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In Vitro Effects of Folate Inhibitors on *Toxoplasma gondii*

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Three sulfonamides and four dihydrofolate reductase inhibitors were tested alone and in combination to determine their in vitro effects on two strains of *Toxoplasma gondii* grown in MRC5 fibroblast tissue culture. *Toxoplasma* growth was quantitated by an enzyme immunoassay performed directly on the fixed cultures, and linear regression models were used to quantify the relationship between the optical density values generated by the enzyme-linked immunosorbent assay and the concentrations of the antimicrobial agents in the culture medium. The cytopathic effects of antimicrobial agents on *T. gondii* were examined in Giemsa-stained cultures. Sulfonamides and dihydrofolate reductase inhibitors exhibited similar patterns of inhibition, consisting of an important increase of the inhibitory effect within a narrow range of concentrations. Sulfadiazine, sulfamethoxazole, and sulfisoxazole were all found to have important inhibitory effects on *T. gondii*; the 50% inhibitory concentrations estimated from the regression models were 2.5 μg/ml for sulfadiazine, 1.1 μg/ml for sulfamethoxazole, and 6.4 μg/ml for sulfisoxazole. This inhibition of growth was associated with a reduction of the number of parasitized cells and intracellular parasites that were morphologically normal. With dihydrofolate reductase inhibitors, including pyrimethamine, trimethoprim, trimetrexate-glycuronate, and piritrexim, a strong inhibition of *Toxoplasma* growth was observed, which was associated with striking morphological changes of the parasites. The 50% inhibitory concentrations were 0.04 μg/ml for pyrimethamine, 2.3 μg/ml for trimethoprim, 0.16 ng/ml for trimetrexate-glycuronate, and 6.9 ng/ml for piritrexim. When sulfonamides and dihydrofolate reductase inhibitors were used in combination, a synergistic effect was observed with sulfadiazine combined with pyrimethamine, trimetrexate-glycuronate, and piritrexim; sulfisoxazole combined with pyrimethamine; and trimethoprim combined with sulfamethoxazole. These results were analyzed in comparison with human pharmacokinetics data.

Sulfonamides and dihydrofolate reductase (DHFR) inhibitors are at present the most effective agents against *Toxoplasma gondii*, since they act synergically on this parasite (7–9, 17, 24). In human toxoplasmosis, pyrimethamine (PYR) associated with sulfadiazine (SDZ) is the most usual treatment, with documented efficacy in toxoplasmic encephalitis (11, 15), but this regimen is associated with frequent and severe adverse reactions, especially in patients with acquired immunodeficiency syndrome (11, 15). The development of new therapeutic alternatives requires a better knowledge of the effect of antimicrobial agents on *T. gondii*. We previously reported the usefulness of an enzyme immunoassay performed on infected tissue cultures for sensitive analysis of the in vitro effects of antimicrobial agents on *T. gondii* (5, 6); with this method, the relationship between the inhibitory effect of a drug and its concentration in the culture medium can be characterized by regression analysis. In the present study, this in vitro method was used to assess the inhibitory effects of three commonly used sulfonamides (SDZ, sulfamethoxazole [SMZ], and sulfisoxazole [SSX]) and four DHFR inhibitors (trimethoprim [TMP], piritrexim [PIRI], trimetrexate [TMTX], and PYR), tested alone or in combination.

MATERIALS AND METHODS

Parasites. Two virulent *Toxoplasma* strains were used: the RH strain and the C strain, which was isolated from a congenitally infected placenta (4). Parasites were maintained in mice, and tachyzoites were harvested from the peritoneal fluid of mice infected intraperitoneally 2 days earlier. After parasites were washed with minimum essential medium (Eurobio, Paris, France) containing 10% fetal calf serum (Flow Laboratories, France) and supplemented with ampicillin (100 μg/ml) and kanamycin (200 μg/ml), they were counted and adjusted to a concentration of $3 \cdot 10^4$ parasites per ml.

