Veterinary Parasitology 197 (2013) 113–121

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Parasiticidal activity of a novel synthetic peptide from the core α-helical region of NK-lysin

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ARTICLE INFO

Article history:
Received 7 December 2012
Received in revised form 10 March 2013
Accepted 13 April 2013

Keywords:
Avian
Coccidiosis
Eimeria
Neospora
Alternative to antibiotics
NK-lysin

ABSTRACT

NK-lysin is an anti-microbial peptide that plays a critical role in innate immunity against infectious pathogens through its selective membrane disruptive property. We previously expressed and purified a full-length chicken NK-lysin (cNKL) recombinant protein, and demonstrated its *in vitro* anti-parasitic activity against the apicomplexan protozoan, *Eimeria*, the etiologic agent of avian coccidiosis. This study evaluated the *in vitro* and *in vivo* anti-parasitic properties of a synthetic peptide (cNK-2) incorporating a predicted membrane-permeating, amphipathic α-helix of the full-length cNKL protein. The cNK-2 peptide exhibited dose- and time-dependent *in vitro* cytotoxic activity against *E. acervulina* and *E. tenella* sporozoites. The cytotoxic activity of 1.5 μM of cNK-2 peptide against *E. acervulina* following 6 h incubation was equal to that of 2.5 μM of melittin, the principal active component of apitoxin (bee venom) that also exhibits anti-microbial activity. Even greater activity was detected against *E. tenella*, where 0.3 μM of cNK-2 peptide was equivalent to 2.5 μM of melittin. Against *Neospora caninum* tacyzoites, however, the cytotoxic activity of cNK-2 peptide was inferior to that of melittin. Transmission electron microscopy of peptide-treated *E. tenella* sporozoites revealed disruption of the outer plasma membrane and loss of intracellular contents. *In vivo* administration of 1.5 μM of cNK-2 peptide increased protection against experimental *E. acervulina* infection, as measured by greater body weight gain and reduced fecal oocyst shedding, compared with saline controls. These results suggest that the cNK-2 synthetic peptide is a novel anti-infective peptide that can be used for protection against avian coccidiosis during commercial poultry production.

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1. Introduction

Compared with other commercial livestock, chickens are the most efficient feed-to-meat converters, and the relative global production of poultry products is increasing at a faster rate compared with pork, cattle, and sheep (Godfray et al., 2010). Many challenges lie ahead with this increasing demand for poultry meat and eggs that will only be solved by a paradigm shift in current management and production practices (Lee and Lillehoj, 2011).

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[1] This work was carried out during sabbatical leave at the National Academy of Agricultural Science, Rural Development Administration, Suwon, Gyeonggi, Republic of Korea.

0304-4017/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.vetpar.2013.04.020

Table 1
cNKL synthetic peptides.

Peptide	Amino acid sequence[a]	Molecular weight (Da)	p*I*	Hydrophobicity ⟨H⟩[b]	Hydrophobic moment ⟨μH⟩[b]	Net charge at pH 7.4 (z)[b]
cNK-1	GDDPDEDAINNALNKVCSTG	2048	3.7	0.066	0.263	−4
cNK-2	RRQRSICKQLLKKLRQQLSDALQNNDD	3269	10.3	0.017	0.425	4
cNK-3	VCSTGRRQRSICKQLLKKLRQQ	2630	11.6	0.159	0.444	7
cNK-4	AINNALNKVCSTGRRQRSICKQLLKKLRQQ	3469	11.6	0.161	0.469	8

[a] Hong et al. (2006).
[b] Gautier et al. (2008).

Among the more important of these problems are the use of antibiotic growth promoters (AGPs) and the emergence/re-emergence of infectious diseases (Lillehoj and Lee, 2012).

Avian coccidiosis is an economically important enteric disease of commercial poultry caused by several species of *Eimeria* protozoa (Shirley and Lillehoj, 2012). Avian coccidiosis is estimated to cost the global poultry industry over $3 billion annually due to the combined expenses associated with vaccination, in-feed coccidiostatic drugs, and impaired growth performance (Lillehoj and Lillehoj, 2000; Shirley and Lillehoj, 2012). This high economic expenditure is expected to increase given the prohibitively high costs of new drug development and the shift toward drug-free poultry production in response to growing consumer concerns of chemical food residues.

