Toxoplasma gondii: Susceptibility and Development of Resistance to Anticoccidial Drugs In Vitro

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Anticoccidial drugs were evaluated for activity and for the development of resistance in a model of *Toxoplasma gondii* growing in human fibroblast cultures. Of 13 anticoccidial drugs tested, 9 had selective antitoxoplasma activity (50% inhibitory concentration, in micrograms per milliliter): decoquinate (0.005), arprinocid-*N*-oxide (0.015), robenidine (0.03), the aryl triazine CP-25,415 (0.2), toltrazuril (0.4), clopidol (1), dinitolmide (Zoalene; Dow) (10), and the carboxylic acid ionophores monensin (0.001) and salinomycin (0.04). Glycarbylamide, amprolium, nicarbazin, and the 6-(*p*-bromophenoxy)-7-chloro analog of halofuginone (Stenorol; Roussel-UCLAF) (CP-63,567) were toxic for the fibroblasts. Since *Eimeria tenella* has a similar drug susceptibility profile, anticoccidial drugs can be viewed as a potential source of new antitoxoplasma therapies. The development of resistance has limited the usefulness of most of these drugs as anticoccidial agents; in coccidia, resistance to all except the ionophores occurs readily in vivo. We explored the development of resistance in *T. gondii* by attempting to select mutants in vitro from parasites mutagenized with ethylnitrosourea. Mutants that had 20- to 50-fold-reduced susceptibility to decoquinate, arprinocid-*N*-oxide, and CP-25,415 were obtained. Ionophore-resistant *T. gondii* mutants were also selected in vitro; however, there was only a twofold difference in susceptibility between these mutants and the wild type. For three drugs (clopidol, robenidine, and toltrazuril), we were unable to select resistant mutants. For experimental anticoccidial drugs, there is currently no in vitro method for assessing the risk of development of resistance in *Eimeria* species. Our results suggest that *T. gondii* may offer a useful surrogate for this assessment.

In this study, we determined the potency of a variety of anticoccidial drugs against *Toxoplasma gondii* and attempted to develop drug-resistant mutants in vitro. The goals of the study were to assess (i) the extent to which the many drug discoveries in the anticoccidial field can benefit the search for new antitoxoplasma compounds and (ii) whether the ability to continuously propagate *T. gondii* can be used to advantage in the discovery and evaluation of potential new anticoccidial agents.

The sporozoan parasite *T. gondii* is a common tissue parasite in humans (10). It causes toxoplasmosis, which may be life threatening in immunocompromised individuals (20). The AIDS epidemic has focused recent attention on the importance of toxoplasmic encephalitis. Available treatments include pyrimidine and sulfonamide antifolates and clindamycin. However, the side effects of these agents limit their usefulness (15). Investigational drugs include naphthoquinones (1), macrolides (6), tetracyclines (4, 5), and cytokines (22, 36). In contrast to the lack of well-tolerated and effective antitoxoplasma treatments, many different drug classes have been developed against coccidia of the genus *Eimeria*, which cause coccidiosis (23). Coccidia are ubiquitous sporozoan parasites that are of significance in the intensive rearing of poultry. The constant demand for new anticoccidial drugs and the number of drugs that have been introduced are explained by the commercial importance of coccidiosis, the early establishment of efficient laboratory models for testing anticoccidial activity in vitro and in vivo (28, 29), and the high rate of resistance development in the field (7). We were interested in learning to what extent anticoccidial drugs might be a source of potential antitoxo-

plasma agents. Drug resistance has been a major problem for anticoccidial drugs. For this reason, we also determined whether resistance development in vitro in *T. gondii* mirrors anticoccidial drug resistance development in the field.

The discovery and evaluation of potential anticoccidial drugs is efficient up to the point of testing for resistance development. The importance of understanding the resistance profile for a new anticoccidial drug is underlined by the number of anticoccidial drugs that have suffered resistance development in the field (7). Since there are no established techniques for the continuous propagation of *Eimeria* species in vitro, studies on the development of resistance must be conducted by the laborious technique of passaging parasites in medicated animals. In contrast, *T. gondii* can be propagated continuously in tissue culture, allowing the selection of drug-resistant mutants in vitro. The present study addresses whether in vitro testing for resistant mutants of *T. gondii* might be a useful supplement to the more resource-demanding in vivo anticoccidial drug resistance cycling method as a way to evaluate the likelihood that the development of resistance will compromise the usefulness of potential new anticoccidial drugs.