In vitro studies. The assessment of the in vitro inhibitory effects of antimicrobial agents on *Toxoplasma* growth was performed as previously described (5), with minor modifications. Briefly, the cultures of MRC5 fibroblasts (BioMérieux, Lyon, France) were prepared in 96-well tissue culture plates (Nunc, Roskilde, Denmark) and grown to confluence. Each well of the plate, except eight control wells, was inoculated with 50 μl of the parasite suspension (i.e., 1,500 parasites). After 4 h, antimicrobial agents at various concentrations were added to the wells; after 72 h, the cultures were fixed with cold methanol. *Toxoplasma* growth was evaluated by an enzyme-linked immunosorbent assay (ELISA) performed directly on the infected cultures, with a rabbit anti-*T. gondii* immunoglobulin G as the first antibody, a phosphatase-labeled anti-rabbit immunoglobulin G as the second antibody, and *p*-nitrophenylphosphate as the substrate (Sigma Chemical Co., St. Louis, Mo.). After development of the coloration, the substrate was transferred into new ELISA plates for spectrophotometric readings at 405 nm; blank readings were made on the eight control wells. The culture plates were immediately washed with distilled water, stained with Giemsa, and examined microscopically. All of the assays were repeated twice and were performed with tachyzoites of the two stains under the same conditions.

Antimicrobial agents. PYR (Sigma), PIRI (Burroughs Wellcome Co., Research Triangle Park, N.C.) TMP (Produits Roche, Neuilly, France), SDZ (Laboratoires Théra-

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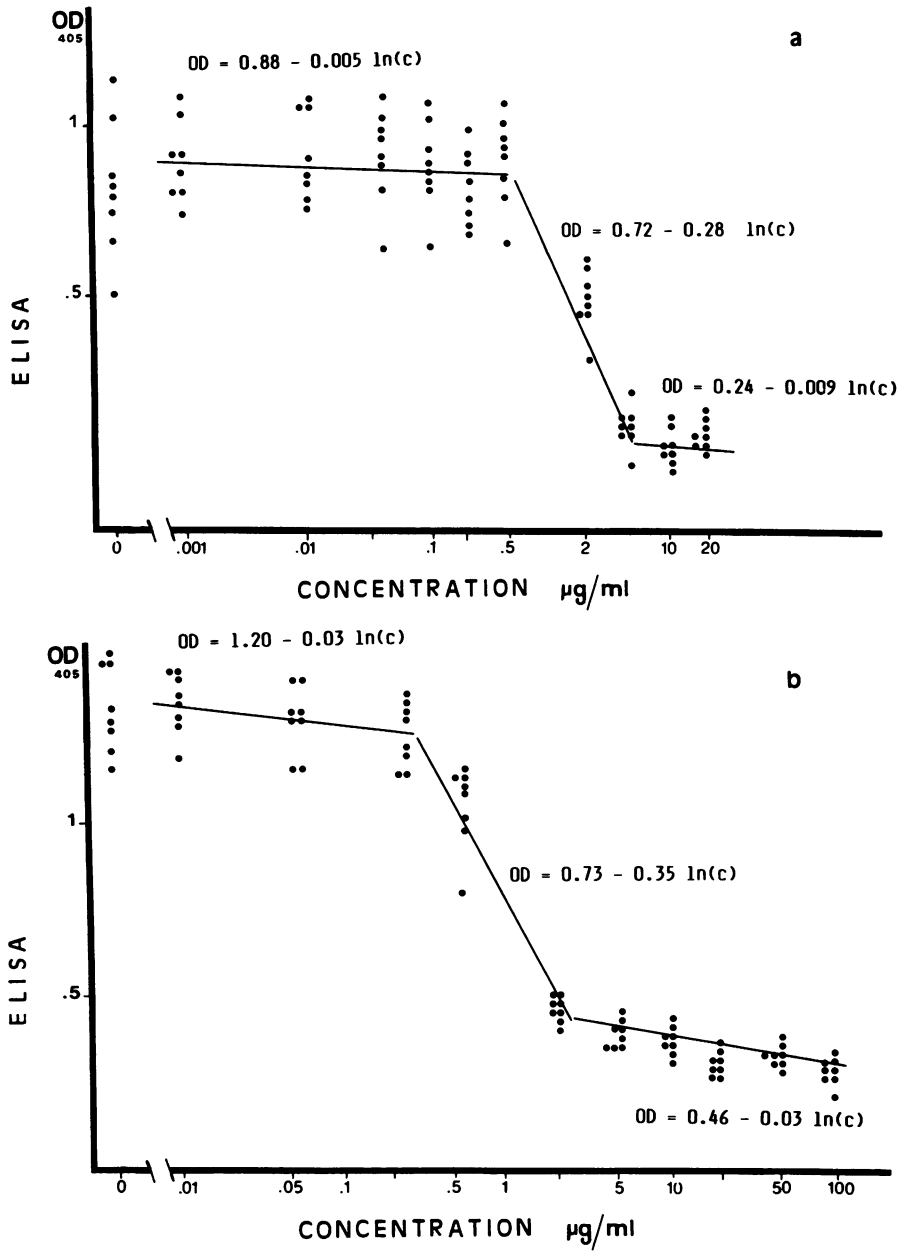


plix, Paris, France), SMZ (Produits Roche), and SSX (Laboratoires Abbott, Rungis, France) were initially dissolved in 50% methanol–50% acetone at a concentration of 5 mg/ml (PIRI, PYR) or 10 mg/ml (SDZ, SSX, SMZ). TMTX glycuronate (TMTX-G; Parke Davis Laboratories, Ann Arbor, Mich.) was dissolved in sterile distilled water at a concentration of 12.5 mg/ml.