Feed-based AGPs have undoubtedly improved poultry production since their introduction over 60 years ago (Yegani and Korver, 2008). Nevertheless, these beneficial effects have not been realized without unintended negative consequences. Bager et al. (1997) first reported an association between the use of the glycopeptide antibiotic, avoparcin, as a growth promoter and the occurrence of vancomycin-resistant *Enterococcus faecium* on poultry and pig farms in Denmark. Since then, an increasing body of literature has documented the appearance of antibiotic-resistant pathogenic bacteria in AGP-fed chickens, and there is a growing consensus that these pathogens may be spreading to human populations. Indeed, multiple studies prior to and following that of Bager et al. (1997) have described direct transmission of antibiotic-resistant bacteria from chickens to humans (Cooke et al., 1971; Levy et al., 1976; Ojeniyi, 1989; Singh et al., 1992; Al Ghamdi et al., 1999). In addition, other studies have suggested that the benefit of increased growth performance in AGP-fed chickens does not offset the added cost of antibiotic usage (Graham et al., 2007). As a result, interest has shifted toward the development of antibiotic-free strategies for poultry production including, for example, drug-free organic farming (Lillehoj and Lee, 2012). Removal of antibiotics from poultry production, however, is correlated with an increasing incidence in some infectious diseases. The occurrence of avian necrotic enteritis due to *Clostridium perfringens* infection has grown in countries that have restricted the use of AGPs (Van Immerseel et al., 2004, 2009; Cooper and Songer, 2009; Van der Sluis, 2000).

Anti-microbial peptides (AMPs) offer one strategy to maintain poultry health in the absence of antibiotic usage. AMPs in general are relatively small polypeptides, ranging from 12 to 50 amino acids in length with estimated molecular weights of 2–6 kDa. These peptides possess potent, broad spectrum antibiotic activity against Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and enveloped viruses (Yount et al., 2006). Far less is known about the ability of AMPs to kill protozoa parasites (Jacobs et al., 2003; Gelhaus et al., 2008). One such AMP was originally isolated from porcine intestine and named NK-lysin as a peptide of possible natural killer (NK) cell origin (Andersson et al., 1995). In previous reports, we described the cloning, expression, and characterization of a chicken NK-lysin (cNKL) recombinant protein (Hong et al., 2006, 2008). Although the deduced amino acid sequence of cNKL possessed less than 20% identity to mammalian NK-lysin molecules, it retained critical charged amino acids and an α-helical secondary structure that has been proposed to be responsible for membrane insertion and lysis of target cells. Recombinant cNKL mediated *in vitro* cytotoxic activity against *E. acervulina* and *E. maxima* parasites, as well as against an avian retrovirus-transformed B cell line. The current study was undertaken to evaluate the *in vitro* cytotoxic activity of synthetic peptides corresponding to predicted α-helices of cNKL against *Eimeria* and *Neospora* apicomplexan protozoa, and to assess those peptides with *in vitro* activity for their ability to modulate host resistance to experimental avian coccidiosis *in vivo*.

2. Materials and methods

2.1. Peptide synthesis

Four synthetic peptides (Table 1) corresponding to cNKL amino acids (aa) 19–38 (cNK-1), aa 39–65 (cNK-2), aa 34–55 (cNK-3), and aa 26–55 (cNK-4) were synthesized with an amidated COOH-terminus and purified to greater than 80% homogeneity by reversed phase high performance liquid chromatography (Bio Basic Inc, Ontario, Canada). All peptides were dissolved in 0.1% trifluoroacetic acid to prepare stock solutions that were diluted in the appropriate assay medium prior to analysis.

2.2. Cytotoxic activity of cNKL synthetic peptides for *E. acervulina* and *E. tenella* sporozoites and *N. caninum* tachyzoites

E. tenella and *E. acervulina* sporozoites were obtained by excystation of sporulated oocysts (Lee et al., 2011). Freshly sporulated oocysts were disrupted with 0.5 mm glass beads for 5–7 s using a Mini-beadbeater (BioSpec Products, Bartlesville, OK). The released sporocysts were purified by isopycnic centrifugation on a Percoll gradient (Dulski and Turner, 1988), washed in ice-cold PBS, and treated at 41 °C