MATERIALS AND METHODS

General materials and methods. The methods used in these studies were those described by Pfefferkorn et al. (32). The cloned RH strain of *T. gondii* was used with human fibroblasts as the host cells (33). The growth medium was Eagle minimal essential medium with 10% fetal bovine serum and penicillin-streptomycin. Infections were carried out in minimal essential medium with 1% fetal bovine serum. Parasite inocula were prepared by scraping infected cultures and then shearing them with a pipet or a 25-gauge needle. To avoid

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unintentional bias in the interpretation of test results, drugs were coded at Pfizer Inc. and sent under code to Dartmouth Medical School, where antitoxoplasma efficacy and resistance testing was conducted. The code was broken only after the experiments and data analysis were complete. The compounds selected for study were commercialized agents, with the exception of CP-25,415 and CP-63,567. The former is an aryl triazine related in structure to the anticoccidial agents clazuril and diclazuril, and its anticoccidial activity was reported previously (9). CP-63,567 is the 6-(p-bromophenoxy)-7-chloro analog of halofuginone, as described by Glazer (11–13).

Dose responses. Dose-response curves against *T. gondii* were constructed for 13 anticoccidial drugs. Drug titrations were performed in 24-well tissue culture plates (32). Cultures were treated with various drug dilutions at the time of infection. Parasite growth was measured 2 days later by the incorporation of [^{3}H]uracil (20 Ci/mmol; Moravek Biochemicals) during a 4-h incubation in medium that contained 1.2 μCi of [^{3}H]uracil per ml. Quadruplicate wells were used for each drug concentration. All data are expressed as a percentage of the incorporation in infected unmedicated control cultures, which incorporated between 5,000 and 10,000 cpm. Uninfected wells showed no significant incorporation. For the comparison of antitoxoplasma potency with anticoccidial potency, the concentration producing 50% inhibition of parasite growth (IC$_{50}$) was determined for *Eimeria tenella*. Values for decoquinate, monensin, salinomycin, robenidine, and clopidol were taken directly from Ricketts (34), and values for the other anticoccidial drugs were obtained as described in reference 34.

Toxicity. The incorporation of [^{3}H]thymidine into human fibroblasts was used to measure drug toxicity. Subconfluent uninfected host cell monolayers in 24-well plates were treated with various drug concentrations for 2 days. The cultures were then labeled for 4 h with 8 μCi of [*methyl*-^{3}H]thymidine per ml (6 Ci/mmol; Moravek Biochemicals). At the time of labeling, the unmedicated control cultures were still subconfluent, and the fibroblasts were actively proliferating. All results are expressed as a percentage of the incorporation in the unmedicated control cultures.

Mutagenesis. Infected cultures were treated for 4 h at 37°C with concentrations of ethylnitrosourea (typically 200 μg/ml) sufficient to kill 90 to 95% of parasites. The effectiveness of the mutagenesis was confirmed by demonstrating that the mutagenized stocks contained $\geq$10 mutants resistant to 10 μM fluorodeoxyuridine per 10^6 parasites (31).

Resistance development. Mutagenized parasites were grown in host cells for 3 days in the absence of ethylnitrosourea to allow the phenotypic expression of induced mutations. Then, fibroblast monolayers in 150-cm^2 flasks were infected with 4 × 10^7 mutagenized *T. gondii* and treated with selective drug concentrations calculated to cause 75 to 95% inhibition of parasite growth. The parasites were subcultured with minimal dilutions at 1- to 2-day intervals until resistant mutants emerged. When robust parasite growth was observed, the selective drug concentrations were increased in twofold steps. At intervals, the IC$_{50}$s for the resistant mutants were determined.

RESULTS

Antitoxoplasma activity of anticoccidial drugs. Three categories of drug response were found. For nine of the drugs, selective antitoxoplasma effects were observed at concentrations that did not show toxicity for the host cell. Results

for toltrazuril, clopidol, dinitolmide, and robenidine are shown in Fig. 1, and those for decoquinate, CP-25,415, monensin, and salinomycin are shown in Fig. 2. Arprinocid-*N*-oxide produced results similar to those found in an earlier study (32), with an antitoxoplasma IC$_{50}$ of 0.015 μg/ml and 50% toxicity for human fibroblasts at 0.25 μg/ml. For three of the drugs (glycarbylamide, amprolium, and the halofuginone analog CP-63,567), antitoxoplasma activity coincided with toxicity for the host cell, and there was no indication of selectivity. An example of this type of dose response is shown in Fig. 1 for glycarbylamide. The remaining drug in the study, nicarbazin, showed a biphasic dose response (Fig. 1).

