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## **Anti-toxoplasma effect of pyrimethamine, trimethoprim and sulphonamides alone and in combination: implications for therapy**

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The aim of the present study was to determine the in-vitro susceptibility of *Toxoplasma gondii* to dihydrofolate reductase inhibitors and sulphonamides alone, and in combination. It was found that pyrimethamine had the most potent anti-*Toxoplasma* activity, while sulphadiazine, sulphamethoxazole and sulphametrole were approximately equally effective, but only at a high concentration. The 50% inhibitory concentration of sulphamethoxazole was determined using different fixed concentrations of pyrimethamine or trimethoprim. It was found that a ten-fold increment of the concentration of pyrimethamine, reduced the IC<sub>50</sub> of sulphamethoxazole 1000 times. The influence of trimethoprim on the IC<sub>50</sub> of sulphamethoxazole was less concentration dependent. Addition of dihydrofolate or tetrahydrofolate did not influence the IC<sub>50</sub> of pyrimethamine. In conclusion, the present dose recommendations for sulphonamides, when combined with pyrimethamine, may be unnecessarily high. There may be a justification for dose reduction of sulphonamides in order to prevent side effects.

### **Introduction**

Cerebral toxoplasmosis is a common opportunistic infection in patients infected with human immunodeficiency virus (HIV) (Schärtlander *et al.*, 1992). The combination of pyrimethamine and sulphadiazine is the standard treatment, with documented efficacy for *Toxoplasma* encephalitis. However, the use of this combination is hampered by the frequent occurrence of adverse reactions in HIV-seropositive individuals (Haverkos, 1987; Wanke *et al.*, 1987; Leport *et al.*, 1988; Danneman *et al.*, 1992). Side-effects, especially rash and fever, occur in up to 60% of the AIDS patients treated with a sulphonamide. Pyrimethamine is mostly associated bone marrow depression (Jacobsen *et al.*, 1992, 1994), but this toxicity may be prevented or reversed by the concomitant use of folic acid (Frenkel *et al.*, 1957; DePaillerets *et al.*, 1975). The incidence of these adverse reactions is dose dependent, both for sulphonamides and pyrimethamine (Leport *et al.*, 1988; van der Ven *et al.*, 1991). It is therefore important to select the lowest possible doses for treatment in HIV infected patients.

Sulphadiazine and pyrimethamine exert their effect on two different steps in folic acid metabolism. Sulphonamides function as anti-metabolites for dihydropteroate

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synthetase (DHPS), while pyrimethamine is an inhibitor of dihydrofolate reductase (DHFR) (Hitchings, 1973; Allegra *et al.*, 1990). Trimethoprim is another DHFR inhibitor and the combination with sulphamethoxazole is used in HIV-seropositive patients for the treatment and prophylaxis of *Pneumocystis carinii* pneumonia. This combination also has an anti-*Toxoplasma* effect (Grossman & Remington, 1979; Carr *et al.*, 1992). Trimethoprim is also available in combination with sulphametrole but less is known about this formulation. Sulphametrole has been associated with less toxicity, especially in patients with glucose-6-phosphate dehydrogenase deficiency (Plummer *et al.*, 1983). Unlike DHPS, DHFR is present both in *T. gondii* and mammalian cells, but the affinity of pyrimethamine for *Toxoplasma* DHFR is much higher compared with mammalian DHFR. Mammalian cells possess a carrier-mediated active transport system responsible for the uptake of preformed folates, while *T. gondii* lacks this system (Allegra *et al.*, 1987).

The aim of the present study was to determine the susceptibility of *T. gondii* to DHFR inhibitors and sulphonamides alone and in combination. We also addressed the question to what extent a dose increase of pyrimethamine could lead to a reduction of the sulphonamide dose with similar anti-*Toxoplasma* effect. Since higher dosages of DHFR inhibitor could possibly require the use of folic acid, the influence of folic acid was also studied.