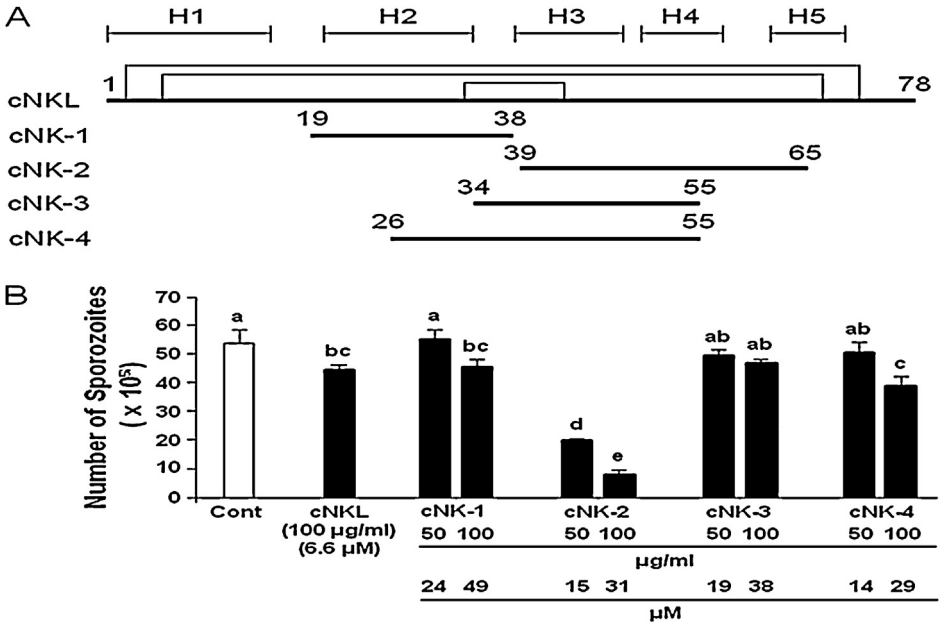

Fig. 1. (A) The relative positions of the cNK-1, cNK-2, cNK-3, and cNK-4 synthetic peptides within the full-length cNKL sequence. α-Helices (H) and disulfide bonds are as described in Sordillo et al. (2005). (B) Cytotoxic activities of the cNK-1, cNK-2, cNK-3, and cNK-4 synthetic peptides against *E. acervulina* sporozoites. Parasites (5.0×10^6) were incubated at 41 °C for 6 h with the medium control (Cont), the full-length cNKL protein, or the synthetic peptides at the indicated concentrations, stained with trypan blue, and viable cells were enumerated microscopically. Each bar represents the mean ± SEM value ($n = 3$). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

for 30 min with 0.25% trypsin and 0.014 M taurocholic acid (Sigma, St. Louis, MO) to release sporozoites. The sporozoites were collected by filtration, washed 3 times with Hank's Balanced Salt Solution (HBSS) at 3000 × *g* for 10 min at 4 °C, and resuspended to 1.0×10^6/ml in 10% RPMI-1640 medium (Sigma). Green monkey kidney CV1 cells were infected with the NC-1 isolate of *N. caninum* tachyzoites (Tuo et al., 2005) in RPMI-1640 medium supplemented with 2.0% fetal calf serum and 50 μg/ml gentamicin at 37 °C. Infected cells were collected by centrifugation and lysed by sequential passage through 20- and 27-gauge needles. Tachyzoites were collected by centrifugation at 1000 × *g* for 20 min at 4 °C, purified by centrifugation over a 40% Percoll gradient at 2000 × *g* for 30 min at 4 °C, washed 3 times with PBS, and resuspended at 5.0×10^6/ml in 10% RPMI-1640 medium. The sporozoites and tachyzoites were incubated at 41 °C for 1, 3, 6, or 24 h with HBSS alone, 2.5 μM of melittin (HPLC grade, Sigma) as a positive control (Dempsey, 1990), or various concentrations of cNK-1, cNK-2, cNK-3, or cNK-4 synthetic peptides. Parasites were stained with trypan blue and viable cells were enumerated microscopically.

2.3. Transmission electron microscopy

E. tenella sporozoites (5.0×10^6/ml) in 10% RPMI-1640 medium were incubated at 41 °C for 24 h with HBSS or 1.5 μM of cNK-2 synthetic peptide. The sporozoites were collected by centrifugation for 2 min at 5000 × g, fixed at room temperature for 2 h in 2.5% glutaraldehyde and 0.05 M cacodylate, pH 7.2, post-fixed for 2 h in 2% buffered osmium tetroxide, dehydrated in an a graded ethanol series, and infiltrated with Spurr's low-viscosity embedding resin. Gold sections (90 nm thickness) were cut on a Reichert/AO Ultracut microtome with a Diatome diamond knife, mounted on 400 mesh Formvar-coated Ni grids, stained with 4.0% uranyl acetate and 3.0% lead citrate, and viewed at 80 kV in a HT7700 Hitachi transmission electron microscope (Hitachi High Technologies America, Inc., Dallas, TX).

2.4. In vivo administration of cNK-2 peptide

Broiler eggs at 18 days of embryogenesis were divided into 4 groups of 20 eggs each. Uninfected and *E. acervulina*-infected negative control groups were mock inoculated with HBSS alone using an Intelliject system (AviTech, Easton, MD) as described (Lee et al., 2010). *E. acervulina*-infected experimental groups were inoculated with a single dose (0.3 μM) or 5 consecutive doses (1.5 μM) of the cNK-2 peptide. Three days later, all eggs were hatched and the chickens were housed in Petersime brooder units. At 1 day post-hatch, male birds in the experimental groups were injected intraperitoneally with an identical dose of cNK-2 peptide as they received *in ovo*. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

2.5. E. acervulina infection and measurements of body weight gain and fecal oocysts shedding

At 1 day post-hatch, birds were uninfected or orally infected with 500 sporulated, live *E. acervulina* oocysts. Body weights were measured between 0 and 5, and

between 5 and 9 days post-infection (S.H. Lee et al., 2012). For the determination of fecal oocyst shedding, birds were placed in oocyst collection cages and fecal samples were collected between 5 and 9 days post-infection (S.H. Lee et al., 2012). Oocyst numbers were determined using a McMaster chamber according to the formula: total oocysts per bird = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/2.