Comparison of antitoxoplasma and anticoccidial potencies. The antitoxoplasma potency observations made in this study were compared with the results of previous anticoccidial activity testing in an in vitro model of *E. tenella* growing in MDBK cells. The results, shown in Fig. 3, indicate that *T. gondii* is similar to *E. tenella* in its susceptibility to anticoccidial agents.

Development of *T. gondii* resistance to anticoccidial drugs. Attempts were made to isolate mutants resistant to eight anticoccidial drugs that showed selective and reasonably potent antitoxoplasma activity. Dose-response curves for the parental strain and strains selected with CP-25,415, decoquinate, monensin, and salinomycin are shown in Fig. 2. The CP-25,415-resistant mutant had a 50-fold reduction in drug susceptibility, the decoquinate-resistant mutant a 20-fold reduction, and the ionophore-resistant mutants had a 2-fold reduction. With arprinocid-*N*-oxide, a mutant with 20-fold-reduced susceptibility was isolated (data not shown), confirming the results of a previously published study (32). No resistant mutants were obtained by selection with robenidine, toltrazuril, or clopidol.

DISCUSSION

In the present study, all anticoccidial drugs tested inhibited the growth of *T. gondii*. In a few instances (glycarbylamide, amprolium, and an analog of halofuginone), the dose response for parasite inhibition was the same as that for host cell toxicity, but for most of the anticoccidial drugs, selective antitoxoplasma activity was observed. We are not aware of previous reports of antitoxoplasma activity of anticoccidial triazines, dinitolmide, or clopidol; however, antitoxoplasma activity has been reported for polyether ionophores (26), arprinocid in the form of the in vitro-active *N*-oxide metabolite (32), robenidine and nicarbazin (3), decoquinate (27), and toltrazuril (14). These results indicate substantial similarities in drug susceptibility between the genera *Eimeria* and *Toxoplasma*. This conclusion is supported by evidence from others who have found activity against both parasites for several different drugs. Antifolates, such as synergistic combinations of pyrimethamine with sulfonamides, are the mainstay of antitoxoplasma treatment, and the anticoccidial activity of these drugs has been appreciated for over 50 years (19). Clindamycin is in clinical use against toxoplasmosis, and the lincosamides exhibit activity against *Eimeria* species (30). Several experimental antiprotozoal drugs, including naphthoquinones (16) and L-651,582 (17), share anticoccidial and antitoxoplasma activities.