Serial dilutions were then prepared in minimum essential medium-fetal calf serum, and 25 µl of each dilution was added to the culture to yield the following final concentrations: SDZ, 0.001, 0.01, 0.05, 0.1, 0.2, 0.5, 2, 10, and 20 µg/ml; SMZ, 0.01, 0.05, 0.2, 0.5, 2, 5, 10, 20, 50, and 100 µg/ml; SSX, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, and 100 µg/ml; TMP, 0.01, 0.05, 0.2, 0.5, 1, 2, 5, 20, 50, and 100 µg/ml; TMTX-G, 0.001, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 5 ng/ml; PIRI-G, 0.05, 0.1, 1, 5, 10, 50, 100, and 500 ng/ml. PYR, which was previously found to have a significant inhibitory effect at 0.05 µg/ml (5), was tested at 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µg/ml to better characterize its inhibitory effect at low concentrations.

Antimicrobial agents were studied in combination in a two-dimensional test. The following concentrations were tested: SDZ (0.1 and 0.5 µg/ml) combined with PYR (0.005 and 0.02 µg/ml), PIRI (0.2 and 1 ng/ml), and TMTX-G (0.02 and 0.1 ng/ml); SMZ (0.2 and 1 µg/ml) combined with TMP (0.1 and 0.5 µg/ml); and SSX (0.5 and 2 µg/ml) combined with PYR (0.005 and 0.02 µg/ml).

In a preliminary experiment, methanol and acetone, used for dissolution of antimicrobial agents, were found to have no significant inhibitory effect on *Toxoplasma* growth for concentrations up to 1:50 (vol/vol) in the culture medium. In the different experiments, these solvents were always at a final concentration of less than 1:100.

Statistical analysis. The effects of antimicrobial agents at

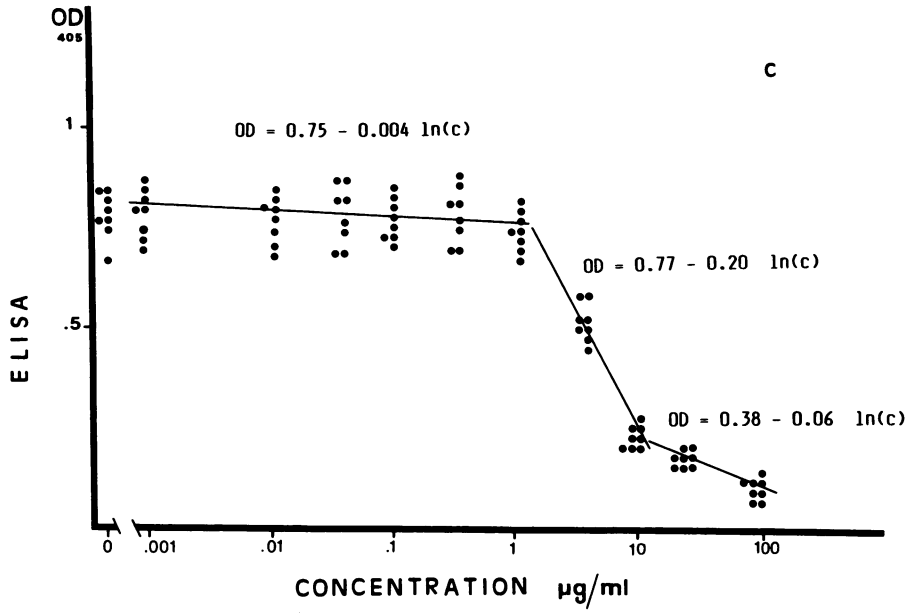


FIG. 1. A_{405}s (y axis) for ELISA with infected monolayers versus concentrations of the following sulfonamide antimicrobial agents (x axis): SDZ (a), SMX (b), and SSX (c).

various concentrations were described by data plotting. We assumed that optical density (OD) is a function of the logarithm of the drug concentration. Although these curves displayed a sigmoid shape, we preferred to model the relationship with three linear regression models that fit the data better (19). This allowed us to described each drug effect as a sequence of three lines: line 1 indicated the absence of inhibitory effect (slope not significantly different from 0), line 2 indicated a marked increase of inhibition, line 3 indicated a residual effect for higher concentrations. Data plotting was used to select pertinent concentrations for each line; some concentrations were used to estimate two adjacent regression lines.