### Materials and methods

The in-vitro tests were performed as described elsewhere (Schoondermark-van de Ven *et al.*, 1995). Briefly, confluent HEp-2 cells were prepared in 96-well tissue culture plates (Costar, Cambridge, MA, USA). At the start of the assay, *T. gondii* parasites of the RH strain were transferred to fresh culture medium with or without antimicrobial agents at a concentration of  $10^5$  parasites per mL. The HEp-2 cells were overlaid with 150  $\mu$ L of the parasite suspension (parasite-to-cell ratio, 0.7), and incubated in air with 5% CO<sub>2</sub> at 90% humidity and 36°C for 90 h. Each test included eight wells without parasites and eight wells with parasites but no drugs. Each drug concentration was tested eight-fold, and each test was performed three times. After incubation, parasite growth was quantified using an enzyme-linked immunosorbent assay, which was performed directly on *T. gondii*-infected HEp-2 cells, as described before (Schoondermark-van de Ven *et al.*, 1995). After incubation with human antiserum against *T. gondii*, horseradish peroxidase-labeled rabbit anti-human immunoglobulin (Dakopatts, Copenhagen, Denmark) was added and the colour reaction with *o*-phenylenediamine (Sigma, St Louis, MO) with perhydrol 30% H<sub>2</sub>O<sub>2</sub> (Merck, Darmstadt, Germany) was quantified in a spectrophotometer.

Pyrimethamine and trimethoprim were obtained from Wellcome (Kent, England), sulphadiazine from OPG (Utrecht, the Netherlands), sulphamethoxazole from Hoffman laRoche (Basel, Switzerland), and sulphametrole from Nycomed (Vienna, Austria).

The 50% inhibitory concentrations (IC<sub>50</sub>) were determined by testing the drugs at the following concentrations: pyrimethamine: 5, 1, 0.5, 0.4, 0.3, 0.2, 0.1, 0.075, 0.05 and 0.01 mg/L; trimethoprim: 100, 50, 20, 15, 10, 5, 2, 1, 0.5 and 0.1 mg/L; and sulphadiazine, sulphamethoxazole and sulphametrole: 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 mg/L. The IC<sub>50</sub> of sulphamethoxazole was also determined in combination with a fixed dose of pyrimethamine and trimethoprim. In these experiments, the concentrations of pyrimethamine were 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.025, 0.05,

