Parasitol Res (1991) 77:595–599
0044325591000958

Parasitology Research

Evaluation of anticoccidial drugs in chicken embryos

M.Q. Xie*, T. Fukata, J.M. Gilbert, and L.R. McDougald**

Department of Poultry Science, University of Georgia, Athens, GA 30602, USA

Accepted April 23, 1991

Abstract. Infections of *Eimeria tenella* in chicken embryos were used to compare the anticoccidial activity of ten drugs. The minimal inhibitory concentration (MIC) and minimal toxic concentration (MTC) were affected by the time of inoculation into the embryos and by the chemical nature of the compounds. Some compounds (nicarbazin, amprolium) had no effect on the development of coccidia when they were injected into embryos after the day of infection. Drugs that act early in the life cycle of coccidia (robenidine, clopidol, decoquinate, diclazuril, halofuginone, monensin, salinomycin, and lasalocid) were active at 5–125 μg/embryo when they were injected on the day of infection. The ionophores and halofuginone were highly toxic to embryos; most synthetic compounds were nontoxic. The incubation of merozoites in drug suspensions prior to the infection of embryos did not result in embryo toxicity, but the resultant MICs were much higher than those obtained when drugs were injected directly into the embryos. Several products were essentially inactive. Neither nicarbazin nor amprolium prevented oocyst formation. The widely divergent endpoints for the MIC and MTC of anticoccidials in embryos seriously limits the application of this technique as a screen for anticoccidial drugs.

The discovery that sporozoites of *Eimeria tenella* would colonize cells in culture or would infect chicken embryos led to the development of in vitro models to test anticoccidial drugs (Long and Millard 1973; McDougald and Galloway 1973; Ryley 1968; Strout and Ouellette 1973). It soon became apparent that there are serious limitations to the use of embryos to screen unknown compounds for anticoccidial activity. The coccidia have a complex life cycle involving many metabolically distinct stages of development that react differently to drugs. The concentration of drug required for activity varies widely between and within classes of drugs, and the drugs may be toxic to the embryo. Some drugs are inactive in vitro because the system lacks enzymes that are necessary for conversion of the molecule to an active form (Latter and Wilson 1979).

With the aim of discovering the conditions under which embryo infections might be used to evaluate unknown compounds for anticoccidial activity, we characterized the anticoccidial and embryo-toxic properties of ten representative anticoccidial compounds, which were introduced either at the time of infection or at 1–3 days postinfection. As an alternative for avoidance of embryo toxicity, merozoites of *E. tenella* were incubated in suspensions of drugs in vitro prior to their inoculation into embryos.

Materials and methods

Preparation of sporozoites and merozoites

The Wisconsin strain of *Eimeria tenella* was used throughout the study. Oocysts obtained from infected chickens were sterilized by treatment with sodium hypochlorite (McDougald and Galloway 1973). Sterilized oocysts were ground with glass beads to release sporocysts, which were then treated with a solution of trypsin and taurodeoxycholic acid (2.5%, 0.75%) at 40° C for 30–40 min. Freed sporozoites were washed twice in Hanks' balanced salt solution (HBSS) and stored at 4° C until needed. Second-generation merozoites were collected from embryos as previously described (Xie et al. 1990). Purified merozoites obtained after filtration of the crude suspension through four layers of cheesecloth were washed twice by centrifugation in HBSS.

Anticoccidial drugs

Purified analytical standards of the drugs were obtained from the manufacturer (Table 1). As most of the products were insoluble in water or buffer, a suspension was prepared after the dry compound had been wetted with a small amount of a 10% solution of Tween-20 (McDougald and Galloway 1973).

Present addresses: * Veterinary Medicine Institute, Guangdong Academy of Agricultural Sciences, P.R. of China
** Department of Veterinary Medicine, University of Osaka Prefecture, Osaka, Japan

Offprint requests to: L.R. McDougald

Table 1. Commercial and experimental anticoccidial drugs tested in vitro

Generic name	U.S. trade name (commercial sponsor)	Chemical type	Feed level (ppm)
Lasalocid	Avatec (Roche, Inc.)	Ionophore	75–125
Monensin	Coban (Elanco)	Ionophore	100–121
Salinomycin	Bio-Cox (Agri-Bio)	Ionophore	44–66
Halofuginone	Stenorol (Hoechst)	Quinazoline	3
Diclazuril	Clinicox (Pitman-Moore)	Triazine	1
Robenidine	Robenz (Cyanamid)	Bis-guanidine	33
Clopidol	Coyden (Rhone-Poulenc)	Pyridinol	123
Decoquinate	Deccox (Rhone-Poulenc)	Quinolone	33
Nicarbazin	Nicarb (MSD-AGVET)	Carbanilide	125
Amprolium	Amprol (MSD-AGVET)	Thiamine antagonist	125