For each drug we chose to estimate the OD corresponding to a concentration of 0 by extrapolating line 1 (noninhibitory concentration) on the y axis; the 50% inhibitory concentration (IC_{50}) was obtained by reporting 50% of this value in the equation of line 2. The confidence interval of the IC_{50} was derived from the confidence interval of line 2.

The inhibitory effects of antimicrobial agents in combination, assessed by the two-dimensional test, were studied by using a two-way analysis of variance, including the estimation of an interaction coefficient (23). Drugs were considered to act synergically when the interaction coefficient was significantly different from 0, i.e., the mean OD with the combined drugs was significantly lower than the OD expected from the simple additive effects of both drugs.

Both BMDP and SAS statistical libraries were used.

RESULTS

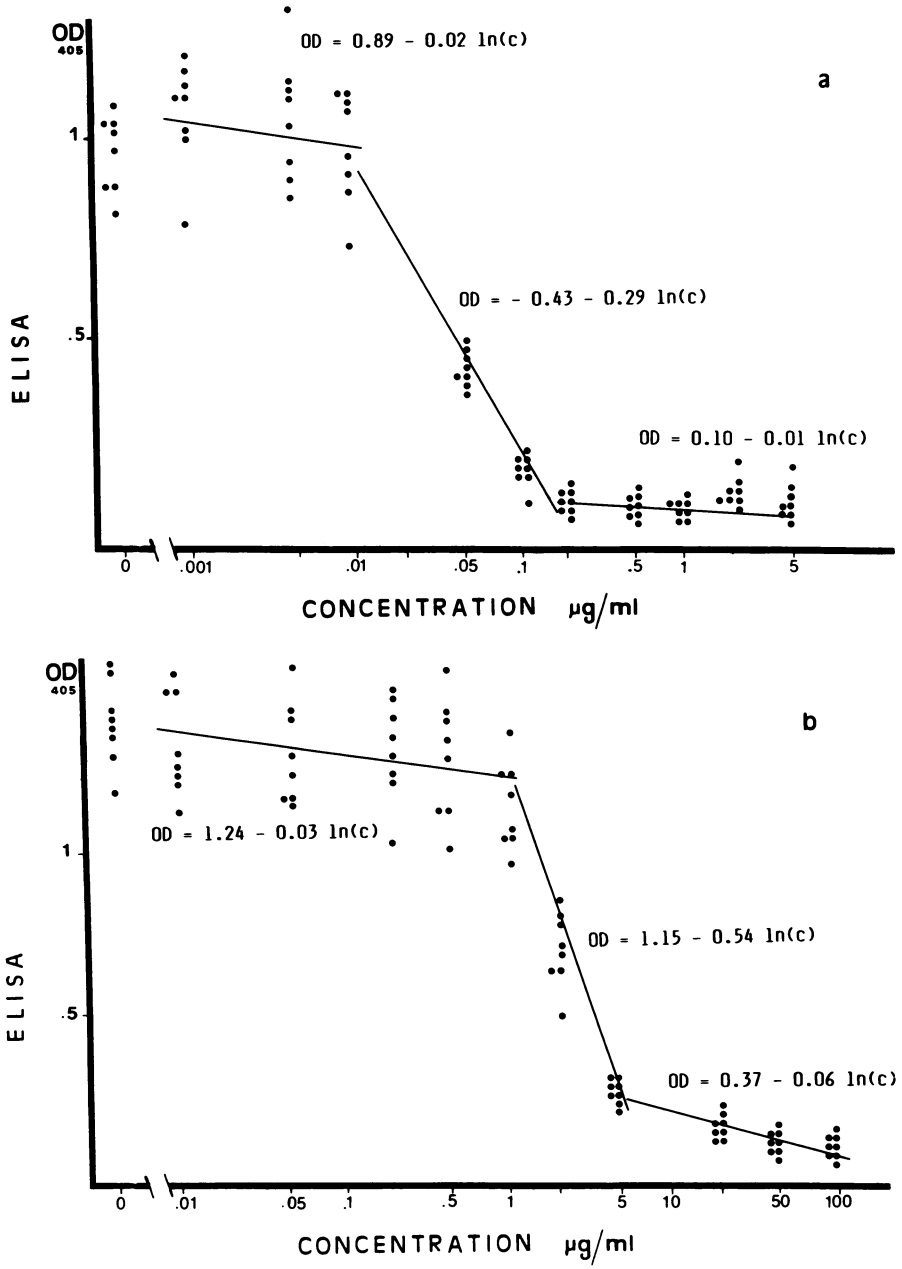
Sulfonamides and DHFR inhibitors at various concentrations were found to have important inhibitory effects on *Toxoplasma* growth. For each antimicrobial agent, the relationships between ODs (which are correlated with the number of *Toxoplasma* organisms in the cultures) and the concentrations are presented in Fig. 1 and 2; the combined effects of sulfonamides and DHFR inhibitors are presented in Tables 1, 2, and 3. All of the results presented below were obtained from cultures inoculated with tachyzoites of the RH strain; with tachyzoites of the C strain, the patterns of inhibition observed with the different agents were similar to those observed with the RH strain and are not presented individually. Similarly, the cytopathic effects of antimicrobial agents on *Toxoplasma* organisms were similar with both strains.