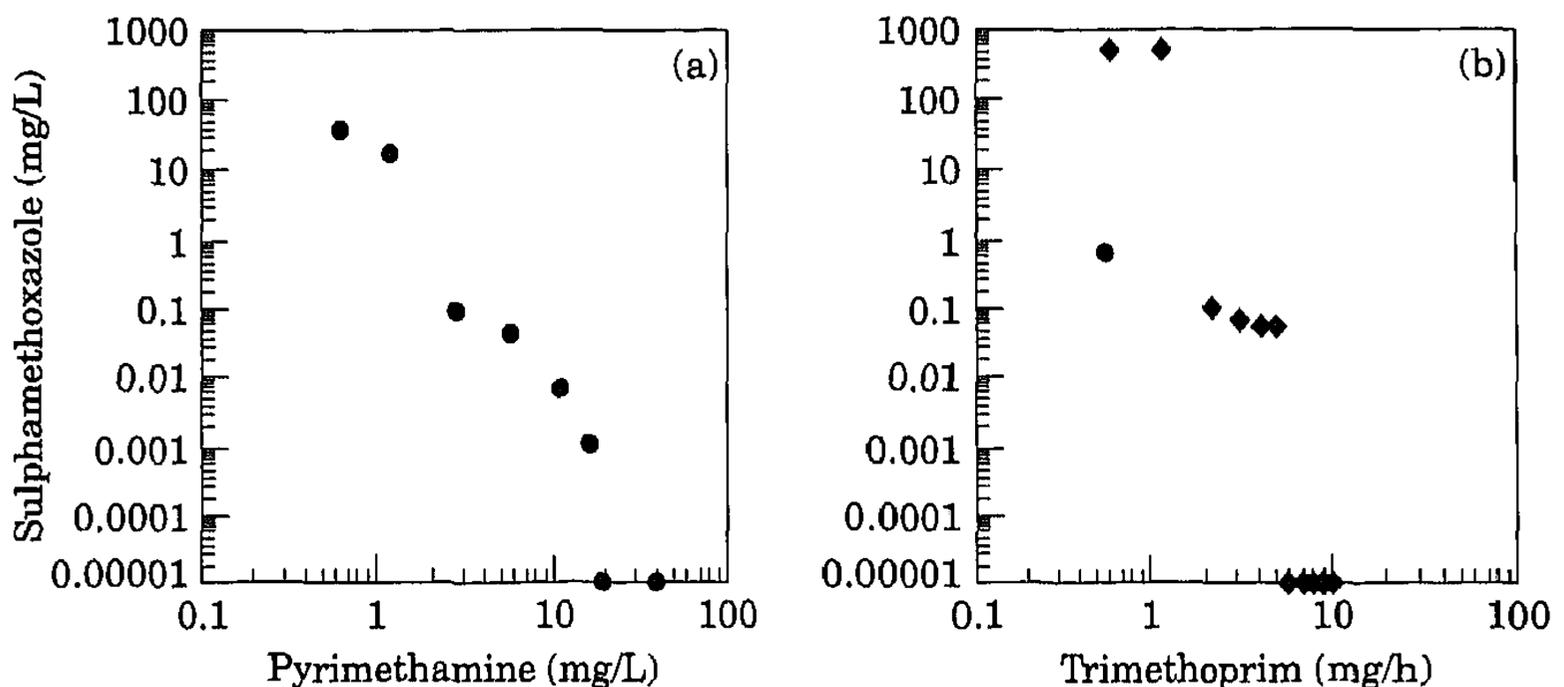
**Table.** In-vitro effect (50% inhibitory concentrations) of different drugs on *T. gondii*

	IC <sub>50</sub> (mg/L)
Trimethoprim	5–10
Pyrimethamine	0.2
Sulphadiazine	600–700
Sulphamethoxazole	400–500
Sulphametrole	600–700

0.1, 0.15, 0.2, 0.4 mg/L, and trimethoprim concentrations were 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 10 mg/L. The corresponding IC<sub>50</sub> of sulphamethoxazole was quantified by testing sulphamethoxazole at the following concentrations: 200, 100, 50, 20, 10, 5, 1, 0.5, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 mg/L. The addition of 0.3 mg/L or 1 mg/L tetrahydrofolate, or 1 mg/L dihydrofolate to the culture medium was also studied for the effects on the IC<sub>50</sub> of pyrimethamine.

### Results

The IC<sub>50</sub> of pyrimethamine, trimethoprim and the three sulphonamides is given in the Table. It was found that pyrimethamine has the most potent anti-*Toxoplasma* activity and trimethoprim was approximately 25–50 times less active *in vitro*. The sulphonamides were about equally active but on a weight basis are 40–3500 times less active compared with the tested DHFR inhibitors. Sulphamethoxazole was used to test the combination with a DHFR inhibitor. Figure (a) presents the IC<sub>50</sub> of sulphamethoxazole in combination with various concentrations of pyrimethamine and trimethoprim, and shows that a ten-fold increment of the concentration of pyrimethamine reduced the IC<sub>50</sub> of sulphamethoxazole 1000 times. Figure (b) demonstrates that the anti-*Toxoplasma* effect of the combination trimethoprim-sulphamethoxazole was not as dose related. The addition of tetrahydrofolate or



**Figure.** IC<sub>50</sub> of sulphamethoxazole for *T. gondii* at different concentrations of pyrimethamine (a) or trimethoprim (b).

dihydrofolate to the culture medium did not change the  $IC_{50}$  for pyrimethamine (data not shown).