Table 2. Titration of anticoccidial activity and toxicity of compounds in chicken embryos

Compound	Test number	Range tested (μg/embryo)	Day of treatment	Endpoints (μg/ml)[a]	
				Toxicity	Activity
Lasalocid	1	1–1000	2	1,000	NA
	2	50–1000	1	500	100
	3	100–400	1	300	100
	4	25–200	0	NT	100
Salinomycin	1	1–1000	2	100	>100
	2	10–500	1	100	100
	3	60–150	1	90	90
	4	25–100	0	75	50
Monensin	1	1–1000	2	100	NA
	2	10–500	1	100	100
	3	60–150	1	75	90
	4	30–75	0	75	>75
Halofuginone	1	10–10000	3	100	10
	2	0.1–100	3	100	10
	3	20–80	3	60	40
	4	5–40	0	10	5
Diclazuril	1	0.1–100	3	NT	NA
	2	0.1–100	1	NT	100
	3	20–80	1	NT	60
	4	10–60	0	NT	10
Robenidine	1	10–2000	3	NT	NA
	2	10–2000	1	NT	100
	3	20–80	1	NT	20
	4	5–30	0	NT	20
Clopidol	1	100–2000	1	NT	1000
	2	600–900	1	NT	NA
	3	125–1000	0	NT	125
	4	125–1000	0	NT	125
Decoquinate	1	10–2000	1	NT	1,000
	2	200–800	1	NT	400
	3	125–1000	0	NT	125
Nicarbazin	1	10–10,000	3	NT	NA
	2	10–10000	1	NT	NA
	3	10–10000	0		NA
Amprolium	1	100–2000	1	NT	>2000
	2	1200–1800	1	NT	>1800
	3	250–2000	0	NT	2000

[a] Embryo mortality was used to determine the endpoints. The minimal inhibitory concentration (MIC) was the lowest tested level that did prevented mortality due to coccidiosis. The minimal toxic concentration (MTC) was the lowest tested level that caused mortality. The embryo mortality due to coccidiosis in unmedicated controls was 82%–100% (average, 96.9%). NT, Nontoxic within tested levels; NA, nonactive within tested levels

Chicken embryos

White Leghorn chicken embryos were used following their incubation at 38° C for 11 days. Penicillin and streptomycin (1000 units/1000 µg) were injected into the allantoic cavity on the 10th day of incubation.

Infection and drug treatment

Sporozoites (100000/embryo) were inoculated through the allantoic membrane of 11-day-old embryos. Drugs were diluted to appropriate concentrations in HBSS and inoculated in 0.1 ml suspension either simultaneous with the sporozoites or at 1, 2, or 3 days following sporozoite inoculation. Merozoites were incubated in drug suspensions for 30 min at 40° C and then cleaned of drug by centrifugation and resuspension in HBSS. The merozoites were inoculated at 10^7/embryo in 11-day-old embryos.

Endpoints of activity and toxicity. The dose of sporozoites given to embryos was sufficient in most cases to cause 80%–100% mortality in unmedicated treatments. The endpoint of anticoccidial activity in most cases was the prevention of mortality. The lowest level tested that prevented mortality was called the minimal inhibitory concentration (MIC). If embryos treated with anticoccidial drugs died without signs of coccidiosis and at a different time, they were recorded as toxic deaths. The lowest drug level tested that caused toxic death was recorded as the minimal toxic concentration (MTC).

Merozoite infections did not result in mortality due to coccidiosis. In this case, the urates and epithelial casts were collected from the allantoic cavity at 60 h postinoculation and accumulated oocysts were counted in a McMaster chamber. The effectiveness of drugs was based on reductions in the number of oocysts.

Experimental design. Groups of ten embryos were used for each treatment. Doses of each anticoccidial product in the first test included 1, 10, 100, and 1000 µg/embryo. Subsequent tests (2, 3, and 4) included a restricted range of concentrations clustered around the apparent endpoint of activity. Embryos were treated with drugs either simultaneously with sporozoite inoculation with sporozoites or at 1, 2, or 3 days thereafter. When sporozoites were used as the inoculum, the tests were terminated on the 6th day postinoculation and the numbers of live and dead embryos were tabulated. The deaths were attributed to coccidiosis if there was excessive bleeding into the allantoic cavity or to drug toxicity if there was no blood. When merozoites were used as the inoculum, the tests were terminated at 60 h postinoculation and the anticoccidial activity was determined based on the reduction in oocyst counts in the urates and epithelial cast material.