Inhibitory effects of sulfonamides. (i) SDZ (Fig. 1a). A significant inhibitory effect was observed for SDZ concentrations greater than 0.5 μg/ml. Between 0.5 and 5 μg/ml, this effect was summarized by regression line L2: OD = 0.72 − 0.28 ln(c), where c is concentration. In the interval of 5 to 20 μg/ml, the slope of the regression line L3 was not significantly different from 0 ($P = 0.59$), which indicates that the inhibitory effect was reached at 5 μg/ml. The IC_{50} was estimated to be 2.5 μg/ml with a 5% confidence interval (2.2 to 3 μg/ml).

(ii) SMZ (Fig. 1b). SMZ showed no significant inhibition over the concentration range of 0.01 to 0.2 μg/ml. A progressive increase of the inhibitory effect was observed for 0.5 and 2 μg/ml, which was summarized by regression line L2: OD = 0.73 − 0.35 ln(c). Within the interval of 2 to 100 μg/ml, the regression model showed a slight increase of the inhibitory effect, since the slope of the regression line L3 was significantly different from 0 ($P < 0.01$). The IC_{50} was estimated to be 1.1 μg/ml with a 5% confidence interval (1 to 1.3 μg/ml).

(iii) SSX (Fig. 1c). SSX had no effect at concentrations between 0.001 and 1 μg/ml. The inhibitory effect increased rapidly at 2 and 10 μg/ml and then more gradually within the interval of 10 to 100 μg/ml (slope of L3 was significantly different from 0; $P < 0.01$). The IC_{50} was estimated to be 6.4 μg/ml with a 5% confidence interval (4.9 to 8.5 μg/ml).

Inhibitory effects of DHFR inhibitors. (i) PYR (Fig. 2a). PYR was ineffective for concentrations between 0.001 and 0.01 μg/ml. An important increase of the inhibitory effect was observed for concentrations of ≥0.05 μg/ml; line L2 summarizes this effect from 0.01 to 0.2 μg/ml. No significant



increase of inhibition was observed for concentrations greater than 0.2 µg/ml (slope of L3 was not significantly different from 0). The IC_{50} was estimated to be 0.04 µg/ml with a 5% confidence interval (0.02 to 0.05 µg/ml).

(ii) TMP (Fig. 2b). TMP was not inhibitory for concentrations ranging between 0.01 and 1 µg/ml. A marked increase of the inhibitory effect was noted in a narrow dose range, i.e., 2 to 5 µg/ml; for higher concentrations, between 5 and 100 µg/ml, the inhibitory effect increased very progressively (slope of L3 was significantly different from 0; $P < 0.01$). The IC_{50} was estimated to be 2.3 µg/ml with a 5% confidence interval (2 to 2.6 µg/ml).

(iii) TMTX-G (Fig. 2c). With TMTX-G, a very important inhibitory effect was observed at 0.2 ng/ml, whereas concentrations up to 0.1 ng/ml had no significant effect. For concentrations greater than 0.2 ng, the slope of the regression line L3 was significantly different from 0 ($P < 0.01$), indicating a progressive increase of the inhibitory effect within the range of 0.2 to 5 ng/ml. The IC_{50} was estimated to be 0.16 ng/ml with a 5% confidence interval (0.12 to 0.21 ng/ml).

(iv) PIRI (Fig. 2d). PIRI was not inhibitory for concentrations ranging between 0.05 and 1 µg/ml. A marked increase of inhibition was observed for 5 and 10 µg/ml; for higher concentrations, this effect increased more slightly ($P < 0.01$). The IC_{50} was estimated to be 6.9 ng/ml with a 5% confidence interval (5.8 to 8.2 ng/ml).