2.6. Statistical analysis

Each sample was analyzed in triplicate or quadruplicate. Statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL) and all data were expressed as mean ± SEM values. Comparisons of the mean values were performed by one-way ANOVA, followed by Duncan's multiple-range test, and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Identification of the cNK-2 synthetic peptide with cytotoxic activity against E. acervulina

Prior studies demonstrated that porcine NK-lysin adopts a secondary protein structure consisting of 5 α-helices separated by 3 loops, and that synthetic peptides corresponding to the helix-2/loop/helix-3 motif possess anti-bacterial activity (Andreu et al., 1999; M.O. Lee et al., 2012). Subsequently, a 27-amino acid synthetic peptide (NK-2) incorporating helix-3 of porcine NK-lysin exhibited anti-microbial activity against a variety of bacterial, fungal, and parasitic pathogens (Andrä and Leippe, 1999; Jacobs et al., 2003; Andrä et al., 2007; Gelhaus et al., 2008; Jena et al., 2011). Therefore, four sequential or overlapping peptides corresponding to the homologous helix-2/loop/helix-3 region of cNKL were prepared (Table 1) and tested for *in vitro* cytotoxic activity against *E. acervulina* sporozoites. Fig. 1A illustrates the relative positions of the cNK-1, cNK-2, cNK-3, and cNK-4 peptides within the full-length cNKL protein sequence. Peptide cNK-2, which corresponds exactly to the porcine NK-2 peptide, demonstrated the greatest cytotoxic activity against *E. acervulina* sporozoites compared with the other peptides (Fig. 1B).

3.2. Cytotoxic activity of cNK-2 peptide against E. tenella

To extend the cytotoxic activity of the cNK-2 peptide to another *Eimeria* species, *E. tenella* sporozoites were incubated with cNK-2 for 24 h and parasite viability was determined. Peptide treatment of *E. tenella* sporozoites reduced viability by 27% (0.3 μM) and 71% (1.5 μM), while *E. acervulina* viability was reduced by 10% and 86% at these 2 concentrations (Fig. 2). By comparison, 2.5 μM of melittin, the cytolytic component of apitoxin (bee venom) that also exhibits anti-microbial activity (Dempsey, 1990), reduced *E. tenella* viability by 32% and *E. acervulina* viability by 80%.

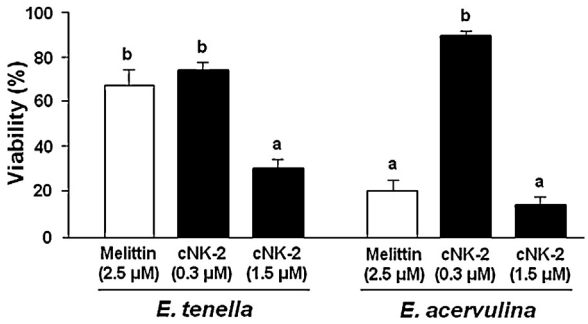

Fig. 2. Cytotoxic activity of cNK-2 synthetic peptide against *E. tenella* and *E. acervulina* sporozoites. Parasites (5.0×10^6) were incubated at 41 °C for 6 h with medium control, melittin (2.5 μM), or the indicated concentrations of cNK-2, stained with trypan blue, and viable cells were enumerated microscopically. The percent parasite viability was calculated as [(parasite number in control group − parasite number in treatment group)/(parasite number in control group) × 100%]. Each bar represents the mean ± SEM value ($n = 3$). Bars not sharing the same letters within each panel are significantly different according to the Duncan's multiple range test ($P < 0.05$).

3.3. Dose–response and time-dependent cytotoxic activity of cNK-2 peptide against E. tenella

The cNK-2 synthetic peptide exhibited dose–response and time-dependent *in vitro* cytotoxic activity against *E. tenella* sporozoites (Fig. 3). Greater cytotoxic activity was detected following 24 h incubation with 0.50 μM of cNK-2 peptide compared with 2.5 μM of melittin. At 1.0 μM of cNK-2 peptide, greater sporozoite killing was observed at 1, 3, 6, and 24 h incubation compared with melittin. Fig. 4 illustrates the effects of cNK-2 peptide on the ultrastructure of *E. tenella* sporozoites. Transmission electron microscopy of parasites treated with 1.5 μM of peptide for 24 h revealed focal disruption of the outer plasma membrane (indicated by the arrows in Fig. 4B) and a prominent area of high electron density occupying the majority of the cell's interior (Fig. 4B, asterisk). It was also evident that cNK-2 peptide-treated sporozoites exhibited a conspicuous absence of the normal intracellular organelles that were evident in medium control-treated parasites, including the nucleus, apical complex, rhopteries, and dense granules (Fig. 4A).

