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In Vitro Effects of Sulfadiazine and Its Metabolites Alone and in Combination with Pyrimethamine on *Toxoplasma gondii*

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Sulfadiazine and the metabolites N4-acetyl-sulfadiazine, 4-OH-sulfadiazine, 5-OH-sulfadiazine, 5-OH-glucuronide-sulfadiazine, and 5-OH-sulfate-sulfadiazine were tested separately and in combination with pyrimethamine for the inhibitory activity on *Toxoplasma gondii* growth in vitro. Except for N4-acetyl-sulfadiazine, all sulfa compounds possessed anti-*Toxoplasma* activity. The addition of 0.05 pg of pyrimethamine per ml, a concentration which in itself is not inhibitory, potentiated the microbial activity of sulfadiazine and its metabolites 100 fold.

The combination of sulfadiazine and pyrimethamine is frequently used for the treatment of *Toxoplasma gondii* infections (9, 14). Both drugs act synergistically in the metabolic pathway of folic acid, which in turn is required for the biosynthesis of purines, pyrimidines and certain amino acids (4).

Studies on the antimicrobial activity of sulfadiazine metabolites are scarce. Pharmacokinetic studies of sulfadiazine so far have been performed by the method of Bratton and Marshall (1). This method, however, cannot discriminate between different hydroxylated metabolites of sulfadiazine. By using high-performance liquid chromatography (HPLC), a variety of hydroxy metabolites of sulfadiazine have been discovered in different animal species (11, 12, 19). Recently, HPLC determination of sulfadiazine in the plasma and the urine of rhesus monkeys revealed the presence of five metabolites of sulfadiazine: N4-acetyl-sulfadiazine (N4-acetyl), 4-OH-sulfadiazine (4-OH), 5-OH-sulfadiazine (5-OH), 5-OH-glucuronide-sulfadiazine (5-OH-Gluc), and 5-OH-sulfate-sulfadiazine (5-OH-Sulf), (16a). It was thought previously that rhesus monkeys and humans were similar in their metabolization of sulfadiazine (10). However, the three 5-OH metabolites have not been found in humans.

A previous study with rhesus monkeys on the efficacy of pyrimethamine and sulfadiazine for the treatment of congenital *T. gondii* infection (15) raised the question of whether the metabolites of sulfadiazine possess antiparasitic activity. Nouws et al. (13) demonstrated that a hydroxylated metabolite of sulfadiazine had antimicrobial activity against *Escherichia coli* at 2.5% of the activity of the parent drug, N4-acetyl, on the other hand, showed no antimicrobial activity against this bacterium.

In the present study the anti-*Toxoplasma* activities of sulfadiazine and the metabolites that have been found in rhesus monkeys were investigated in vitro. The activities of these drugs were also studied in combination with pyrimethamine.

Anti-*Toxoplasma* activity was measured by quantitation of parasite growth using an enzyme-linked immunosorbent assay, which was performed directly on *T. gondii*-infected Hep-2 cells. The in vitro tests were performed as described previously by Derouin and Chastang (3). Hep-2 cells were prepared in 96-well tissue culture plates (Costar, Cambridge, Mass.) and grown to confluence. At the start of the assay, *T. gondii* parasites of the RH strain were transferred to fresh culture medium at a concentration of 10⁶ parasites per ml. Culture medium consisted of Dulbecco's modified eagle's medium (Gibco, Breda, The Netherlands) with 10 mM NaHCO₃ and 4.5 g of glucose per ml, supplemented with 5% fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, 0.5 mg of gentamicin per ml, 1 mM sodium pyruvate, and the desired concentrations of drugs to be studied. The Hep-2 cells were overlaid with 150 µl of a parasite suspension (parasite-to-cell ratio, 10:35). The parasites were grown at 5% CO₂, 90% humidity, and a temperature of 35°C for 90 h. Each test included eight wells without parasites (background staining of the Hep-2 cells) and eight wells with parasites but no drugs (positive control). Each drug concentration was tested eightfold, and each test was performed three times. The Hep-2 cells were stained with 0.2% trypan blue and examined microscopically for their viability.