From the foregoing results, it is clear that there is considerable overlap in drug targets between *T. gondii* and *E. tenella*. In the present study, we found that the potencies of a number of agents against the two parasites were similar. This result supports the concept that compounds with utility
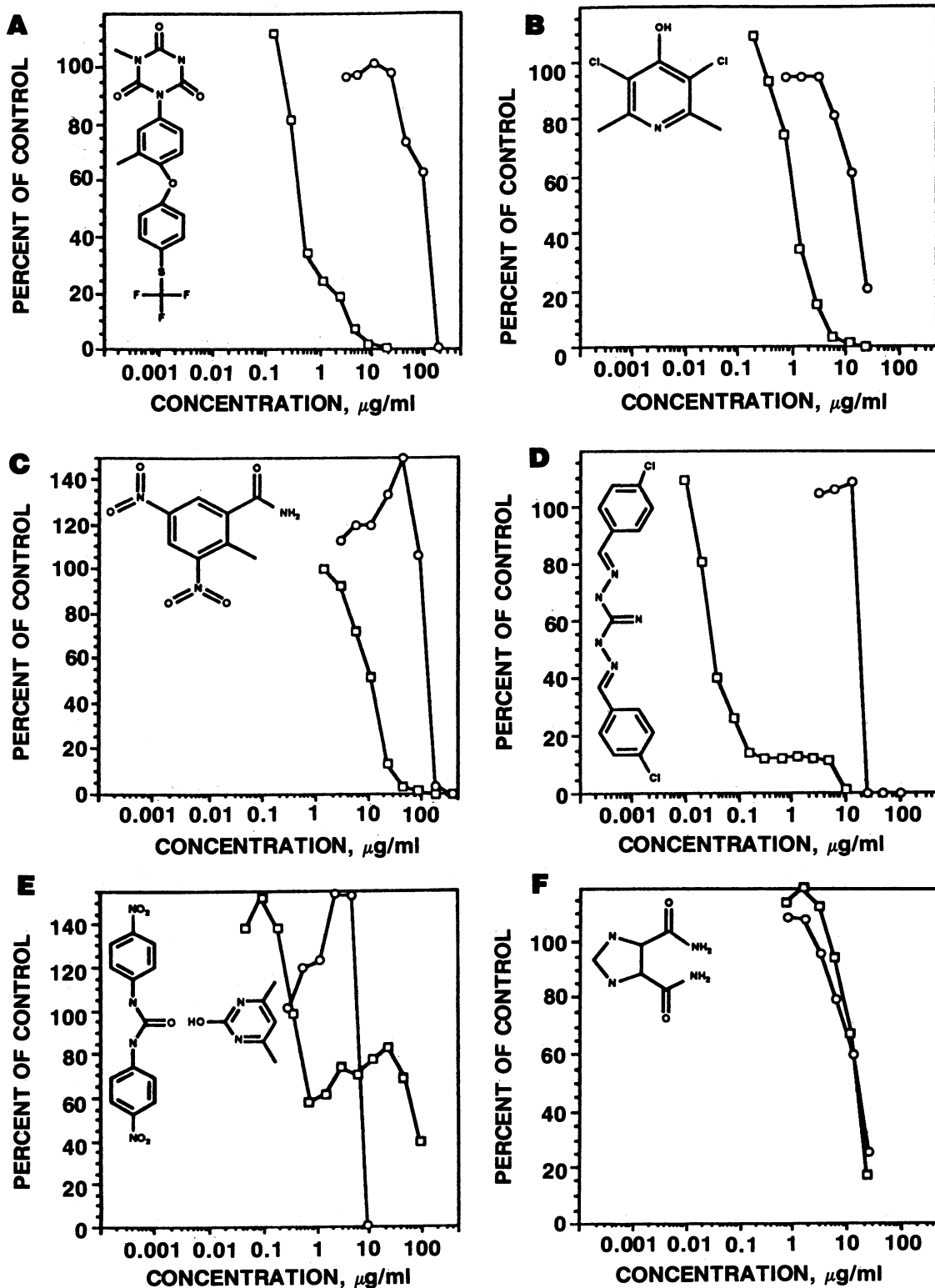
FIG. 1. Dose-response curves for six anticoccidial drugs (A, toltrazuril; B, clopidol; C, dinitolmide; D, robenidine; E, nicarbazin; and F, glycamide) against human fibroblasts (○) and *T. gondii* (□). Values represent the incorporation of radiolabel expressed as a percentage of that in untreated control wells.
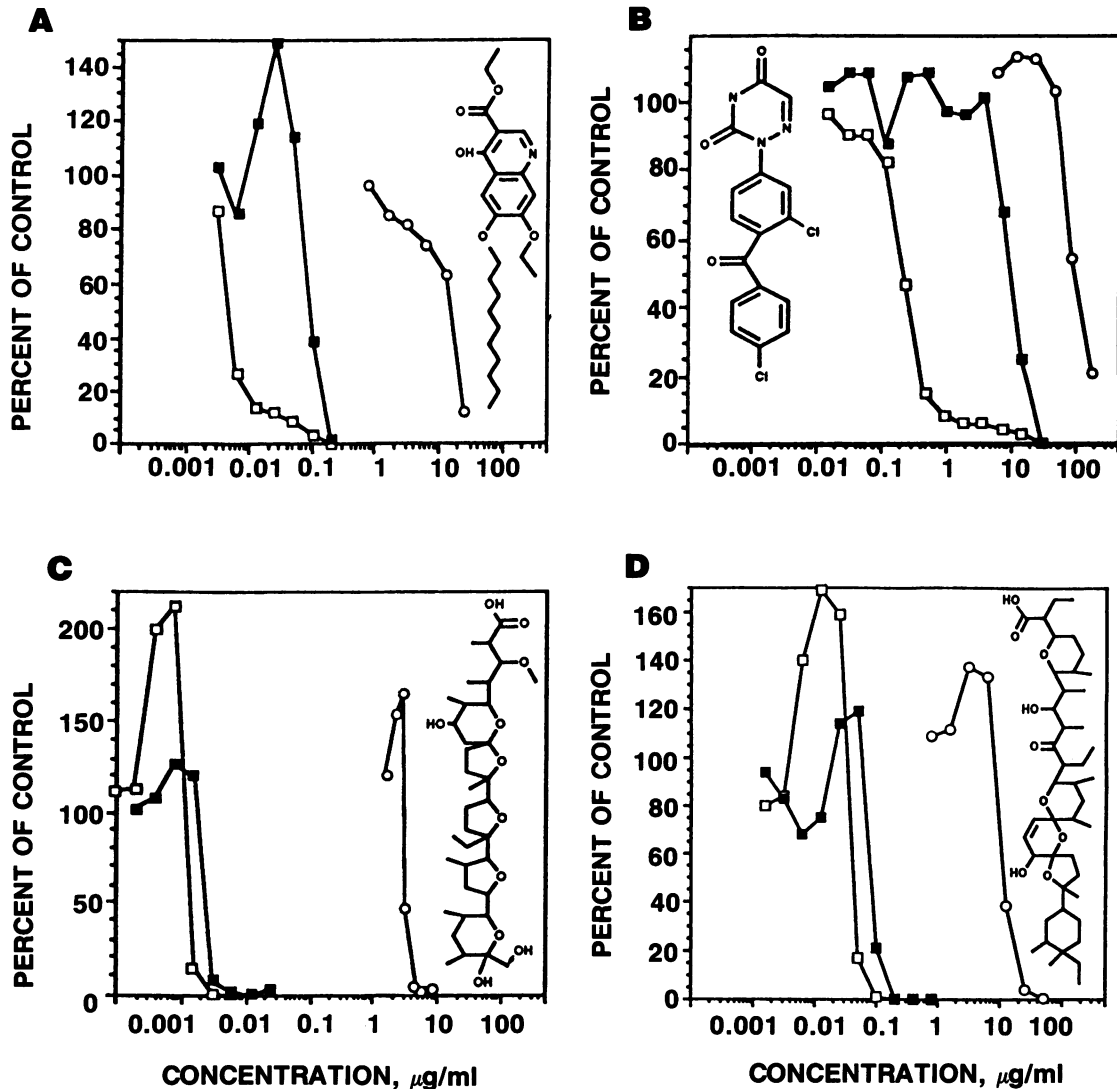
FIG. 2. Dose-response curves for four anticoccidial drugs (A, decoquinate; B, CP-25,415; C, monensin; and D, salinomycin) against human fibroblasts (O), the *T. gondii* parental strain (□), and *T. gondii* strains passaged in the presence of drug (■). Values represent the incorporation of radiolabel expressed as a percentage of that in untreated control wells.