### Discussion

All the tested compounds possessed antimicrobial activity against *T. gondii* but the highest in-vitro activity was observed using DHFR inhibitors and in particular pyrimethamine.  $IC_{50}$  and attainable plasma concentrations may, however, only be compared cautiously. The  $IC_{50}$  of pyrimethamine (0.2 mg/L) in this in-vitro system is in the range of plasma concentrations obtained after a once weekly dose of 75 mg pyrimethamine (Opravil *et al.*, 1994). Trimethoprim has a higher  $IC_{50}$  (5–10 mg/L) and concentrations in this range are only reached when patients are treated with the relatively high dose of trimethoprim of 20 mg/kg body weight daily (Hart *et al.*, 1991). The  $IC_{50}$ s of the three sulphonamides are all within the same range and are well above the concentrations found in patients treated with a high dose of sulphonamides of 100 mg/kg body weight daily (Hart *et al.*, 1991).

The  $IC_{50}$  of sulphamethoxazole of 500 mg/L was reduced considerably when a DHFR inhibitor was added to the incubation medium. The addition of 0.005 mg/L pyrimethamine reduced the  $IC_{50}$  of sulphamethoxazole to 50 mg/L, and even to 0.05 mg/L when 0.05 mg/L pyrimethamine was used, indicating strong potentiation. These findings are in accordance with previous in-vitro studies assessing anti-*Toxoplasma* activity of various drug combinations (Harris *et al.*, 1988; Derouin & Chastang, 1989). In these studies a limited amount of concentration combinations of pyrimethamine and sulphadiazine were investigated. By assessing more concentrations, the present study confirms the presence of synergism and shows that a ten-fold increment of the concentration of pyrimethamine reduced the  $IC_{50}$  of sulphamethoxazole 1000 times. Because of the strongly potentiating effect of pyrimethamine, sulphonamides should remain effective at much lower doses than presently recommended. It is likely that dose reduction of sulphonamides will not reduce efficacy but will only reduce toxic side effects. Trimethoprim combined with a sulphonamide is not as potent as a combination with pyrimethamine. This is a reason to be cautious about reducing co-trimoxazole dosages in HIV patients on PCP prophylaxis. The protective effect of 960 mg co-trimoxazole daily is already questionable (Mallolas *et al.*, 1993) and further dose reduction may hamper concomitant *Toxoplasma* protection.

Pyrimethamine serves to trap cellular folates in the non-functional dihydro state. The accumulation of dihydrofolate reduces the effectiveness of the pyrimethamine. The effect of newly formed dihydrofolate is eliminated by the simultaneous application of a sulphonamide and by feedback inhibition of accumulated dihydrofolate on its synthesis (Hitchings, 1973). This may explain why an increment in the concentration of pyrimethamine dramatically reduces the need for a sulphonamide with similar effect on *T. gondii*. The affinity of *Toxoplasma* DHFR for trimethoprim is less compared with pyrimethamine (Chio *et al.*, 1993). This may explain the observation that the anti-*Toxoplasma* effect of the combination trimethoprim-sulphamethoxazole is not as dose dependent.

The present findings confirm the suggestion of other authors (Harris *et al.*, 1988) that the dose of pyrimethamine may be too high for the treatment of *T. gondii* infection. When haematological toxicity occurs during pyrimethamine treatment, concomitant use of folic acid will not reduce the effectiveness of the anti-*Toxoplasma* therapy. This is

in contrast with a report on the treatment of *P. carinii* pneumonia in AIDS patients with trimethoprim-sulphamethoxazole, where adjunctive folinic acid was associated with increased therapeutic failure (Safrin, Lee & Sande, 1994).

At present, the recommended concentrations of sulphonamides, when co-administered with pyrimethamine or trimethoprim, do not account for the potentiating effect of the combination and may therefore be too high. This could lead to unnecessary toxicity in HIV infected patients. However, the present data were obtained by in-vitro studies and their significance for the treatment of AIDS patients needs to be demonstrated. It should be noticed that serum drug concentrations may not be relevant when treating cerebral infections with drugs that differ in their penetration of the blood-brain barrier. Furthermore, the absorption of drugs may be variable, particularly as AIDS patients often have gastrointestinal pathology. Therefore more investigations are needed to validate these results in animals and in carefully designed pharmacokinetic studies in humans.

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