W.K., 2013. Zebrafish in pharmaceutical industry research: finding the best fit fleming. Drug Discov. Today Dis. Models 10, e43–e50.
Goitsuka, R., Chen, C.L., Benyon, L., Asano, Y., Kitamura, D., Cooper, M.D., 2007. Chicken cathelicidin-B1, an antimicrobial guardian at the mucosal M cell gateway. Proc. Natl. Acad. Sci. U. S. A. 104, 15063–15068.
Goldsmith, P., 2004. Zebrafish as a pharmacological tool: the how, why and when. Curr. Opin. Pharmacol. 4, 504–512.
Hancock, R.E., Sahl, H.G., 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24, 1551–1557.
Herbomel, P., Thisse, B., Thisse, C., 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126, 3735–3745.
Hilchie, A.L., Wuerth, K., Hancock, R.E., 2013. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat. Chem. Biol. 9, 761–768.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.
Le Guyader, D., Redd, M.J., Colucci-Guyon, E., Murayama, E., Kissa, K., Briolat, V., Mordelet, E., Zapata, A., Shinomiya, H., Herbomel, P., 2008. Origins and unconventional behavior of neutrophils in developing zebrafish. Blood 111, 132–141.
Lisanby, M.W., Swiecki, M.K., Dizon, B.L., Pflughoeft, K.J., Koehler, T.M., Kearney, J.F., 2008. Cathelicidin administration protects mice from *Bacillus anthracis* spore challenge. J. Immunol. 181, 4989–5000.
Meijer, A.H., van der Vaart, M., Spaink, H.P., 2014. Real-time imaging and genetic dissection of host-microbe interactions in zebrafish. Cell. Microbiol. 16, 39–49.
Molhoek, E.M., van Dijk, A., Veldhuizen, E.J., Haagsman, H.P., Bikker, F.J., 2011. Improved proteolytic stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization. Peptides 32, 875–880.
Morash, M.G., Douglas, S.E., Robotham, A., Ridley, C.M., Gallant, J.W., Soanes, K.H., 2011. The zebrafish embryo as a tool for screening and characterizing pleurocidin host-defense peptides as anti-cancer agents. Dis. Models Mech. 4, 622–633.
Nijnik, A., Madera, L., Ma, S., Waldbrook, M., Elliott, M.R., Easton, D.M., Mayer, M.L., Mullaly, S.C., Kindrachuk, J., Jenssen, H., Hancock, R.E., 2010. Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. J. Immunol. 184, 2539–2550.
Ordas, A., Raterink, R.J., Cunningham, F., Jansen, H.J., Wiweger, M.I., Jong-Raadsen, S., Bos, S., Bates, R.H., Barros, D., Meijer, A.H., Vreeken, R.J., Ballell-Pages, L., Dirks, R.P., Hankemeier, T., Spaink, H.P., 2015. Testing tuberculosis drug efficacy in a zebrafish high-throughput translational medicine screen. Antimicrob. Agents Chemother. 59, 753–762.
Redd, M.J., Kelly, G., Dunn, G., Way, M., Martin, P., 2006. Imaging macrophage chemotaxis in vivo: studies of microtubule function in zebrafish wound inflammation. Cell Motil. Cytoskelet. 63, 415–422.
Ridges, S., Heaton, W.L., Joshi, D., Choi, H., Eiring, A., Batchelor, L., Choudhry, P., Manos, E.J., Sofla, H., Sanati, A., Welborn, S., Agarwal, A., Spangrude, G.J., Miles, R.R., Cox, J.E., Frazer, J.K., Deininger, M., Balan, K., Sigman, M., Muschen, M., Perova, T., Johnson, R., Montpellier, B., Guidos, C.J., Jones, D.A., Trede, N.S., 2012. Zebrafish screen identifies novel compound with selective toxicity against leukemia. Blood 119, 5621–5631.
Rubinstein, A.L., 2006. Zebrafish assays for drug toxicity screening. Expert Opin. Drug Metab. Toxicol. 2, 231–240.
Scott, M.G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., Wang, A., Lee, K., Doria, S., Hamill, P., Yu, J.J., Li, Y., Donini, O., Guarna, M.M., Finlay, B.B., North, J.R., Hancock, R.E., 2007. An anti-infective peptide that selectively modulates the innate immune response. Nat. Biotechnol. 25, 465–472.
Trede, N.S., Langenau, D.M., Traver, D., Look, A.T., Zon, L.I., 2004. The use of zebrafish to understand immunity. Immunity 20, 367–379.
van der Sar, A.M., Appelmelk, B.J., Vandenbroucke-Grauls, C.M., Bitter, W., 2004. A star with stripes: zebrafish as an infection model. Trends Microbiol. 12,

451–457.
van der Sar, A.M., Musters, R.J., van Eeden, F.J., Appelmelk, B.J., Vandenbroucke-Grauls, C.M., Bitter, W., 2003. Zebrafish embryos as a model host for the real time analysis of *Salmonella typhimurium* infections. Cell. Microbiol. 5, 601–611.
van Dijk, A., Molhoek, E.M., Veldhuizen, E.J., Bokhoven, J.L., Wagendorp, E., Bikker, F., Haagsman, H.P., 2009. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. Mol. Immunol. 46, 2465–2473.
van Dijk, A., Veldhuizen, E.J., van Asten, A.J., Haagsman, H.P., 2005. CMAP27, a novel chicken cathelicidin-like antimicrobial protein. Vet. Immunol. Immunopathol. 106, 321–327.
van Leeuwen, L.M., van der Kuip, M., Youssef, S.A., de Bruin, A., Bitter, W., van Furth, A.M., van der Sar, A.M., 2014. Modeling tuberculous meningitis in zebrafish using *Mycobacterium marinum*. Dis. Models Mech. 7, 1111–1122.
Veldhuizen, E.J., Brouwer, E.C., Schneider, V.A., Fluit, A.C., 2013. Chicken cathelicidins display antimicrobial activity against multiresistant bacteria without inducing strong resistance. PLoS One 8, e61964.
Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G., Merrifield, R.B., 1990. All-D amino acid-containing channel-forming antibiotic peptides. Proc. Natl. Acad. Sci. U. S. A. 87, 4761–4765.
Welling, M.M., Hiemstra, P.S., van den Barselaar, M.T., Paulusma-Annema, A., Nibbering, P.H., Pauwels, E.K., Calame, W., 1998. Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. J. Clin. Investig. 102, 1583–1590.
White, R.M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C.E., Zon, L.I., 2008. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2, 183–189.
Willett, C.E., Cortes, A., Zuasti, A., Zapata, A.G., 1999. Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev. Dyn. 214, 323–336.
Xiao, Y., Cai, Y., Bommineni, Y.R., Fernando, S.C., Prakash, O., Gilliland, S.E., Zhang, G., 2006. Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. J. Biol. Chem. 281, 2858–2867.
Zon, L.I., Peterson, R.T., 2005. In vivo drug discovery in the zebrafish. Nat. Rev. Drug Discov. 4, 35–44.