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Research paper

Anticoccidial activity of novel triazine compounds in broiler chickens

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ABSTRACT

The objective of present studies was to evaluate and compare the anticoccidial activity of triazine compounds in broiler chickens infected with *E. tenella, E. necatrix, E. acervulina, E. maxima*, and two field mixed *Eimeria* species. The anticoccidial efficacy was evaluated using the anticoccidial index (ACI). The results showed that Aminomizuril (AZL) and Ethanamizuril (EZL) were active metabolites of nitromezuril, which demonstrated excellent effectiveness against *E. tenella, E. necatrix, E. acervulina, E. maxima*, and the field *Eimeria* isolates in broiler chickens at a dosage of 10 mg/kg in feed. The anticoccidial activities of AZL and EZL at dose 10 mg/kg were roughly equivalent to the parent nitromezuril at a dosage of 3 mg/kg in feed. The decrease in metabolite anticoccidial activity is probably due to an increasing polarity of compounds in the metabolic processes. The sensitivity of two field *Eimeria* isolates to triazines EZL, diclazuril and toltrazuril was tested using 4 indices including anticoccidial index (ACI), percent of optimum anticoccidial activity (POAA), reduction of lesion scores (RLS) and relative oocysts production (ROP). Results showed that the sensitivity of EZL treatment against the two field *Eimeria* isolates were relatively superior to that of diclazuril and toltrazuril treatment. The field *Eimeria* isolates from Gansu Province was determined to be slightly, moderately and highly resistant to EZL, diclazuril and toltrazuril respectively. The field *Eimeria* isolates from Zhejiang Province was slightly, highly and slightly resistant to EZL, diclazuril and toltrazuril respectively. The results above indicated that the anticoccidial activity of metabolites was lower than that of the parent nitromezuril and there was partial cross-resistance among triazines EZL, diclazuril and toltrazuril. However the field *Eimeria* isolates had higher sensitive to EZL than the triazines diclazuril and toltrazuril. It was suggested that the site of C4 substituents of phenol of triazine anticoccidials may have important biological functions and the metabolite EZL would be a potential novel anticoccidial agent worthy of more attention.

1. Introduction

Avian coccidiosis is one of the most important diseases affecting the poultry industry. The annual loss directly or indirectly caused by *Eimeria* species in the raising fowls is estimated to reach up US $800 million (Williams, 1998). Coccidiosis control depends mainly on prophylactic chemotherapy with anticoccidial drugs. Hence, developing new drugs would be an effective method to control coccidiosis (Peek and Landman, 2011). Triazine compounds have been used widely for years to combat the detrimental effects of veterinary protozoan parasites. Triazines antiprotozoal drugs commonly used in veterinary clinic include toltrazuril, diclazuril, ponazuril and clazuril (Alnassan et al., 2013; Stock et al., 2018). Ponazuril, formerly known as toltrazuril sulfone, was an active metabolite of toltrazuril against protozoans and was currently approved as a new drug for the treatment of equine protozoal myeloencephalitis caused by *Sarcocystis neurona* in horses (Mackay et al., 2008). Clazuril is also a triazine coccidioidal drug related to diclazuril. Fenbendazole, an active metabolite of febantel, is a broad spectrum benzimidazole anthelmintic. It can be seen that active metabolites are one of the major ways of new drug development.

Nitromezuril is a novel triazine compound, of which 3 mg/kg in feed has an excellent efficacy for preventing coccidiosis in broiler chickens

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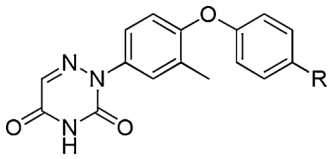

Fig. 1. The structures of nitromezuril, aminomizuril and ethanamizuril, when R = NO_2 named nitromezuril, R = NH_2 named aminomizuril (AZL), and R = CH_3CONH named ethanamizuril (EZL).