After 90 h of incubation, the cultures were washed twice with wash buffer (136 mM NaCl, 27 mM KCl, 1.4 mM KH₂PO₄, 8 mM Na₂HPO₄·2H₂O, 0.1% phenol red, and 0.05% Tween 20), fixed with cold methanol, and air dried. The cells were incubated with a human antiseraum against *T. gondii* at 37°C for 2 h. The cells were subsequently incubated overnight at 4°C with a horseradish peroxidase-labeled rabbit anti-human immunoglobulin G, 1:10,000 (Dakopatts, Copenhagen, Denmark). The substrate was 100 µl of 4-mg/ml o-phenylenediamine (Sigma, St. Louis, Mo.) with 15-µl/ml perhydroil 30% H₂O₂ (Merck, Darmstadt, Germany). The color reaction was terminated after a 5-min incubation at room temperature with 100 µl of 0.5 M H₂SO₄. One hundred fifty microliters of the substrate was then transferred to a 96-well plate for spectrophotometric analysis at 492 nm. Blank readings were performed on the eight control wells without parasites. Pyrimethamine was obtained from the Wellcome Foundation (Kent, England), sulfadiazine was obtained from the Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands), and N4-acetyl was obtained from Astra (Södertälje, Sweden). The metabolites of sulfadiazine, 4-OH, 5-OH, 5-OH-Gluc, and 5-OH-Sulf were isolated from rhesus monkey urine by preparative HPLC (16a).

5-OH-Gluc and 5-OH-Sulf were dissolved in culture me-

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**FIG. 1.** Structural formulas of sulfadiazine and its metabolites N4-acetyl and hydroxy derivatives.

Sulfadiazine and N4-acetyl were dissolved in culture medium at a concentration of 10 mg/ml by adjusting the pH to 8.4 with 1 M NaOH. When the drugs were completely dissolved, the pH was slowly reduced to 7.5 with 1 M HCl. 5-OH and 4-OH were dissolved in 100% N,N-dimethylformamide at a concentration of 10 mg/ml and subsequently diluted in culture medium. Pyrimethamine was dissolved in methanol-acetone (50:50, vol/vol) at a concentration of 0.1 mg/ml and further diluted in culture medium. Methanol and acetone had no inhibitory effect on *Toxoplasma* growth or toxic effect on the monolayer cells, at the concentrations that were used in the experiments. *N,N*-dimethylformamide, alone or in combination with methanol and acetone, also had no inhibitory or toxic effect at concentrations <1%. *N,N*-dimethylformamide never reached concentrations of more than 0.5%.

Fifty percent inhibitory concentrations (IC50s) were determined for the sulfa compounds both alone and in combination with 0.05 µg of pyrimethamine per ml. At this concentration pyrimethamine alone showed no inhibitory effect. Fifty percent inhibitory concentrations were determined by statistical analysis using a linear regression model as described by Derouin and Chastang (3). In brief, the optical densities (OD) obtained from the experiments were displayed as sigmoid curves when expressed as a function of the logarithms of the drug concentrations. The middle part of the sigmoid curve, showing the increase of inhibition, approximated a linear regression line. This line was adjusted to the basic OD measurements for three successive concentrations. Assuming a constant coefficient of variation, a weighted least-squares method was applied. The mean OD measurements corresponding to a concentration of 0 (noninhibitory concentration) and to a maximum inhibitory concentration (background) served as the limiting values. The IC50 was obtained by inserting the OD value halfway between these limiting values in the equation of the adjusted regression line.

The structures of sulfadiazine and its metabolites which were investigated for their in vitro effects on *T. gondii* growth are shown in Fig. 1. IC50 could not be determined for the sulfa compounds separately, because the maximum concentrations of the drugs that could be dissolved had only a partial or negligible inhibitory effect on parasite growth. Besides this, sulfadiazine and N4-acetyl were toxic for HEp-2 cells at concentrations higher than 1,000 µg/ml. As a consequence, the concentration ranges that were tested did not result in a sigmoid curve. The maximal concentrations that were tested are shown in Table 1.