Combinations of sulfonamides and DHFR inhibitors. The inhibitory effects of the different combinations of antimicrobial agents were assessed by using two concentrations of each drug which alone were not found inhibitory. Several combinations of antimicrobial agents had significant inhibi-

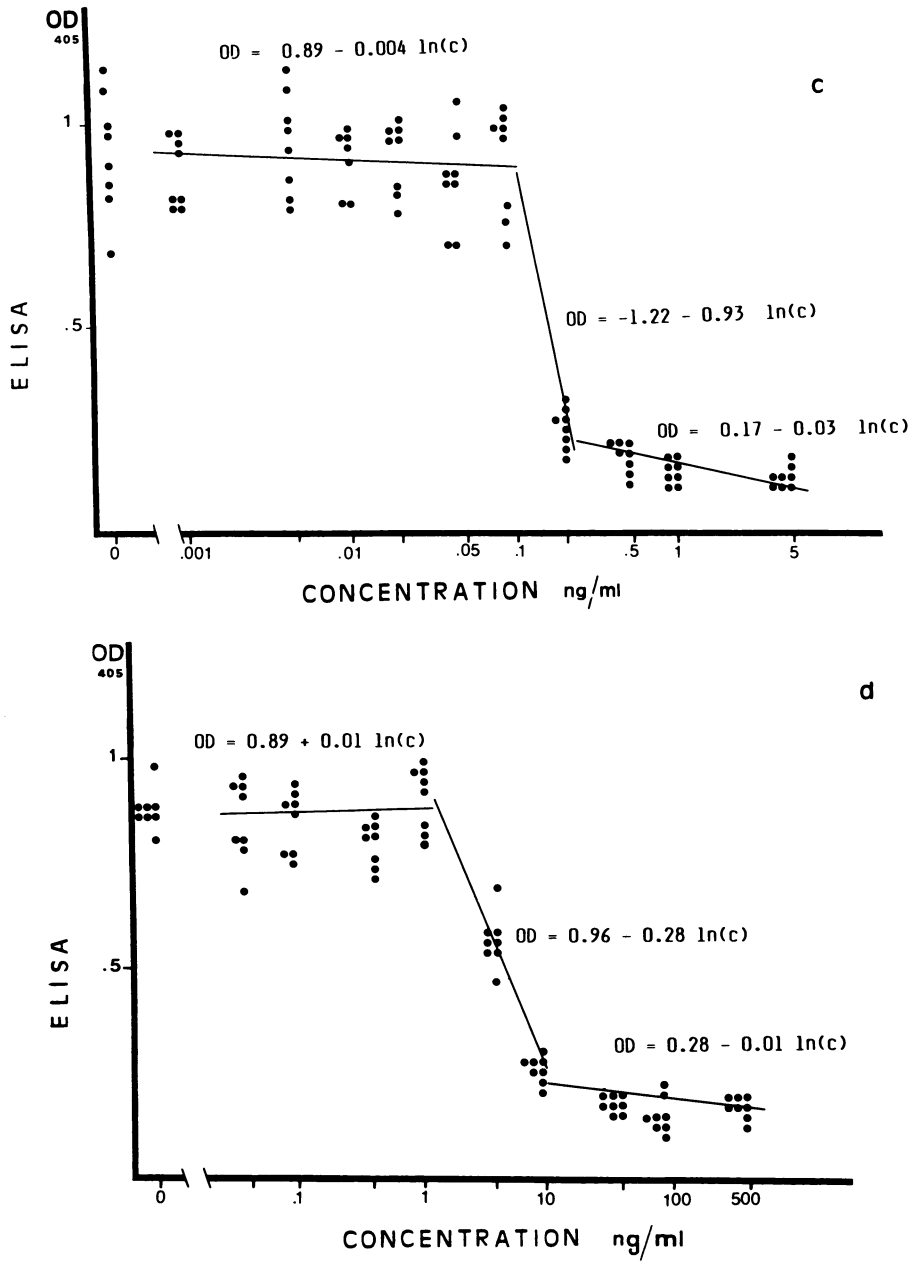


FIG. 2. A_{405}s (y axis) for ELISA with infected monolayers versus concentrations of the following DHFR inhibitors: PYR (a), TMP (b), TMTX-G (c), and PIRI (d).

tory effects on *T. gondii*. With SDZ, a significant interaction effect was observed at the concentration of 0.1 μg/ml when SDZ was combined with PYR at 0.02 μg/ml or TMTX-G at 0.1 ng/ml and when SDZ at 0.5 μg/ml was combined with PYR at 0.005 and 0.02 μg/ml, PIRI at 1 ng/ml, or TMTX-G at 0.1 ng/ml (Table 1). With TMP combined with SMZ, a significant interaction effect was observed, which was maximum with SMZ at 1 μg/ml plus TMP at 0.5 μg/ml (Table 2). SSX was inhibitory at 2 μg/ml when combined with PYR at 0.02 μg/ml (Table 3).