(Fei et al., 2013). The action stage of nitromezuril is probably during the entire endogenous stage of the parasites and the schizogony stage is intrinsically more vulnerable (She et al., 2017). Three metabolites of nitromezuril were identified by LC and LC/MS analysis with *in vivo* and in *vitro* drug metabolism models, of which Aminomizuril (AZL, 2-(3-Methyl-4-(4-aminophenoxy)phenyl)-1,2,4-triazine-3,5(2H,4H)-dione) and Ethanamizuril (EZL, 2- (3-methyl-4- (4-acetylaminophenoxy) phenyl)-1,2,4-triazin-3,5 (2H, 4 H)-dione) were two main metabolites. The structures of nitromezuril, AZL and EZL are shown in Fig. 1. These proposed metabolites of nitromezuril were both novel triazine compounds that would be expected to play a critical role in pharmacodynamics and safety evaluations (Zhang et al., 2014). To better understanding the structure basis of anticoccidial activities of triazine compounds and new anticoccidial drugs development, chicken battery trials were conducted to evaluate the anticoccidial efficacy of two metabolites of nitromezuril against *Eimeria tenella, E. necatrix, E. acervulina*, *E. maxima* in laboratory and compare the sensitivity of triazine compounds EZL, diclazuril and toltrazuril against two field mixed *Eimeria* species from typical broiler farms.

2. Materials and methods

2.1. Birds and feed

One-day-old Pudong yellow broiler chicks were purchased from a local hatchery. The birds were reared on wire-floored batteries under hygienic conditions with *ad libitum* access to water and a standard diet without drug supplements. Electric radiators and ventilation fans were used to maintain the recommended temperature, and 24 h light was maintained. The protocol was conformed to the guidelines of the Institutional Animal Care and Use Committee of China, and was approved by the Ethics Committee of the Veterinary Research Institute.

2.2. Drugs and parasite

Diclazuril (> 98%), Nitromezuril (> 98%), Aminomizuril (AZL, > 98%) and Ethanamizuril (EZL, > 98%) were provided by the Shanghai Veterinary Research Institute, Chinese Academy of Agriculture Sciences. Toltrazuril (Baycox®, 2.5% oral suspension, batch no: CN28550) was purchased from Bayer (Sichuan) corporation, China. Oocysts of *E. tenella, E. necatrix, E. acervulina* and *E. maxima* were maintained in the Key Laboratory of Animal Parasitology of the Ministry of Agriculture. Oocysts of field *Eimeria* strains were isolated from broiler chicken farms located in Gansu and Zhejiang provinces, China. These field *Eimeria* isolates used in this experiment were propagated for one generation, and the method of propagation has been described by Suo and Li (1998). Species identification of field oocysts were performed by polymerase chain reaction amplification method reported by Vrba et al. (2010). Oocysts from Gansu were a mixture of *E. tenella, E. necatrix, E. mitis, E. brunetti, E. acervulina*, *E. praecox*, and oocysts from Zhejiang were a mixture of *E. tenella, E. acervulina* and *E. mitis, E. brunetti, E. praecox*. The sporulated oocysts were stored at 4 °C in 2.5% potassium dichromate, and these cells were washed with phosphate buffered saline (PBS, pH = 7.2) for three times before use and diluted to the required concentrations in 1 mL solutions. The infected chicks were fed with the 1 mL solutions of sporulated oocysts respectively, whereas the uninfected chicks were sham-inoculated with PBS alone.

2.3. Experimental design

At approximately 2 weeks of age, the chickens were weighed and allocated to cages using a restricted randomization procedure that approximately equalized their initial weights. All chickens were given an unlimited standard diet and water. Constant light was provided for the remainder of the experiment. The detailed experimental designs are described below.

2.3.1. Comparison of activity of nitromezuril metabolites at different dosages

440 chicks were equally divided into eleven groups (A1, A2, …A11) of 40 chicks each group with 4 replications(10 chicks per cage/replication). Groups A1、A2、A3 and A4 were treated with standard feed supplemented with AZL at a chosen level 3, 6, 9 and 12 mg/kg, respectively. Groups A5、A6、A7 and A8 were treated with standard feed supplemented with EZL at a chosen level 3, 6, 9 and 12 mg/kg, respectively. Group A9 were treated with 3 mg/kg nitromezuril in feed as drug control. Groups A10 and A11 received standard feed and served as the infected non-medicated control (INC) and the non-infected non-medicated control (NNC), respectively. The chickens in groups A1 to A10 were infected with 8×10^4 sporulated oocysts of *E. tenella* by oral gavage, whereas group A11 (NNC) were inoculated with PBS alone.