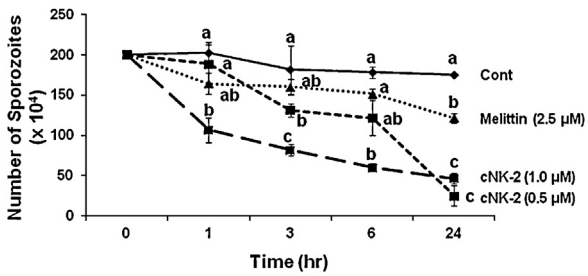

Fig. 3. Dose–response and time course of cytotoxic activity of cNK-2 synthetic peptide against *E. tenella* sporozoites. Parasites (5.0×10^6) were incubated at 41 °C for the indicated times with medium control (Cont), melittin (2.5 μM), or the indicated concentrations of cNK-2, stained with trypan blue, and viable cells were enumerated microscopically. Each bar represents the mean ± SEM value ($n = 3$). Bars not sharing the same letters are significantly different according to the Duncan's multiple range test ($P < 0.05$).

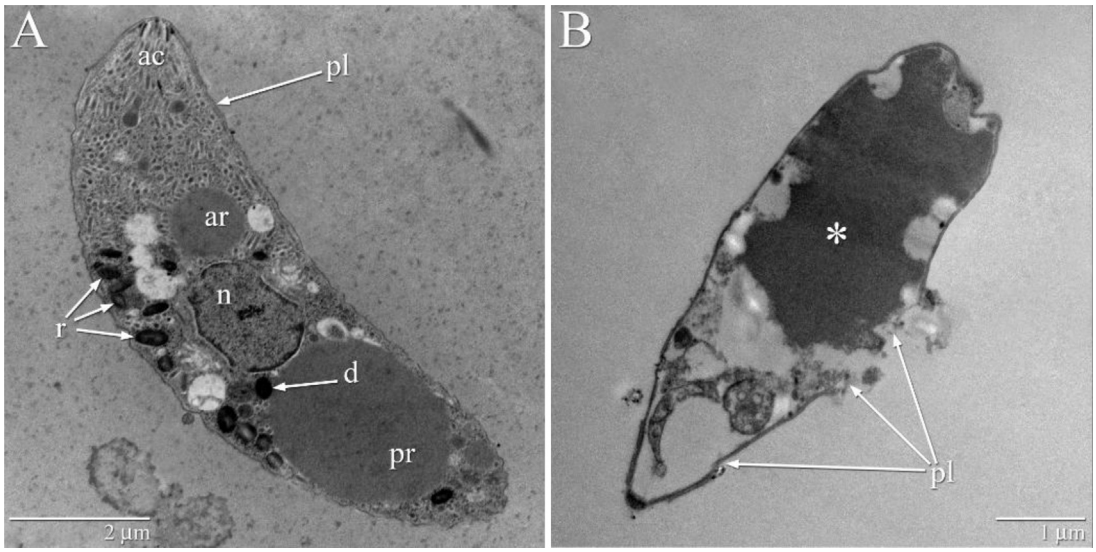

Fig. 4. Ultrastructural effects of cNK-2 synthetic peptide on *E. tenella* sporozoites. Parasites (5.0×10^6) were incubated at 41 °C for 24 h with (A) medium control or (B) 1.5 μM of cNK-2 and examined by transmission electron microscopy. The asterisk in (B) denotes a prominent area of high electron density occupying the majority of the area of the cNK-2 peptide-treated cell. n, nucleus; ac, apical complex; r, rhopteries; d, dense granule; pl, outer plasma membrane; ar, anterior refractile body; pr, posterior refractile body.

3.4. Cytotoxic activity of cNK-2 peptide against N. caninum

Treatment of *N. caninum* tachyzoites with 0.5 μM or 1.0 μM of cNK-2 peptide for 1 h reduced viability by 52% and 70%, respectively (Fig. 5). Following 3 h incubation, parasite viabilities were reduced by 73% and 79% at these two peptide doses. The viability of melittin-treated *N. caninum* tachyzoites was almost completely abolished at both time points.

3.5. Effect of cNK-2 peptide on protection against live E. acervulina infection

Chickens were given 0.3 μM or 1.5 μM of cNK-2 peptide at 18 days of embryogenesis and an identical booster dose at 1 day post-hatch, and body weight gains and fecal oocyst shedding were measured as parameters of resistance to avian coccidiosis following experimental infection with *E. acervulina*. No significant difference in body weights were observed between untreated and peptide-treated chickens at 1 day post-hatch, suggesting that *in ovo* administration of the peptide did not affect overall embryonic development (Unpublished observation). Following *E. acervulina* infection, untreated control birds had significantly reduced body weight gain compared with uninfected, untreated controls (Fig. 6). However, *in ovo* plus intraperitoneal treatment with 0.3 μM or 1.5 μM of cNK-2 peptide prior to infection was associated with significantly increased weight gains between 0 and 5 days post-infection (Fig. 6A) and between 5 and 9 days post-infection (Fig. 6B), compared with untreated, infected controls. No differences were seen between the lower and higher peptide doses on body weight gains at either time interval. As shown in Fig. 6C, treatment with 1.5 μM of cNK-2 peptide, but not 0.3 μM, significantly reduced fecal oocyst shedding between 5 and

