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Evaluation of the effect of peptidyl membrane-interactive molecules on avian coccidia

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Abstract This study examined the lytic effect of seven different synthetic peptidyl membrane-interactive molecules (Peptidyl-MIMs) on sporozoites of five different species of *Eimeria* infecting chickens and merozoites of two different species that infect chickens. All Peptidyl-MIMs (pMIMs) demonstrated antiparasitic effects at concentrations of 1–50 μ*M* during incubation periods varying from 1 to 20 min. In addition, electron microscopy showed that ultrastructural degeneration of the pellicle of sporozoite stages of the parasites occurred within 5–10 min of exposure to 5-μ*M* concentrations of three different pMIMs. Pore-like openings were seen in the pellicle of the sporozoites at the ultrastructural level, which indicated that the pMIMs had the same mechanism of action on the parasites as that reported from studies done on bacteria. A reduction in lesion scores was seen in chickens treated orally with 10-, 50-, or 75-μ*M* concentrations of two different proteolytic stabilized (methylated) pMIMs after challenge with three different species of avian coccidia in battery-cage trials. Collectively these data indicate that pMIMs may be useful in the control of coccidiosis in poultry.

Abbreviations *MBHA* 4-Methyl benzhydral amine · *Peptidyl-MIMs* Synthetic peptidyl membrane-interactive molecules · *pMIMs* Peptidyl-MIMs · *PBEL* Parasite Biology and Epidemiology Laboratory

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Introduction

The control of coccidiosis, an intestinal disease caused by *Eimeria* spp., has been a vital component in modern poultry production practices. Use of anticoccidial compounds, usually mixed in the feed, has allowed the rearing of large flocks of chickens in growout houses with little or no effect on bird performance by the parasite. However, increasing resistance of coccidial species to all anticoccidials cleared for use in the poultry industry is now posing a threat to this practice and has the potential of producing a catastrophic economic impact on the poultry industry (Chapman and Hacker 1994). Because these medication programs may no longer be fully effective, alternative methods for control of avian coccidia are sorely needed.

One approach toward improved control of avian coccidia may be the use of synthetic analogues of antimicrobial peptides such as peptidyl membrane-interactive molecules (Peptidyl-MIMs; Demeter Biotechnologies, Ltd.). Antimicrobial peptides are constituents of the defense systems of all animals and plants (Gabay 1994) and form pores in the cell membranes of pathogens. These pores allow ion diffusion across the membrane and eventually cause lysis of the pathogen. Synthetic antimicrobial peptides have demonstrated lytic activity against a variety of protozoan parasites (Jaynes et al. 1988; Arrowood et al. 1991; Barr et al. 1995) and can be engineered to enhance their specificity.

The objectives of the present study were (1) to determine whether newly designed Peptidyl-MIMs (pMIMs) would have a lytic effect on sporozoites and merozoites of *Eimeria* spp. that infect chickens, (2) to examine changes caused by pMIMs at the ultrastructural level, and (3) to test the potential of pMIMs in controlling *Eimeria* infection in chickens.

Materials and methods

Peptidyl membrane-interactive molecules

The pMIMs used in these trials were 22–34 amino acids in length and were synthesized and purified as previously described (Arrowood et al. 1991). In brief, peptides were synthesized on a Biosearch Sam 2 peptide synthesizer using MBHA (4-methyl benzhydral amine) resin with a -COOH-terminal amide. Peptides were purified by high-performance liquid chromatography (HPLC; 95% or greater purity was achieved). Respective molecular weights of the acetate salts were: DP1M — 3,126.27, 1D1 — 3,364.20, 1D2 — 3,126.27, 2D1 — 3,364.20, 4E1 — 2,611.87, 5C1 — 5,228.20, and P1E — 3,540.50. pMIMs DP1M and 1D1 were stabilized by methylation to prevent proteolytic digestion prior to their use in in vivo experiments. After stabilization the lots used for such experiments were first verified to lyse sporozoites effectively in vitro. Peptides were dissolved in phosphate-buffered saline (PBS) supplemented 0.5% glucose (Saline A) to make a 50-μM stock solution. Peptide solutions were dispensed in 2.5-ml aliquots and were stored at −20 °C.