Results

Titration of anticoccidial activity and toxicity in chicken embryos inoculated with sporozoites

The endpoints for the activity and toxicity of the compounds depended on the day of treatment and the chemical nature of the compounds (Table 2). The ionophores (lasalocid, salinomycin, or monensin) were active at 50–100 µg/embryo but were also very toxic in this concentration range. Most compounds were considerably more active when they were injected into embryos at the time of infection (day 0) or during the 1st day thereafter. The toxic endpoint was often very near the activity endpoint, especially for the ionophores (see Fig. 1 for lasalocid, a typical ionophore). The synthetic organic compounds were generally nontoxic to embryos and were active at concentrations of 5–2000 µg/embryo. Halofuginone was the most active in vitro (5 µg) but was also highly toxic (10 µg). Nicarbazin exhibited no activity or cytotoxicity in embryos, and amprolium showed only marginal activity.

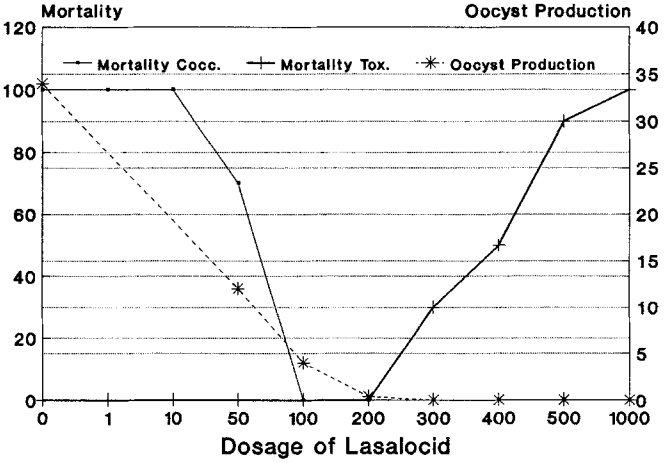

Fig. 1. Anticoccidial activity and embryo toxicity of a representative ionophore (lasalocid)

Effects of anticoccidial drugs on free merozoites. There was no toxicity to embryos when second-generation merozoites had been incubated in suspensions of test compounds prior to their inoculation into embryos. The effect of ionophores on the formation of oocysts in embryos was directly proportional to the concentration of the test compound (Table 3). Most of the synthetic organic compounds also reduced oocyst formation, but neither amprolium nor nicarbazin seemed to have any significant effect on merozoite development in embryos.

Discussion

Chick embryos and cell cultures infected with *Eimeria* offer the best opportunity to study the effects of drugs and other substances on the development of coccidia. The unique modes of action against specific stages of development can easily be demonstrated in cell cultures (Ryley and Wilson 1975; Strout and Ouellette 1973). These interesting features of drug/parasite interaction tend to limit the utility of both the embryo and cell cultures for testing unknown compounds for anticoccidial activity (drug screening). For poorly understood reasons, the endpoint of activity of a compound in vitro often differs greatly from that in vivo (McDougald and Galloway 1973; Ryley and Wilson 1972, 1976), even in relation to other compounds in the same class. In some cases, a compound must be metabolized within the animal to an active form. Such compounds often show no activity in vitro (Latter and Wilson 1979).

Delay of drug treatment by even 1 day usually increased the dose necessary for complete control. Nicarbazin, which is thought to be active against developing

Table 3. Effects of anticoccidial drugs on free merozoites prior to inoculation into embryos

Compound	Concentration (μg/ml)	Oocysts/embryo ($\times 10^3$)
Lasalocid	0	738.5
	10	166.7
	100	25
	250	4.8
	300	0.8
	350	0
	400	0
Salinomycin	0	690.9
	0.01	700
	0.1	345.6
	1	200
	10	103.5
	50	30
	100	25
	200	0
Monensin	0	475
	10	69
	100	4.2
	120	0
Halofuginone	0	400
	10	380
	50	60
	100	10
	500	0
Diclazuril	0	750
	10	550
	50	38.3
	100	3.3
	200	0
Robenidine	0	440
	10	435
	50	412.5
	100	45
	500	15
	600	0
Clopidol	0	440
	10	420
	100	405
	500	315
	1000	210

Table 3. (continued)