2.3.2. Anticoccidial activities of nitromezuril metabolites at dose of 10 mg/kg in feed

144 chickens were equally divided into four groups B1, B2, INC and NNC of 36 chickens each group with 4 replications(9 birds per cage/replication). Chickens in group B1and B2 were continuously administered a regular diet containing 10 mg/kg AZL and EZL, respectively. The chickens in groups B1, B2 and INC were infected with 8×10^4 sporulated oocysts of *E. tenella* by oral gavage, whereas NNC were inoculated with PBS alone.

2.3.3. Anticoccidial activities of nitromezuril metabolites against four Eimeria species

390 chickens were caged 10 each and divided into 3 groups (C1, C2 and C3) with 120 chickens in each group, and the remaining 30 chickens served as shared NNCs. Chickens in groups C1, C2 and C3 were further equally subdivided into four subgroups of 30 chickens each subgroup. The chickens in the four subgroups were infected by oral gavage with 8×10^4 sporulated oocysts of *E. tenella*, 10×10^4 sporulated oocysts of *E. necatrix*, 15×10^4 sporulated oocysts of *E. acervulina*, and 10×10^4 sporulated oocysts of *E. maxima*, respectively. Groups C1 and C2 were continuously administered a regular diet containing 10 mg/kg AZL and EZL, respectively, whereas Group C3 was administered a regular diet as the INCs.

2.3.4. Anticoccidial activities of EZL against field Eimeria isolates

144 birds were divided into 4 groups (D1, D2, D3 and D4) with 32 birds in each group, and the remaining 16 chickens served as shared NNCs. Chickens in groups D1, D2, D3 and D4 were further equally subdivided into two subgroups of 16 chickens each subgroup. The chickens in the two subgroups were infected by oral gavage with 15×10^4 sporulated oocysts isolated from Gansu and Zhejiang, respectively. Groups D1and D2 were continuously administered a regular diet containing 10 mg/kg EZL and 1 mg/kg diclazuril, respectively. Groups D3 was continuously administered regular water containing 25 mg/L toltrazuril. Group D4 was administered a regular diet and water as the INCs.

2.4. Efficacy evaluation

The anticoccidial activity was assessed by measuring the weight gain (WG), intestine lesion scores (LS), faecal oocycsts and mortality caused by coccidiosis, which followed the guidelines for evaluating the efficacy of anticoccidial drugs in chickens and turkeys by Holdsworth et al. (2004). The overall efficacy was mainly evaluated by the anticoccidial index (ACI), where ACI = (survival rates + relative weight gain) - (lesion score + oocyst index). The ACI greater than 180 was considered to be an excellent activity, 160–179 was moderate activity, 120–159 was limited activity, and lower than 120 was inefficacy (Suo and Li, 1998).

The drug sensitivity against field species was based on the calculated percentage of optimum anticoccidial activity (POAA), reduction of lesion scores (RLS), relative oocyst production (ROP) and ACI (Suo and Li, 1998). The ACI greater than 160 was judged to be sensitive, lower than 160 was resistant. The POAA greater than 50% was considered to be sensitive, lower than 50% was resistant. The RLS greater than 50% was sensitive, lower than 50% was resistant. The ROP greater than 15% was resistant, lower than 15% was sensitive. When all of four indices above were indicated resistance, the group was judged to be "completely resistant." The term "highly resistant" meant that only three of four indices indicated resistance, "moderately resistant" meant only two of the four indices indicated resistance, and "slightly resistant" meant only one of the four indices indicated resistance.

2.5. Statistical analysis

Statistical analyses were performed using statistical software SPSS 19.0. Data were expressed as mean ± SEM, and analyzed by Tukey's multiple range test and Kruskal-wallis test. Differences between groups in the multilevel analyses were considered statistically significant at $P < 0.05$.

3. Results

3.1. Comparison of activity of nitromezuril metabolites at different dosages

The different parameters of metabolites of Nitromezuril against *E. tenella* at different dosages are listed in Table 1. Chicken death caused by coccidiosis was observed in groups A6 and INC, while the mean LS in the INC was 2.2. AZL and EZL added in feed at dose 6 mg/kg and above could increase the WG and reduce the LS and faecal oocysts significantly ($P < 0.05$). The ACI of group A1 to A4 treated with AZL at a dosage of 3, 6, 9 and 12 mg/kg in feed were 136, 171, 188 and 188 respectively. The ACI of groups A5 to A8 treated with EZL were 165, 175, 186 and 185 for different dosages respectively, whereas that of the nitromezuril control (A9) and INC were 187 and 106, respectively. The anticoccidial activity based on the ACI was both excellently effective at dose 9 and 12 mg/kg in feed. There was no significant difference of the ACI between groups medicated with AZL and EZL at the same dose ($P > 0.05$).