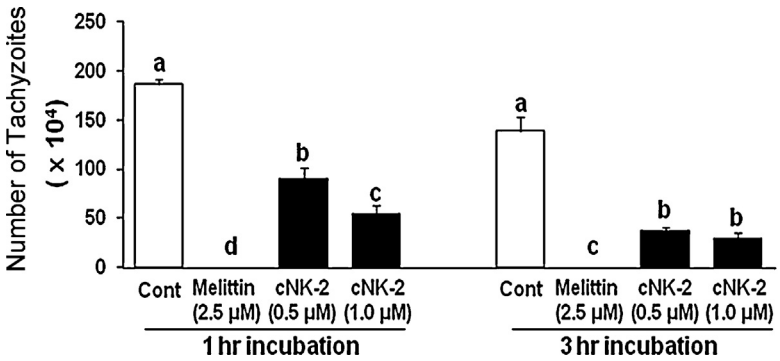

Fig. 5. Cytotoxic activity of cNK-2 synthetic peptide against *N. caninum* tachyzoites. Parasites (5.0×10^6) were incubated at 41 °C for 3 h with medium control (Cont), melittin (2.5 μM), or the indicated concentrations of cNK-2, stained with trypan blue, and viable cells were enumerated microscopically. Each bar represents the mean ± SEM value ($n = 3$). Bars not sharing the same letters within each panel are significantly different according to the Duncan's multiple range test ($P < 0.05$).

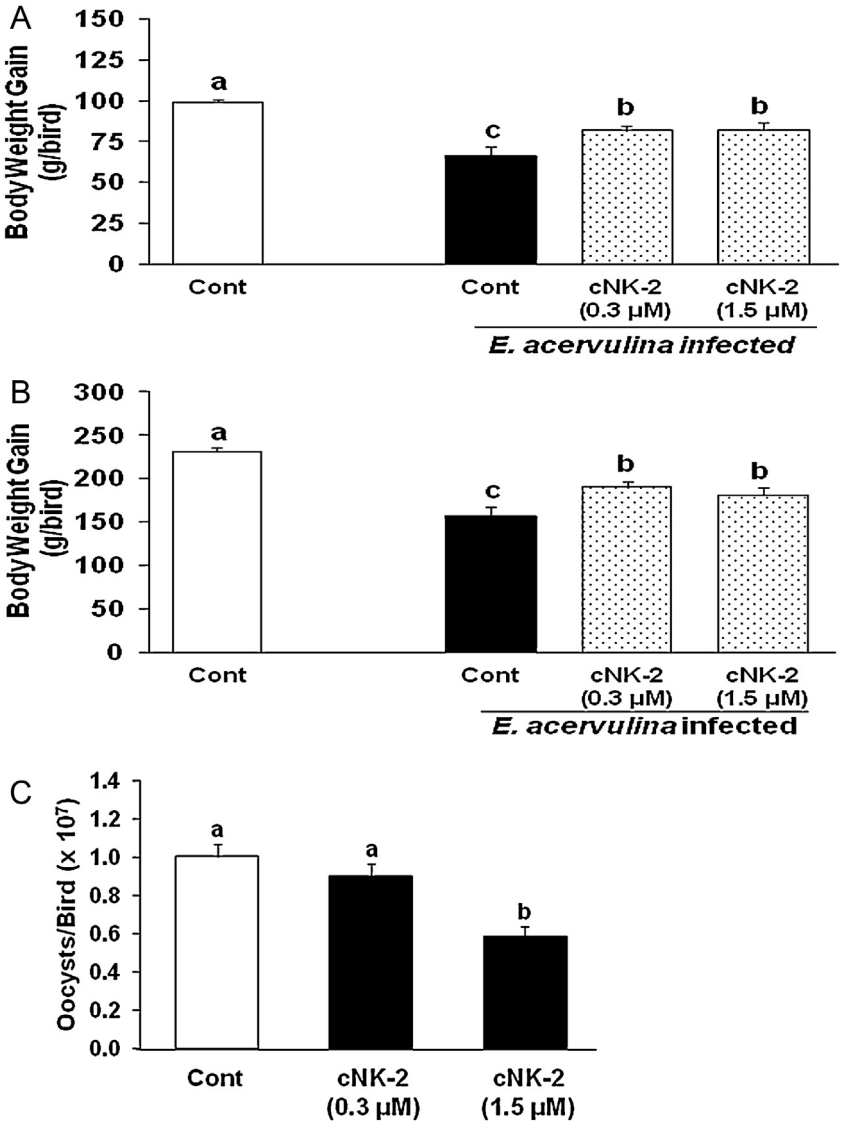

Fig. 6. Effect of *in vivo* cNK-2 synthetic peptide on body weight gain and fecal oocyst shedding following *E. acervulina* infection. Chickens were injected *in ovo* with 100 μl of PBS (Cont) or the indicated concentrations of cNK-2 at 18 days of embryogenesis and given an identical dose at 1 day post-hatch. Chickens were orally infected with 500 sporulated oocysts of *E. acervulina* at 1 day post-hatch. Body weight gains were determined between (A) 0 and 5 days and (B) 5 and 9 days post-infection. Fecal oocyst numbers were determined between 5 and 9 days post-infection (C). Each bar represents the mean ± SEM value ($n = 15$). Bars not sharing the same letter within each panel are significantly different according to the Duncan's multiple range test ($P < 0.05$).