In vitro studies on lysis

All dilutions of the stock pMIMs, sporozoites, and merozoites were made in Saline A. Sporozoites of *Eimeria acervulina* (PBEL isolate 12), *E. brunetti* (PBEL isolate 32), *E. maxima* (PBEL isolate 68), *E. necatrix* (PBEL isolate 56), and *E. tenella* (PBEL isolate 80) were freshly excysted (Patton and Brigman 1979). Merozoites were obtained fresh from intestinal tissue of infected chickens (Jenkins and Dame 1987). Approximately 5×10^5 sporozoites or merozoites were added to 0.5 ml prewarmed pMIMs (37 °C), which was shaken gently to mix, and 50-µl samples of each treatment group were examined microscopically at 5, 10, 20, or 30 min. Visual assessment of killing of the samples was scored from 0 to 4 as follows: 0 — no killing was evident, and all parasites within the sample appeared normal; 1 — less than 30% of parasites were killed, showing internal disruption or a shriveled appearance, whereas all others appeared normal; 2 — more than 50% of parasites were killed, showing internal disruption or a shriveled appearance, whereas some of those remaining appeared less motile and less refractile; 3 — all parasites were dead, showing internal disruption or a shriveled appearance; and 4 — all parasites were dead and many exhibited total membrane disruption.

Sporozoites of the five species of avian coccidia were tested against pMIMs 1D1, 1D2, 2D1, 4E1, 5C1, and P1E to determine which were the most efficacious in killing sporozoites. DP1M killing of *E. acervulina*, *E. maxima*, and *E. tenella* was assessed in the same manner. pMIMs were diluted to concentrations of 1, 5, 10, 25, and 50 μM. Merozoites were obtained from *E. acervulina* or *E. tenella* and were tested against 1-, 5-, 10-, and 25-μM concentrations of the six pMIMs (excluding DP1M).

Electron microscopy

E. tenella sporozoites were prepared as described above and 3×10^6 were exposed to pMIMs 1D1, 4E1, and 5C1 (5-μM concentration) for 2.5-, 5-, 10-, and 20-min intervals. They were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, postfixed in 2% osmium tetroxide , dehydrated in a graded series of ethanol, and embedded in Epon 812 (Augustine et al. 1992). All specimens were prestained overnight with 0.1% uranyl acetate in 70% ethanol at 4 °C during the dehyration procedure. Specimens were sectioned and viewed under a Phillips 201 electron microscope.

In vivo studies

Experiment 1

Broiler chicks were divided into groups of 10 per cage at 14 days of age and were assigned to the following treatments: unchallenged controls (n = 20), challenged controls (n = 20), and challenged pMIM-treated birds (n = 10). Chicks were challenged with 50,000 oocysts of *E. tenella* or 10,000 oocysts of *E. acervulina* in 1 ml water by oral gavage. Unchallenged chicks were given 1 ml water as a control. Chicks were held for 30 min after challenge, were given 1 ml 50 μM pMIM DP 1 M orally or 1 ml water as a control, and were then individually weighed. At the end of the experiment (6 days postinfection), chickens were weighed, killed by cervical dislocation, and examined for intestinal lesions (Johnson and Reid 1970).

Experiment 2

At 14 days of age, broiler chicks were divided into groups of five to six chicks per cage and treatments were randomly assigned to cages, with three cages being used per treatment. Treatment groups included unchallenged control, challenged control, unchallenged pMIM-treated, and challenged pMIM treated chicks. Challenged groups were given 1-ml aliquots of 10,000 oocysts of *E. maxima* in Saline A via oral gavage. Unchallenged groups were given 1 ml Saline A by oral gavage. Treatment doses were 10-, 25-, 50-, and 75-μM concentrations of pMIM 1D1 in Saline A. All chicks were held for 30 min after challenge, were given 1 ml pMIM or 1 ml Saline A, and were then individually weighed. At the end of the experiment (6 days postinfection), chickens were weighed, killed by cervical dislocation, and examined for intestinal lesions (Johnson and Reid 1970). Uninfected treated birds were examined for any tissue abnormality, sign of toxicity, or intestinal damage.

Statistical analysis

Results of in vivo experiments were analyzed by analysis of variance using Duncan's multiple range-test (P = 0.05) for treatment effects on the percentage of body weight gain postinfection, feed conversion, and intestinal or cecal lesion scores.

Results

Sporozoite lysis

All seven of the pMIMs tested were effective in lysing sporozoites of up to five of the different species of coccidia tested (Table 1). Sporozoites were shriveled in appearance or completely disrupted (ghosts). pMIM 1D1 was most effective against all species, killing the parasites within 5 min at concentrations of 1, 5, and 10 µM. Other pMIMs demonstrated variability in their lytic activity against the several species and required longer incubation periods to destroy the sporozoites. For example, whereas 5C1 acted quickly against *Eimeria brunetti* and *E. tenella*, 20–30 min were required for efficacy against *E. acervulina* , *E. maxima*, and *E. necatrix* (Table 1). An increase in concentration above 10 µM did not necessarily increase lytic activity. Enzyme-stabilized methylated pMIMs DP1M and 1D1 were no less effective in killing parasites than were the unstabilized pMIMs. *E. acervulina* was somewhat resistant to lysis relative to the other species, and complete lysis required 10–20 min at nearly all concentrations of the pMIMs tested. *E. tenella*, in contrast, was readily lysed by all pMIMs tested.