Table 1
Comparison of the activity of metabolites of nitromezuril against *Eimeria tenella* at different dosages.

Groups	Dose (mg/kg)	Mean WG(g) (n = 40)	Mean LS (n = 40)	ACI (n = 4)
A1	3	198 ± 5.9 ^{bc}	1.8 ± 0.1 ^{ab}	136 ± 6.1 ^{c}
A2	6	204 ± 5.1 ^{ab}	1.2 ± 0.1 ^{b}	171 ± 1.3 ^{b}
A3	9	217 ± 5.7 ^{ab}	0.1 ^{c}	188 ± 2.1 ^{ab}
A4	12	216 ± 6.2 ^{ab}	0.0 ^{c}	188 ± 0.9 ^{ab}
A5	3	200 ± 5.5 ^{abc}	1.5 ± 0.1 ^{b}	165 ± 3.2 ^{b}
A6	6	206 ± 6.3 ^{ab}	0.3 ± 0.1 ^{c}	175 ± 9.3 ^{ab}
A7	9	209 ± 6.0 ^{ab}	0.0 ^{c}	186 ± 5.3 ^{ab}
A8	12	207 ± 8.1 ^{ab}	0.0 ^{c}	185 ± 4.6 ^{ab}
A9	3	218 ± 7.5 ^{ab}	0.3 ^{c}	187 ± 2.9 ^{ab}
A10(INC)	0	168 ± 8.1 ^{c}	2.2 ± 0.1 ^{a}	106 ± 10.4 ^{d}
A11(NNC)	0	229 ± 7.0 ^{a}	0.0 ^{c}	200 ± 1.1 ^{a}

^{a,b,c,d,}Means with the same letters are not significantly different ($P < 0.05$).
WG, weight gain; LS, lesion score; ACI, anticoccidial index;
A1-A4, AZL in feed at dose 3, 6, 9 and 12 mg/kg, respectively; A5-A8: EZL in feed at dose 3, 6, 9 and 12 mg/kg, respectively; A9: Nitromezuril in feed at dose 3 mg/kg as drug control; A10: INC, infected non-medicated control; A11: NNC, non-infected non-medicated control.

Table 2
Anticoccidial activities of metabiltes of nitromezuril at dose 10 mg/kg in feed.

Groups	Mean WG(g) (n = 36)	Mean LS (n = 36)	ACI (n = 4)
B1	321 ± 7.3 ^{a}	0.0 ^{b}	188 ± 1.7 ^{a}
B2	331 ± 9.2 ^{a}	0.0 ^{b}	191 ± 0.5 ^{a}
INC	236 ± 9.4 ^{b}	2.3 ± 0.1 ^{a}	93 ± 5.4 ^{b}
NNC	343 ± 10.3 ^{a}	0.0 ^{b}	200 ± 1.1 ^{a}

^{a,b,} Means with the same letters are not significantly different ($P < 0.05$).
WG, weight gain; LS, lesion score; ACI, anticoccidial index;
B1, AZL, 10 mg/kg in feed; B2, EZL, 10 mg/kg in feed; INC, infected non-medicated control; NNC, non-infected non-medicated control.

3.2. Anticoccidial activities of nitromezuril metabolites at dose of 10 mg/kg in feed

The different parameters of metabolites of Nitromezuril against *E. tenella* at dose10 mg/kg in feed are listed in Table 2. The mean LS caused by coccidiosis in the INC was 2.3. Nitromezuril metabolites at dose 10 mg/kg increased the WG and reduced caecal lesion apparently ($P < 0.05$). The ACI of groups medicated with AZL and EZL had no significant difference ($P > 0.05$), whereas the ACI were remarkably higher than that of the INCs ($P < 0.05$).