Compound	Concentration (μg/embryo)	Oocysts/embryo ($\times 10^3$)
Decoquinate	0	764.5
	10	790.9
	100	245.5
	500	190.9
	1000	18.2
Nicarbazin	0	750
	100	700
	1000	516.7
	2000	383.3
Amprolium	0	764.5
	10	738.2
	100	804.5
	1000	777.3

asexual stages (Ball 1959; McLoughlin and Wehr 1960), was ineffective and nontoxic at 10000 μg/embryo when it was injected into embryos after the 1st day of infection. The lack of embryo toxicity found for nicarbazin was especially surprising because of the drug's known toxicity to laying chickens (Ott et al. 1956); contamination of the diet of laying chickens damages the yolk membranes and reduces the hatchability of fertile eggs. Very high doses of amprolium (2000 μg) were required to prevent embryo mortality when treatment was delayed by 1 day postinoculation.

The ionophores presented a special challenge for evaluation in embryos. The endpoint for embryo toxicity (MTC) was frequently very near that for anticoccidial activity (MIC). Treatment of free merozoites in vitro prior to their inoculation into embryos was effective as a means of avoiding embryo toxicity but did not appear to be practical as a substitute for inoculation of sporozoites. Large numbers of merozoites (10^7/embryo) were required, and the anticoccidial products were generally not highly active against this stage. We estimated that 10%–15% of the inoculum could infect cells and proceed to the formation of oocysts after differentiation into macrogametes and microgametes. This method also required time-consuming oocyst counts as an endpoint. The activity of ionophores against merozoites in the present study confirmed the finding of Long and Jeffers (1982) that these compounds show activity against all extracellular stages, although the levels of drug required for antimerozoite action were considerably higher than those previously reported to be effective against sporozoites in cell culture (McDougald and Galloway 1973). Amprolium and nicarbazin exhibited little or no activity against free merozoites. Diclazuril, robenidine, and halofuginone were effective against merozoites only at high doses.

Workers attempting to devise a test for primary evaluation of the anticoccidial activity of unknown compounds have long been frustrated by the idiosyncracies of the various classes of drugs and the lack of correlation of structure/activity relationships in vitro and in vivo. The ideal screen for anticoccidial activity would use a single level of compound inoculated in the same way each time. The widely varying MTCs and MICs of known anticoccidials suggest that titration within a wide range would be necessary for unknown compounds. In the present study, it was not practical to delay the inoculation of test compounds into embryos by even 1 day or to use free merozoites to avoid toxicity to the embryo. These results suggest that the embryo model is more limited in usefulness for the evaluation of unknown compounds than are other models based on cultured cells, but it might be valuable for other types of investigation.

References

Ball SJ (1959) Chemotherapy of cecal coccidiosis in chickens: the activity of nicarbazin. Vet Rec 71:86–91

Latter VS, Wilson RG (1979) Factors influencing the assessment of anticoccidial activity in cell culture. Parasitology 79:169–175

Long PL, Jeffers TK (1982) Studies on the stage of action of ionophorous antibiotics against *Eimeria*. J Parasitol 68:363–371

Long PL, Millard BJ (1973) *Eimeria* infections in chicken embryos: the effect of known anticoccidial drugs against *E. tenella* and *E. mivati*. Avian Pathol 2:111–125

McDougald LR, Galloway RB (1973) *Eimeria tenella*: anticoccidial drug activity in cell cultures. Exp Parasitol 34:189–196

McLoughlin DK, Wehr EE (1960) Stage in the life cycle of *Eimeria tenella* affected by nicarbazin. Poult Sci 39:534–538

Ott WH, Dickinson AM, Feterson AG (1956) Studies on the effect of nicarbazin on reproduction in chickens. Poult Sci 35:1163

Ryley JF (1968) Chick embryo infection for the evaluation of anticoccidial drugs. Parasitology 58:215–220

Ryley JF, Wilson RG (1972) Comparative studies with anticoccidials and three species of chicken coccidia in vivo and in vitro. J Parasitol 58:644–668

Ryley JF, Wilson RG (1975) Laboratory studies with some recent anticoccidials. Parasitology 70:203–222

Ryley JF, Wilson RG (1976) Drug screening in cell culture for the detection of anticoccidial activity. Parasitology 73:137–148

Strout RG, Ouellette CA (1973) *Eimeria tenella*: screening of chemotherapeutic compounds in cell culture. Exp Parasitol 33:477–485

Xie MQ, Gilbert JM, Fuller AL, McDougald LR (1990) A new method for the purification of *Eimeria tenella* merozoites. Parasitol Res 76:566–569