Cytopathic effect on *T. gondii*. Giemsa-stained cultures were examined at a magnification of 1,000. With sulfonamides and DHFR inhibitors, no effect was noted on the fibroblasts at the concentrations studied. With sulfonamides, the inhibitory effect on *T. gondii* was associated with a reduction of the number of the intracellular parasites, but the organisms were morphologically normal. In the cultures with DHFR inhibitors, an important cytopathic effect was observed on the parasites. At the first inhibitory concentrations, i.e., PYR at 0.05 μg/ml, TMP at 2 μg/ml, TMTX-G at 0.2 ng/ml, and PIRI at 5 ng/ml, *Toxoplasma* organisms appeared rounded, and their number was reduced compared with those in nontreated cultures. For higher concentrations, the parasitophorous vacuoles were enlarged and contained distorted parasites, cellular divisions appeared completely inhibited, rosettes were not seen, and the parasites appeared as undivided cytoplasmic formations containing fragmented nuclei. This cytopathic effect was similar in both strains with

TABLE 1. Inhibitory effect of SDZ combined with PYR, PIRI, or TMTX[a]

Drug and concn	Mean A_{405} ± SD at the following SDZ concn (μg/ml):		
	0	0.1	0.5
PYR (μg/ml)			
0	0.829 ± 0.099	0.724 ± 0.063	0.854 ± 0.077
0.005	0.859 ± 0.097	0.872 ± 0.072	0.501 ± 0.043[b]
0.02	0.657 ± 0.095	0.379 ± 0.037[c]	0.168 ± 0.020[b]
PIRI (ng/ml)			
0	0.973 ± 0.097	0.747 ± 0.140	0.805 ± 0.071
0.2	0.850 ± 0.105	0.819 ± 0.086	0.679 ± 0.100
1	0.936 ± 0.060	0.811 ± 0.103	0.097 ± 0.044[b]
TMTX (ng/ml)			
0	0.987 ± 0.060	0.899 ± 0.077	0.891 ± 0.097
0.02	0.926 ± 0.076	0.918 ± 0.041	0.721 ± 0.098
0.1	0.776 ± 0.070	0.146 ± 0.035[b]	0.028 ± 0.015[b]

[a] Each value represents results from 6 or 8 replicate wells. *P* values were calculated for the interacting effect.
[b] $P = 0.001$.
[c] $P = 0.004$.

the different DHFR inhibitors and with the combinations of sulfonamides and DHFR inhibitors.

DISCUSSION

The results clearly demonstrate that sulfonamides and DHFR inhibitors have an important in vitro effect on *T. gondii*, either alone or in combination. With both parasite strains, they exhibited similar patterns of inhibition, consisting of an important increase of the inhibitory effect within a narrow range of concentrations. This finding is consistent with the mode of action of these compounds on folate biosynthesis enzymes, suggesting that a threshold concentration has to be reached to obtain an enzymatic blockade with an inhibition of parasite replication.

With sulfonamides, the inhibitory effect was not associated with alteration of the parasite morphology, suggesting a parasitostatic effect on *T. gondii*. By contrast, all of the DHFR inhibitors induced striking morphological changes, as previously noted by others with TMP (9) and PYR (23); in the Giemsa-stained cultures, parasitophorous vacuoles were enlarged and contained rounded organisms with fragmented nuclei.

The in vitro inhibitory effect of sulfonamides, assessed by ELISA, was significant for SDZ at 2 μg/ml, SMZ at 0.5 μg/ml, and SSX at 5 μg/ml. These results confirm our previous results obtained with sulfadoxine on the inhibitory effect of sulfonamides (5) but contrast with those of other in vitro studies in which sulfonamides were not found inhibitory for *T. gondii* in vitro, even at high concentrations (9, 10, 17, 24); this discrepancy may be related to (i) the long incubation time of the cultures in our assay (72 h), (ii) the sensitivity of the ELISA for the evaluation of *Toxoplasma* growth, or (iii) some difference in the intracellular penetration of the drugs into macrophages or fibroblast cells.