3.3. Anticoccidial activities of nitromezuril metabolites against four Eimeria species

The different parameters of metabolites of Nitromezuril against *E. tenella, E. necatrix, E. acervulina* and *E. maxima* are listed in Table 3. The test showed that the mortality from coccidiosis in the INC grous infected with *E. tenella* and *E. necatrix* were 10% and 3.3%, respectively, whereas groups medicated with AZL and EZL had no death. Groups received AZL and EZL treatment could significantly increase the WG and reduce the LS from four *Eimeria* species ($P < 0.05$). The ACI of AZL

Table 3
Anticoccidial activities of nitromezuril metabolites against four *Eimeria* species.

Groups	Strains infected	SR	Mean WG(g) (n = 30)	Mean LS (n = 30)	ACI (n = 3)
C1	*E.tenella*	30/30	232 ± 7.9 ^{abc}	0.0 ^{b}	192 ± 3.0 ^{a}
	E. necatrix	30/30	228 ± 7.5 ^{abc}	0.1 ± 0.1 ^{b}	186 ± 2.7 ^{a}
	E. acervulina	30/30	218 ± 7.8 ^{abcd}	0.1 ± 0.1 ^{b}	179 ± 4.1 ^{ab}
	E. maxima	30/30	212 ± 7.4 ^{abcd}	0.1 ± 0.1 ^{b}	183 ± 1.5 ^{a}
C2	*E.tenella*	30/30	236 ± 8.3 ^{ab}	0.0 ^{b}	192 ± 5.0 ^{a}
	E. necatrix	30/30	216 ± 8.6 ^{abcd}	0.1 ± 0.1 ^{b}	183 ± 3.8 ^{a}
	E. acervulina	30/30	235 ± 7.5 ^{ab}	0.1 ± 0.1 ^{b}	189 ± 3.5 ^{a}
	E. maxima	30/30	214 ± 6.9 ^{abcd}	0.2 ± 0.1 ^{b}	185 ± 3.8 ^{a}
C3	*E.tenella*	27/30	195 ± 8.7 ^{cd}	1.83 ± 0.2 ^{a}	97 ± 11.3 ^{d}
	E. necatrix	29/30	201 ± 8.0 ^{bad}	0.57 ± 0.2 ^{a}	138 ± 17.0 ^{c}
	E. acervulina	30/30	184 ± 6.1 ^{d}	1.37 ± 0.1 ^{a}	131 ± 11.1 ^{cd}
	E. maxima	30/30	187 ± 9.5 ^{d}	1.33 ± 0.1 ^{a}	142 ± 8.5 ^{bc}
NNC	non-infected	30/30	247 ± 9.0 ^{a}	0.0 ^{b}	200 ± 5.0 ^{a}

^{a,b,c,d} Means with the same letters are not significantly different ($P < 0.05$).
SR, survival rate; WG, weight gain; LS, lesion score; ACI, anticoccidial index;
C1, AZL, 10 mg/kg in feed; C2, EZL, 10 mg/kg in feed; C3, INC, infected non-medicated control; NNC, non-infected non-medicated control.

Table 4
Comparison of the sensitivity of triazine drugs against *Eimeria* field isolates.

Groups	*Eimeria* field isolates	RLS	ROP	POAA	ACI	Conclusion
D1	Gansu	100%(−)	44%(+)	98%(−)	185(−)	slightly resistant
D2		97%(−)	76%(+)	51%(−)	154(+)	moderately resistant
D3		94%(−)	66%(+)	41%(+)	154(+)	highly resistant
D4					97	
D1	Zhejiang	100%(−)	43%(+)	89%(−)	185(−)	slightly resistant
D2		71%(−)	47%(+)	41%(+)	156(+)	highly resistant
D3		100%(−)	46%(+)	66%(−)	171(−)	slightly resistant
D4					108	
NNC	–				200	

RLS, reduction of lesion scores; ROP, relative oocyst production; POAA, percentage optimum anticoccidial activity; ACI, anticoccidial index;
D1, Ethanamizuril, 10 mg/kg in feed; D2, Diclazuril,1 mg/kg in feed; D3, Toltrazuril 25 mg/L in water; D4, INC, infected non-medicated control; NNC, non-infected non-medicated control.

and EZL treatment against *E. tenella, E. necatrix, E. acervulina*, *E. maxima* were 192, 186, 179, 183 and 192, 183, 189, 185, respectively. There was no significant difference against the four *Eimeria* species between Groups medicated with AZL and EZL ($P > 0.05$).