against toxoplasmosis may be identified from among the wide variety of available anticoccidial drugs.

Coccidiosis is highly contagious, and intensive rearing of poultry leads to extensive parasite transmission, unless prevented by continuous drug treatment. Under these conditions, many anticoccidial drugs became useless shortly after their commercial launch because of the rapid development of resistance. We were interested in determining whether resistant *T. gondii* mutants were readily selected by anticoccidial drugs, to judge whether mutant selection in *T. gondii* could predict the likelihood of the development of resistance against a drug in coccidia. The development of resistance in *T. gondii* is also a key aspect of the laboratory profile for an antitoxoplasma agent.

No resistant mutants were selected by robenidine, toltrazuril, or clopidol. In our in vitro studies, each of these agents showed good antitoxoplasma potency combined with a lack of resistance development. This in vitro profile is an attractive one for an antitoxoplasma agent; however, additional

testing would be needed to assess whether this in vitro activity translates to in vivo activity. Furthermore, *Toxoplasma* strains other than RH would need to be evaluated before extrapolation of results from this study, given that the RH strain does not produce cysts and produces an acute rather than a chronic infection. Other agents with this type of profile might well exist among commercial or experimental anticoccidial drugs. With regard to the field experience with these three agents as anticoccidial drugs, resistance was reported 6 years after the introduction of clopidol (18) and 10 years after the introduction of robenidine (21). Toltrazuril was recently introduced and is difficult to compare with the other anticoccidial drugs, as it is used in a limited fashion as an intermittent water treatment rather than extensively as a continuous-feed medication.