TABLE 2. Inhibitory effect of SMZ combined with TMP[a]

TMP concn (μg/ml)	Mean A_{405} ± SD at the following SMZ concn (μg/ml):		
	0	0.2	1
0	0.955 ± 0.026	0.978 ± 0.111	0.639 ± 0.085
0.1	1.057 ± 0.092	1.063 ± 0.111	0.356 ± 0.036[b]
0.5	0.955 ± 0.150	0.567 ± 0.064[b]	0.180 ± 0.028[b]

[a] See footnote *a* of Table 1.
[b] $P = 0.001$.

TABLE 3. Inhibitory effect of SSX combined with PYR[a]

PYR concn (μg/ml)	Mean A_{405} ± SD at the following SSX concn (μg/ml):		
	0	0.5	2
0	0.768 ± 0.056	0.734 ± 0.091	0.749 ± 0.029
0.005	0.754 ± 0.104	0.875 ± 0.108	0.712 ± 0.044
0.02	0.625 ± 0.045	0.605 ± 0.062	0.241 ± 0.011[b]

[a] See footnote *a* of Table 1.
[b] $P = 0.001$.

Our in vitro results obtained with sulfonamides are in partial agreement with in vivo studies. In experimental murine toxoplasmosis, a curative effect has been demonstrated with SDZ, with a comparatively lower efficacy of SSX and SMT (7, 9, 22). Our results, which showed that SDZ, SMT, and SSX are inhibitory in vitro at concentrations that can be obtained in human serum (14, 27), suggest that these drugs should be effective in vivo. The low efficacy of some sulfonamides in vivo could be related to their high degree of binding to protein, which may generate inactive compounds, and/or to their parasitostatic rather that parasiticidal effect on *T. gondii*.

With DHFR inhibitors, our results confirm and extend previous findings based on other models (9, 10, 12, 13, 17, 24). A significant inhibition effect was noted for PYR at 0.05 μg/ml, TMP at 2 μg/ml, TMTX-G at 0.2 ng/ml, and PIRI at 5 ng/ml. Comparison of these in vitro results with pharmacokinetic data, as mentioned in the literature, suggests a potent curative effect of some of these agents. With TMP, the in vitro inhibitory effect is significant at a concentration similar to the peak level obtained in human serum after oral administration of 160 mg (2). Because of the short half-life of TMP, such an inhibitory concentration may not be maintained, which may explain the poor efficacy of this agent. With PYR, a maximum inhibitory effect was obtained with 0.2 μg/ml, i.e., a concentration which can be maintained in human serum with daily or alternate-day administration of 25 mg (1, 18, 25, 26). Because of large individual variations, Weiss et al. (26) suggested that levels in serum should be monitored during therapy; however, we do not consider, like these authors, that a level in serum of 3 μg/ml is necessary for treatment, since the maximum inhibition is reached at 0.2 μg/ml and does not increase for higher concentrations.

PIRI and TMTX, which have been found effective against *Pneumocystis carinii* and *T. gondii* in murine models (3, 12, 20), are of great potential interest since they are inhibitory in vitro for *T. gondii* at very low concentrations. With PIRI, we observed a significant inhibition at a concentration of 5 ng/ml, which is more than 100-fold lower than the concentrations reported in human serum (13). Similarly, TMTX appears to be a promising agent, since our in vitro results showed that its inhibitory effect was very important for a concentration as low as 0.5 ng of TMTX-G (which contains 65% of the basic compound); such a concentration is 1,000-fold lower than those which can be maintained for 24 h in human serum after an oral dose of 60 mg/m^2 (21). These results suggest that the efficacy of these two compounds on acute and chronic toxoplasmosis could be evaluated by using dosages lower than those used for antineoplasic therapy, thus reducing their potential hematological toxicity (16).

When DHFR inhibitors and sulfonamides were combined,

a synergistic effect was assessed by the ELISA for all the combinations tested; an important inhibitory effect was noted for each agent at concentrations 2- to 10-fold lower than those that were found inhibitory when tested individually. This inhibition of growth was associated with a cytopathic effect similar to that observed with DHFR inhibitors alone. Since sulfonamides are not cytopathic alone, even at high concentrations, this suggests that they act primarily by potentiating the effects of DHFR inhibitors.

Finally, our in vitro results confirm that folate inhibitors and, more particularly, DHFR inhibitors are of major interest for treatment of toxoplasmosis. Our experimental design allowed us to characterize the dose-efffect relationship for each antimicrobial agent and to estimate individual parameters, such as IC_{50}, which may prove useful for further in vitro and in vivo evaluation of these compounds.

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