3.4. Anticoccidial activities of EZL against field Eimeria isolates

The various parameters used to evaluate the sensitivity of EZL, diclazuril and toltrazuril against *Eimeria* field isolates are summarized in Table 4. The ACI of groups treated with EZL, diclazuril and toltrazuril against *Eimeria* isolated from Gansu province were 185, 154 and 154, respectively, thereby indicating the high effectiveness of EZL treatment and the limited effectiveness of diclazuril and toltrazuril treatment. The ACI of EZL, diclazuril and toltrazuril against *Eimeria* strains isolated from Zhejiang province were 185, 156 and 171, respectively, accordingly indicating the high effectiveness of EZL treatment, the limited effectiveness of diclazuril treatment and the moderated effectiveness of toltrazuril treatment. Based on the ACI, POAA, RLS and ROP, the resistance of EZL, diclazuril and toltrazuril treatment were assessed to be slightly, moderately and highly resistant for species from Gansu province respectively and slightly, highly and slightly resistant for species from Zhejiang province respectively.

4. Discussion

Triazine drugs commonly used in chickens include diclazuril and toltrazuril. Nitromezuril is a novel anticoccidial triazine compound developed using systematic structure–activity relationship studies of these compounds in our laboratories (Fei et al., 2013). AZL and EZL are also novel triazine compounds, of which anticoccidial efficacy experiments in this study conducted under the same protocol (Chapman, 1998; Holdsworth et al., 2004). The ACI was an overall parameter calculated for each cage and widely adopted in evaluating the chemotherapy effectiveness. Thus, the anticoccidial activities of metabolites of nitromezuril were mostly evaluated by the ACI in the experiments.

The anticoccidial activities were generally measured by the efficacy of drugs at different concentrations in the diet with caged birds (Holdsworth et al., 2004). The anticoccidial activities of metabolites decreased significantly compared to that of nitromezuril at the same dose 3 mg/kg ($P < 0.05$). Nitromezuril metabolites AZL and EZL at dose 9 and 12 mg/kg in feed both exhibited excellent anticoccidial activity. There was no significant difference of the ACI between groups medicated with AZL or EZL at dose 9 and 12 mg/kg and nitromezuril at dose 3 mg/kg ($P > 0.05$). Further anticoccidial activities of nitromezuril metabolites at a dosage of 10 mg/kg revealed that both AZL and EZL increased the WG and reduced caecal lesion significantly ($P < 0.05$). The anticoccidial activity based on the ACI was both excellently effective, and significantly higher than that of the INCs ($P < 0.05$). These results showed that AZL and EZL were active metabolites, and their anticoccidial activities were both lower than that of nitromezuril.

The *Eimeria* species considered to be of economic importance as chicken pathogens are *E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti, E.praecox* and *E. mitis*. The most pathogenic species include *E. tenella and E. necatrix*, whereas *E. acervulina* and *E. maxima* are moderate pathogenic species. These four *Eimeria* species are the major causes of coccidiosis (Chapman, 1976; Ryley, 1980). Therefore, the four species mentioned above were used in the present study to demonstrate the prophylaxis effectiveness of novel triazine compounds AZL and EZL. The test showed that the mortality from coccidiosis in INCs infected with *E. tenella* and *E. necatrix* were only 10% and 3%, respectively. The ACI of groups medicated with AZL and EZL were both 192 in *E. tenella*, 186 and 183 in *E. necatrix*, 179 and 189 in *E. acervulina,* 183 and 185 in *E. maxima*, whereas the corresponding ACI of the INCs were 97, 138, 131, and 142, respectively. It was revealed that AZL and EZL at dose 10 mg/kg in feed have highly efficacy against *E. tenella, E. necatrix, E. acervulina* and *E. maxima.* There was no significant difference between AZL and EZL treatment against the four *Eimeria* species based on the ACI ($P > 0.05$). Consequently the broad-spectrum anticoccidial activities of metabolites were consistent with that of nitromezuril. This study clearly showed that AZL and EZL had highly effects on preventing coccidiosis in broiler chickens at dose 10 mg/kg in feed.