9 days in *E. acervulina*-infected chickens, compared with untreated, infected controls.

4. Discussion

The goal of this study was to identify one or more synthetic peptides with amino acid sequences derived from cNKL that possess anti-protozoan activities against apicomplexan parasites of chickens. The major findings are (a) the cNK-2 peptide representing residues 39–65 of cNKL exhibited dose- and time-dependent *in vitro* cytotoxic activity against *E. tenella* and *E. acervulina* sporozoites, two species of *Eimeria* responsible for avian coccidiosis, as well as against *N. caninum* tacyzoites, (b) transmission electron microscopy of peptide-treated *E. tenella* sporozoites revealed disruption of the outer plasma membrane and loss of intracellular contents, and (c) *in vivo* administration cNK-2 peptide increased protection against experimental *E. acervulina* infection, as measured by greater body weight gain and reduced fecal oocyst shedding, compared with untreated controls. Based on previous studies demonstrating the membranolytic effects of mammalian NK-lysin peptides (Schröder-Borm et al., 2003; Andrä et al., 2007; Yan et al., 2012), these results suggest that the cNK-2 peptide is cytotoxic against *Eimeria* as a consequence of its ability to alter the integrity of the sporozoite outer membrane resulting in the release of its intracellular contents. The cNK-2 peptide also reduced the viability

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cNK-2 (39-65)a                                          RRQRSICKQLLKKLRQQLSDALQNNDD
cN29N (22-51)b                        PDEDAINNALNKVCSTGRRQRSICKQLLKK
cNKLF2 (34-55)c                                     VCSTGRRQRSICKQLLKKLRQQ
pNK-1 (40-63)d                                          ILRGLCKKIMRSFLRRISWDILTG
pNK-2 (39-65)d                                         KILRGVCKKIMRTFLRRISKDILTGKK
pNK-18 (39-56)e                                        KILRGVCKKIMRTFLRRI
pNKLF1 (27-55)f                            VTQAASRVCDKMKILRGVCKKIMRSFLRR
pNKLF2 (34-55)f                                     VCDKMKILRGVCKKIMRSFLRR
pNKL (35-55)g                                        CDKMKILRGVCKKIMRTFLRR
bNKL (35-55)g                                        CSKMRLLKGLCKSIMKKFLRR
bNKL (25-55)g                              TVIEEAASKVCSKMRLLKGLCKSIMKKFLRR
fNK-2 (39-65)h                                         GLLKSLCRKFVKTHLGELIEELTTTDD
cNKL (1-78)i    GIKCRFCVSLVKKVQKIVGDDPDEDAINNALNKVCSTGRRQRSICKQLLKKLRQQLSDALQNNDDPRDVCTTLGLCKG
pNKL (1-78)j    GYFCESCRKIIQKLEDMVGPQPNEDTVTQAASQVCDKLKILRGLCKKIMRSFLRRISWDILTGKKPQAICVDIKICKE
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Fig. 7. Amino acid sequences of synthetic peptides derived from chicken (c), porcine (p), bovine (b), and fish (f) NKLs with demonstrated anti-microbial activity. Amino acids of full-length proteins corresponding to the individual peptides are given in parentheses. Conserved cysteine residues are indicated by boxes to indicate amino acid sequence alignments of the different peptides. [a]This study; [b]M.O. Lee et al. (2012); [c]Linde et al. (2005); [d]Andrä and Leippe (1999); [e]Yan et al. (2012); [f]Andreu et al. (1999); [g]Endsley et al. (2004); [h]Hirono et al., 2007; [i]Hong et al. (2006); [j]Andersson et al. (1995).

of *N. caninum* which is an important coccidian parasite in cattle and dogs with chickens serving as an intermediate host (Lindsay and Dubey, 1989; Costa et al., 2008).

To date, more than 900 AMPs have been identified in prokaryotes and eukaryotes (Yount et al., 2006). Mechanistically, many AMPs act by permeabilization of microbial membranes, thereby directly killing target microorganisms without affecting host cell viability (Hoffmann et al., 1999). The selectivity of some AMPs for microorganisms has been suggested to be related to their ability to discriminate between the different phospholipid compositions of the outer plasma membranes of bacteria and mammalian cells, particularly the presence of anionic phospholipids in the former but not the latter (Schröder-Borm et al., 2003). There is also evidence that AMPs function as modulators of host innate and adaptive immunities (Zhang et al., 2000; Choi et al., 2012). Some of the more well-known members of this evolutionarily conserved family of peptides include those of insects (mastoparan, cecropin, melittin), amphibians (magainin, dermaseptin), and avians and mammals (defensins, granulysin, NK-lysin). Defensins are present in the granules of neutrophils (α-defensins) and mucosal epithelia (β-defensins), while the structurally distinct granulysin and NK-lysin molecules are found within the lytic granules of cytotoxic T lymphocytes and NK cells (Gansert et al., 2003). Granulysin and NK-lysin are members of the saposin-like protein (SAPLIP) family that possess characteristic disulfide bridges and a common tertiary structure, the saposin fold (Munford et al., 1995). Based on their cationic amphipathic structures, granulysin and NK-lysin exhibit pore-forming, membranolytic activity against negatively charged bacterial membranes (Ernst et al., 2000; Linde et al., 2005; Zitvogel and Kroemer, 2010), as well as selective cytotoxic activity against some tumor cells (Wang et al., 2000; Schröder-Borm et al., 2005). Synthetic peptides corresponding to the amphipathic α-helices of these molecules mimic the biological activity of the full-length proteins (Andrä and Leippe, 1999; Andreu et al., 1999; Wang et al., 2000; Jacobs et al., 2003; Endsley et al., 2004; Linde et al., 2005; Schröder-Borm et al., 2005; Hirono et al., 2007; Gelhaus et al., 2008; M.O. Lee et al., 2012; Yan et al., 2012).