Table 1 Time until complete lysis of sporozoites by pMIMs[a]

Species	Peptidyl MIM	Concentration (μ*M*)				
		1	5	10	25	50
Eimeria acervulina	1D1	5	10	10	10	5
	1D2	20	10	10	5	5
	2D1	20	10	5	5	5
	4E1	30+	20	20	20	30+
	5C1	20	20	20	10	10
	P1E	30+	30+	20	20	20
	DP1M	20	10	5	5	5
E. brunetti	1D1	5	5	5	20	20
	1D2	5	5	30+	5	5
	2D1	5	5	5	5	10
	4E1	5	5	5	5	20
	5C1	5	5	5	5	5
	P1E	5	5	5	5	10
E. maxima	1D1	5	5	5	10	10
	1D2	5	5	5	5	10
	2D1	20	10	5	30+	30+
	4E1	5	5	-	20	30+
	5C1	30+	30+	10	30	30
	P1E	5	5	5	5	30+
	DP1M	30+	10	5	5	5
E. necatrix	1D1	5	5	5	5	5
	1D2	30+	30+	20	20	20
	2D1	5	5	5	5	5
	4E1	5	5	5	5	10
	5C1	20	20	20	20	20
	P1E	30+	10	5	5	5
E. tenella	1D1	5	5	5	20	20
	1D2	10	5	5	10	30
	2D1	5	5	5	5	10
	4E1	5	5	5	20	20
	5C1	5	5	5	10	20
	P1E	5	5	5	20	30+
	DP1M	30+	10	10	5	5

[a] Samples were checked at 5, 10, 20, and 30 min after the addition of pMIMs. If complete killing had not occurred at 30 min the time was indicated as 30+ min

Merozoite lysis

E. acervulina and *E. tenella* merozoite lysis was achieved most effectively using 1- to 5-μ*M* concentrations of pMIMs (Table 2). Higher concentrations of pMIMS actually increased the killing time. Like the sporozoites, the merozoites of *E. acervulina* were less sensitive than those of *E. tenella* to lysis by pMIMs.