Eimeria field isolates were found to be almost ubiquitous, averaging two species of *Eimeria* and showing some drug resistance (Godwin and Morgan, 2015; Lan et al., 2017). Studies have indicated that *Eimeria* isolates resistant to certain ionophore were also resistant to other same type compounds (Abbas et al., 2011). It is generally believed that cross-resistance exists between similar structural drugs (Chapman, 1989; Mcdougald et al., 1986). EZL is a novel triazine compound and it is of great significance for whether cross-resistance exists. In the present study, the resistance of triazines EZL, diclazuril and toltrazuril against strains isolated from fields were assessed. Oocysts from Gansu were a mixture of *E. tenella, E. necatrix, E. mitis, E. brunetti, E. acervulina*, *E. praecox*, and oocysts from Zhejiang were a mixture of *E. tenella, E. acervulina* and *E. mitis, E. brunetti, E. praecox*. The results showed that the sensitivity of EZL treatment was relatively superior to the treatment with diclazuril and toltrazuril in terms of ACI for both *Eimeria* field isolates. For the above-mentioned indices ACI, POAA, RLS and ROP, EZL, diclazuril and toltrazuril were determined to be slightly, moderately and highly resistant for species Gansu respectively, and slightly, moderately and slightly resistant for species Zhejiang respectively. The *Eimeria* field isolates showed various levels of resistance to the three triazine compounds, suggesting that partial cross-resistance exists in triazine drugs. This result was not entirely consistent with previous study, of which nitromezuril was highly effective in inducing experimental resistance to *E. tenella*. (Fei et al., 2013). However, the sensitivity of EZL exceeded that of diclazuril and toltrazuril, which suggested that EZL might have effects on seven *Eimeria* strains, and could partially solve the problem of clinical drug resistance.

Accelerated metabolism is usually accompanied by an increase in

polarity, which facilitates drug excretion in the body. Anticoccidial drugs are generally known to be compounds with low solubility and low membrane permeability. Because avian coccidiosis is an intestinal infectious disease caused by obligatory intracellular parasites, compounds with low solubility and low membrane permeability, although less absorbed, tend to have better anticoccidial activity. Triazine anticoccidials diclazuril, toltrazuril and ponazuril are an example (Stock et al., 2018). Triazine compounds AZL and EZL were both active metabolites of nitromezuril, and the decrease of their anticoccidial activity probably due to the increase of the polarity of compounds in the metabolic process.

The molecular structures of compounds AZL, EZL and nitromezuril are only different from the position C4 substituents of phenol, of which 4-nitrophenoxy substitution is nitromezuril, 4-aminophenoxy substitution is AZL, and 4-acetylaminophenoxy substitution is EZL, while the site of C4 substituents of phenol of toltrazuril is 4-trifluoromethylthio. Previous studies found that nitromezuril showed positive in the bacterial reverse mutation trials (Ames) and negative in sperm abnormalities, bone marrow micronucleus and chromosome aberration trials. The mutagenic effects of nitromezuril were different *in vitro* and *in vivo* assays. The proposed metabolic pathways could be expected to play key roles in toxicology (Fei et al., 2015). The Ames test demonstrated that AZL exhibited the same positive results as NZL, but EZL showed negative results (unpublished). These studies suggested that the site of C4 substituents of phenol of triazine compounds might have interesting biological functions, which was one of the important sites for studying the structure-activity relationship and deserves further exploration. Although the anticoccidial activities of AZL and EZL are about the same, the metabolite EZL would be a potential novel anticoccidial agent suitable for more attention.

5. Conclusions

Triazine compounds AZL and EZL with excellent effectiveness against coccidiosis in broiler chickens at a dosage of 10 mg/kg in feed that were active metabolites of nitromezuril. The anticoccidial activities of novel triazine AZL and EZL at dose 10 mg/kg in feed were roughly equivalent to nitromezuril at dose 3 mg/kg in feed, and the reduction of activities probably due to the increased polarity of compounds in the metabolic processes. The novel triazine compound EZL was highly effective against *E. tenella, E. necatrix*, *E. acervulina, E. maxima,* and the field *Eimeria* isolates from Gansu and Zhejiang province. Partial cross-resistance among triazines EZL, diclazuril and toltrazuril were observed. However the sensitivity of the field *Eimeria* isolates to EZL exceeded that of diclazuril and toltrazuril. Our studies suggested that the site of C4 substituents of phenol of nitromezuril might have interesting biological functions. The metabolite EZL would be a potential novel anticoccidial agent worthy of more attention.

Declaration of interests

None.

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