In addition to NK-lysin and granulysin, the SAPLIP family of AMPs includes 6 other sub-families (amoebapores, saposins, pulmonary surfactant proteins, acid sphingomyelinases, acyloxyacyl hydrolases, and aspartic proteases) (Zhai and Saier, 2000). These polypeptides share several structural and functional attributes, including conserved hydrophobic amino acids, 6 cysteine residues forming 3 intra-molecular disulfide bridges (with the exception of 2 bridges in granulysin), 4 or 5 amphipathic α-helices (H1, H2, H3, H4, with or without H5), and the ability to interact with negatively charged lipids (Olmeda et al., 2013). SAPLIP family members possess the characteristic saposin fold, a bipartite, secondary structural element with one leaf of the fold containing helices H1 and H4/5 and the second containing the centrally-located H2 and H3 helices. Based on the 1–6, 2–5, 3–4 pairings formed by the 6 cysteine residues, the H1/H4/5 fold and the H2/H3 fold are each intramolecularly linked through disulfide bridges. Despite our knowledge of their structural properties, however, the structure-function relationship that defines the mechanism of their membranolytic action remains to be determined. One proposal posits that positively charged lysine and arginine residues on the surface of the globular structures of NKL and granulysin, and to a lesser extent amoebapore A, provide an initial interaction with negatively charged groups of membrane phospholipids (Olmeda et al., 2013). This interaction subsequently leads to protein oligomerization, membrane embedment, and formation of lipid bilayer-permeabilizing pore. Alternative mechanisms have also been proposed and it is entirely possible that the anti-microbial effects mediated by these different proteins are mechanistically dissimilar (Bruhn, 2005).

The amino acid sequence of peptide cNK-2 encompasses the majority of the COOH-terminal half of the full-length cNKL protein, and incorporates some or all of the predicted H3, H4, and H5 α-helices (Fig. 1A). At least 11 other synthetic peptides derived from these and other α-helices of chicken, fish, and mammalian NKLs have anti-microbial activity (Fig. 7) (Andrä and Leippe, 1999; Andreu et al., 1999; Endsley et al., 2004; Linde et al., 2005; Hirono et al., 2007; M.O. Lee et al., 2012; Yan et al., 2012). Of these, the

cNK-2 peptide corresponds precisely to the same region of the full-length protein as that of the NK-2 peptide derived from porcine NKL (Andrä and Leippe, 1999) and the JF-NK-2 peptide derived from Japanese flounder NKL (Hirono et al., 2007). However, cNK-2 shares only 14.8% (4/27 amino acids) and 25.9% (7/27) amino acid sequence identifies with the pig and fish NK-2 peptides. Thus, the cNK-2 peptide constitutes a novel anti-microbial synthetic peptide that may be applicable to control apicomplexan infections in chickens. In the case of a different peptide, the chicken and pig NKLF2 peptides possess equivalent cytotoxic activities against the intracellular pathogens, *Mycobacterium smegmatis* and *M. marinum*, despite their low sequence identity (22.7%) and similarity (59.1%) (Linde et al., 2005). Therefore, it is not unreasonable to predict that the cNK-2 peptide also will display anti-microbial activity against a diverse array of pathogenic microorganisms, as has been described for the corresponding porcine NK-2 peptide (Andrä and Leippe, 1999; Jacobs et al., 2003; Andrä et al., 2007; Gelhaus et al., 2008). Future studies evaluating the potential utility of the cNK-2 peptide for treatment of economically costly infectious diseases of poultry and other food animals where conventional control methods are becoming less effective should help to address this prediction.

Acknowledgments

This project was partially supported by an ARS CRIS project (1265-32000-086) and the Next-Generation BioGreen 21 Program (No. PJ008084), Rural Development Administration, Republic of Korea. The authors thank Dr. Duk Kyung Kim and Dr. Seung I. Jang for their expert advice and Margie Nichols, Misun Jeong, Myeong Seon Park, Stacy O'Donnell, Ashley Cox and Heng Tech for their technical assistance.

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