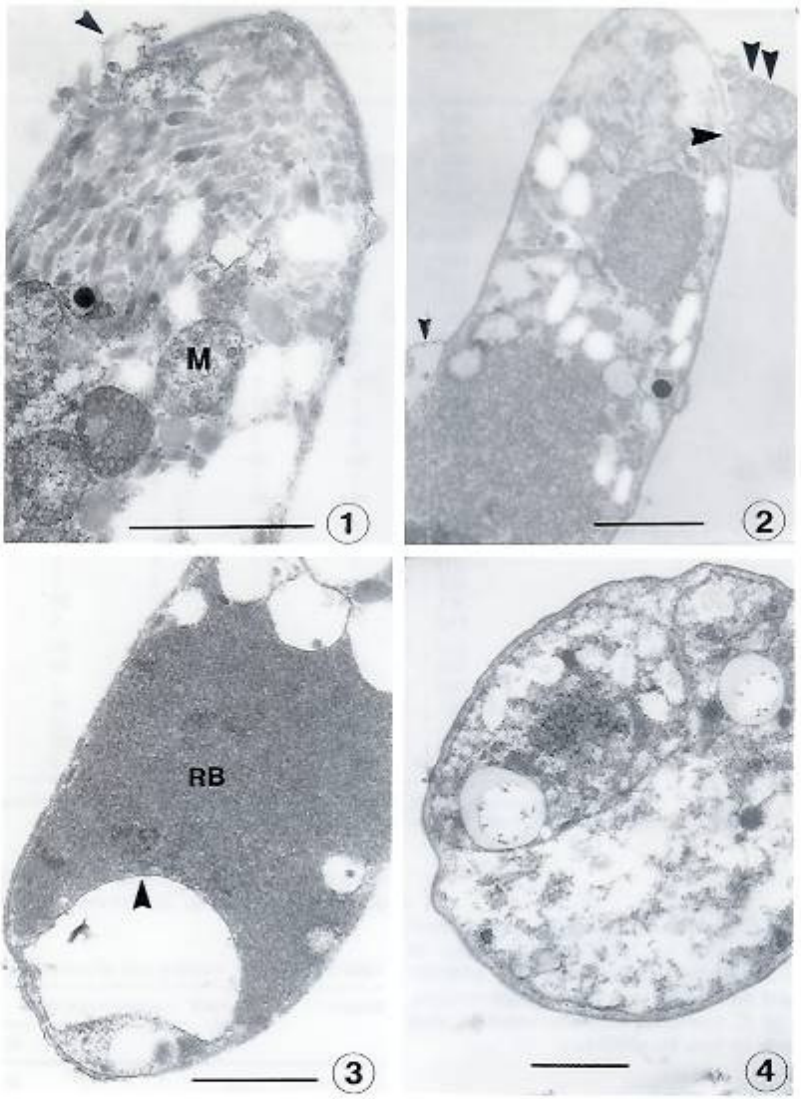
Electron microscopy

Loss of sporozoite membrane continuity was seen ultrastructurally at 5–10 min after incubation with 5-μ*M* concentrations of pMIMs 5C1 , 4E1, and 1D1 (Figs. 1–3). There was a clear disruption of the trilaminar membrane and degeneration of mitochondria (Fig. 1) and, in some specimens, cytoplasm was seen escaping from the sporozoite through a pore-like opening in the membrane pellicle (Fig. 2). Detachment of the outer layer of pellicle was seen in some sporozoites (Fig. 2). Large vacuoles, some of which were membrane-bound, were seen in other specimens of sporozoites at these same time points (Fig. 3). Yet other sporozoites had lost

Table 2 Time until complete lysis of merozoites by pMIMs[a]

Species	Peptidyl MIM	Concentration (μ*M*)			
		1	5	10	25
E. acervulina	1D1	5	5	20+	20+
	1D2	10	5	20+	20+
	2D1	10	10	20+	20+
	4E1	20+	5	20+	20+
	5C1	5	20+	20+	20+
	P1E	5	5	20+	10
E. tenella	1D1	5	5	5	10
	1D2	5	5	10	5
	2D1	5	5	5	5
	4E1	10	5	5	20+
	5C1	5	5	20	5
	P1E	5	5	20	20+

[a] Samples were checked at 5, 10, and 20 min after the addition of pMIMs. If complete killing had not occurred at 20 min the time was indicated as 20+ min



most of their interior organelle organization and were becoming ghost-like in appearance (Fig. 4). No parasite was seen in later-incubation-period samples fixed for electron microscopy, indicating a complete ultrastructural breakdown of the sporozoites.

Chicken studies

Treatment of broiler chickens with a 25-μM concentration of pMIM DPIM at 30 min after challenge with *E. acervulina* or *E. tenella* resulted in a significant decrease in the lesion scores associated with both species (Table 3) as compared with challenged controls in experiment 1. Lesion scores associated with *E. maxima* were also significantly reduced in experiment 2 when the chickens were treated with a 10-μM concentration of pMIM 1D1 (Table 4). Higher concentrations of pMIM 1D1 (25–75 μM) did not consistently reduce lesions for the *E. maxima* infection relative to challenged controls. Weight gains and feed conversions did not differ among treatments in either experiment 1 or experiment 2 (data

Table 3 Effect of pMIM DP1M given orally following infection with 50,000 *E. tenella* oocysts and 10,000 *E. acervulina* oocysts in broiler chickens (mean ± SD)

Treatment	*n*	Lesions[a]		Body weight (g)		% Gain
		Cecal	Upper	Beginning	End	
Uninfected control	20	0 ± 0[d]	0 ± 0[c]	330 ± 32[b]	551 ± 48[b]	67 ± 10[b]
Infected control	20	2.4 ± 0.6[b]	1.0 ± 0[b]	343 ± 30[b]	584 ± 48[b]	70 ± 5[b]
pMIM	10	1.8 ± 1.0[c]	0 ± 0[c]	340 ± 37[b]	589 ± 61[b]	74 ± 7[b]

[a] Gross intestinal lesions were scored on a sliding scale from +1 (mild lesions) to +4 (severe lesions)
[b–d] Mean values within a column having different letters are significantly different ($P < 0.05$)

Table 4 Effect of pMIM D1A2 given 30 min after infection on the severity of infection with *E. maxima* in broiler chickens (means ± SD) (*FC* Feed conversion in g feed/g wt gained)