In an earlier study, *T. gondii* mutants resistant to arprinocid-*N*-oxide were obtained (32); arprinocid is notorious for the rapid development of resistance in coccidia both in the laboratory and in the field. Similar results were obtained
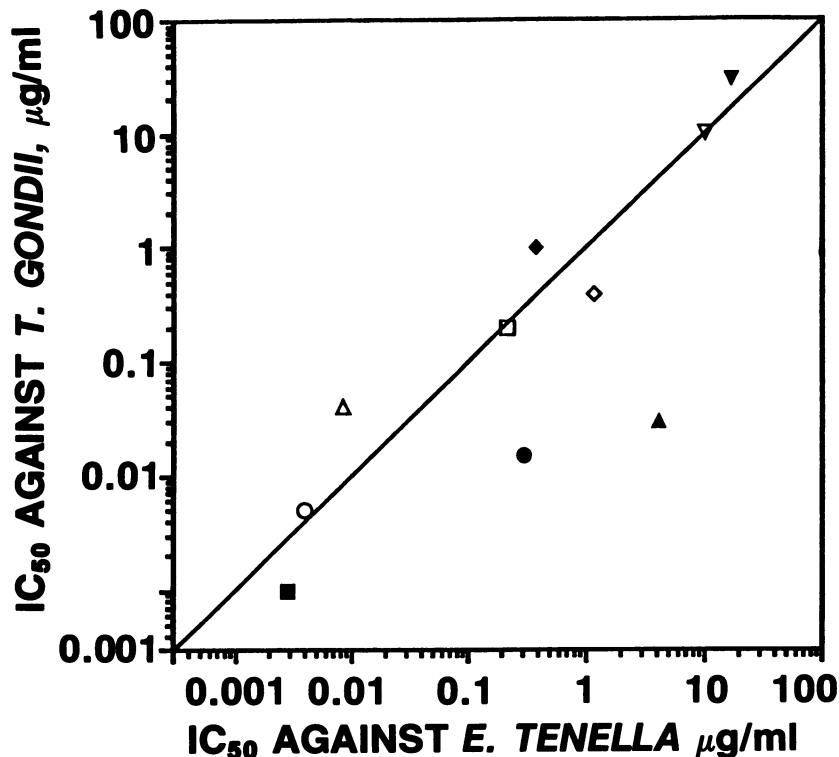
FIG. 3. Comparison of the in vitro potencies against *E. tenella* and *T. gondii* of 10 anticoccidial drugs: decoquinate (○), arprinocid-*N*-oxide (●), CP-25,415 (□), monensin (■), salinomycin (△), robenidine (▲), toltrazuril (◇), clopidol (◆), dinitolmide (▽), and nicarbazin (▼). The line of identity is indicated; $R^2 = 0.909$.

for arprinocid-*N*-oxide in the present study. Likewise, decoquinate yielded a resistant strain of *T. gondii*. Quinolone anticoccidial drugs had only a brief commercial life that was terminated by the development of resistance (25). A strain of *T. gondii* resistant to the triazine CP-25,415 was also obtained. There is little field experience with this series to date; CP-25,415 was not commercialized, and the related triazine diclazuril was only recently launched as an anticoccidial drug. In anticoccidial drug resistance cycling experiments in the laboratory, CP-25,415 resulted in rapid resistance (8a). Hence, for these three classes (aprinocid, quinolones, and triazines) rapid anticoccidial drug resistance development has been observed, and resistant strains of *T. gondii* could be selected. Since the drug target for triazines has not been defined with certainty, the resistant strain of *T. gondii* might offer a starting point for mechanistic studies.

The polyether ionophores present an unusual case. The first anticoccidial ionophore, monensin, was introduced in the United States in 1971, and resistance in the field was reported 10 years later (24). Since that time, the prevalence of strains resistant to ionophores has increased progressively. For example, in a recent study of 15 field isolates, all were classified as ionophore resistant (8). However, the ionophores continue as the most widely used anticoccidial drug class. This observation runs counter to experience with other classes of anticoccidial drugs, for which reports of resistance were promptly followed by a collapse in drug efficacy. The highest degree of resistance that we obtained for *T. gondii* was a twofold reduction in drug susceptibility. One study comparing resistant and susceptible strains in vivo found approximately 2-fold-reduced susceptibility (35),

consistent with our results; however, an in vitro study described field isolates with 10- to 50-fold-reduced susceptibility (2). Ionophore resistance in *Eimeria* species has been proposed to involve an alteration in uptake (2); however, the biochemical basis of resistance has not been defined. Although the strains described in the present study were only twofold resistant, they might still be useful in shedding light on the nature of ionophore resistance in sporozoa.

Our results support the belief that an important human health pathogen and an important animal health pathogen, *T. gondii* and *E. tenella*, respectively, are sufficiently similar in drug susceptibility and resistance development that the advantages of each system can be exploited to the mutual benefit of human and animal health. For *E. tenella*, a particular advantage is the variety of available anticoccidial drugs, and for *T. gondii*, techniques for continuous propagation enable the study of resistance development in vitro. Similarities in drug response may provide useful tools in the search for new chemotherapeutic agents against these two key sporozoan parasites.

ACKNOWLEDGMENTS

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