Concentration (μ*M*)	Lesions[a]	Body wt gain (g)	% Gain	FC
Uninfected control	–	335.2 ± 27.2[b]	81[b]	1.54[b]
Infected control	2.9 ± 0.7[b]	270.1 ± 50.6[c]	68[c]	1.80[b]
75	2.5 ± 0.9[b,c]	290.2 ± 35.0[c]	73[c]	1.63[b]
50	1.9 ± 0.9[c,d]	295.2 ± 46.2[c]	75[c]	1.48[b]
25	2.2 ± 0.9[b,c,d]	286.8 ± 43.2[c]	73[c]	1.72[b]
10	1.8 ± 0.9[d]	277.6 ± 49.5[c]	70[c]	1.73[b]

[a] Gross intestinal lesions were scored on a sliding scale from +1 (mild lesions) to +4 (severe lesions)
[b–d] Mean values within a column having the same superscript letters are not significantly different ($P > 0.05$)

not shown). No gross organ-pathologic change was seen with birds that had been treated with pMIMs at any concentration.

Discussion

pMIMs were highly effective in killing sporozoites and merozoites of avian coccidia, and ultrastructural observations on the sporozoites showed this to be due to disruption of pellicular cell membranes. In addition, administration of pMIMs to chickens effectively reduced lesion scores recorded following infection for three species of avian coccidia.

The cytocidal effect of pMIMs on *Eimeria* sporozoites has also been reported for synthetic antimicrobial peptides acting on other protozoa. Similar peptides have lysed trypomastigotes of *Trypanosoma cruzi* (Jaynes et al. 1988; Barr et al. 1995) and reduced *Cryptosporidium* sporozoite viability as measured by propidium iodide staining (Arrowood et al. 1991).

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Figs. 1–4 Transmission electron micrographs of sporozoites of *Eimeria tenella* exposed for 5–10 min to 5-μ*M* concentrations of pMIMs 1D1, 4E1, and 5C1. *Bars* 1 μm. **Fig. 1** Sporozoite exposed to pMIM 5C1 for 10 min. X34,500. Note the disruption of the membrane pellicle (*arrowhead*) and the degeneration of mitochondria (*M*). **Fig. 2** Sporozoite exposed to pMIM 4E1 for 5 min. X18,700. Note the bulging of the outer membrane of the pellicle (*small arrowhead*) and the cytoplasm escaping (*large double arrowheads*) through a large pore-like opening (*large single arrowhead*) in the membrane pellicle. **Fig. 3** Sporozoite exposed to pMIM 1D1 for 5 min. X25,000. Note the large membrane-bound vacuole (*arrowhead*) in the area of the posterior refractile body (*RB*) of the parasite. **Fig. 4** Sporozoite exposed to pMIM 5C1 for 5 min. X17,500. Note the loss of cytoplasm density and the ghost-like appearance

Naturally occurring antimicrobial peptides (pMIMs) act on targets by forming voltage-regulated channels in the susceptible cells' membrane, an effect that has been reported for targets including bacteria, mycobacteria, viruses, fungi, and spirochetes (Kagan et al. 1994; Martin et al. 1995). The present study, showing ultrastructural disruption of the sporozoite membranes by synthetic antimicrobial peptides, indicates that the synthetic peptides probably retain the same mechanism of action as their naturally derived counterparts. This indicates that the trilaminar membrane of apicomplexan protists is susceptible to pore formation similar to that seen in other classes of pathogens.

The reduced efficacy of pMIMs in killing the parasites at higher concentrations may be related to aggregation of the increased amounts of peptides to each other due to their inherent attaction at the hydrophobic region. In addition, the higher concentration of pMIMs may have produced the correct ratio for efficient aggregation with anions present in either the sporozoite incubation media or the intestine of the bird. Sporozoite membrane has been shown to be readily labeled by cationized ferritin (Augustine and Danforth 1984), and the anionic nature of the membrane would obviously aid in the initial binding of the pMIMs to the parasite to begin the process of lytic destruction. However, in the same ferritin-labeling study, shedding of the label from the sporozoite membrane was seen, which suggests that anion molecules would be present within the incubation media. Thus, the aggregation of higher concentrations of pMIMs in the presence of anion molecules may produce a corresponding loss of pMIM lysing efficacy.

The combined effectiveness against sporozoites and extracellular merozoites indicates that pMIMs may be

useful in controlling coccidiosis in the bird, provided that these peptides are available in the gut of the bird during coccidial exposure. The reduction in lesion scores in pMIM-medicated birds seen following exposure to three different species of coccidia in battery trials shows that these compounds have a potential for therapeutic treatment during infection. Expanded studies on the anticoccidial nature of these compounds, the development of improved systems of delivery to the bird, and their use under conditions more closely resembling that seen in the poultry industry are necessary to assess their potential anticoccidial use.

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