IC50s were determined for sulfadiazine and its metabolites in combination with a constant concentration of 0.05 µg of pyrimethamine per ml. Except for N4-acetyl, the activities of sulfadiazine and its metabolites were enhanced in the presence of pyrimethamine. The concentrations of the sulfa compounds in combination with pyrimethamine were far below those of the drugs alone (Table 1). The IC50 of pyrimethamine alone was 0.2 ± 0.04 µg/ml. The IC50s of pyrimethamine in combination with 25 and 100 µg of the parental drug, sulfadiazine, were 0.02 ± 0.011 and 0.005 ± 0.003 µg/ml, respectively. Concentrations of 25 and 100 µg of sulfadiazine per ml are far below the inhibiting concentration of sulfadiazine alone (Table
TABLE 1. Inhibitory effects of sulfadiazine and its metabolites alone and in combination with pyrimethamine on the growth of *T. gondii*

<table>
<thead>
<tr>
<th>Compound (g)</th>
<th>Without pyrimethamine*</th>
<th>With pyrimethamine (0.05 μg/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfadiazine (250)</td>
<td>&gt;1,000 b</td>
<td>0.5 ± 0.25</td>
</tr>
<tr>
<td>N4-acetyl (292)</td>
<td>&gt;1,000 b</td>
<td>0.5 ± 0.25</td>
</tr>
<tr>
<td>4-OH (267)</td>
<td>&gt;150°</td>
<td>0.6 ± 0.18</td>
</tr>
<tr>
<td>5-OH (267)</td>
<td>&gt;500°</td>
<td>0.5 ± 0.09</td>
</tr>
<tr>
<td>5-OH-Gluc (442)</td>
<td>&gt;2,500°</td>
<td>70 ± 0.32</td>
</tr>
<tr>
<td>5-OH-Sulf (346)</td>
<td>&gt;250°</td>
<td>40 ± 0.34</td>
</tr>
</tbody>
</table>

* The data are means ± standard errors for three experiments.

b *IC₅₀ not determined because the drug was toxic for HeP-2 cells at higher concentrations.

c *IC₅₀ not determined because the maximum solubility of the drug was reached.

d *IC₅₀ not determined because no more drugs were available.

1), but enhanced activity was found when these concentrations were tested in combination with pyrimethamine.

Metabolites of sulfadiazine which possess a free *para*-amino-phenyl group are able to interfere in the folinic acid synthesis of *T. gondii*. The hydroxylated metabolites 4-OH and 5-OH have anti-*Toxoplasma* activities similar to that of the parental drug when used in combination with a concentration of pyrimethamine that is by itself not inhibitory. The metabolites 5-OH-Gluc and 5-OH-Sulf have lower anti-*Toxoplasma* activities. On the molar level, both components are 100 to 150 times less active than the parental drug. The extent of antimicrobial activity depends on the penetration of the metabolite into the parasitic cell, as has also been suggested for *E. coli* (13). 5-OH-Gluc and 5-OH-Sulf both dissolved very well in the culture medium. Conjugation of the OH group increases the hydrophilic character of the metabolite and results in an enhanced excretion from the body in vivo. The hydrophilic character may cause poor penetration through the lipophilic parasitic membrane. The N4-acetyl metabolite possesses no free *para*-amino-phenyl group and is therefore unable to interact with the folinic acid synthesis.

Sulfadiazine was found to inhibit *T. gondii* growth in vitro only at high concentrations. This finding is in agreement with those of other in vitro studies (7, 8, 16) but contrasts with the results of Derouin and Chastang (3). Since the assay we have used in this study is similar to the assay as described by Derouin and Chastang (3), the discrepancy is difficult to explain. Genetic differences have been found between RH strains from different laboratories (6). A variation in sensitivity to antimicrobial agents between different *Toxoplasma* RH strains cannot be excluded. Another explanation may be the 10-fold-higher concentration of parasites used in our study. When sulfadiazine or its metabolites were combined with pyrimethamine, enhanced activity was found for all the combinations tested, except for N4-acetyl. Again, the *IC₅₀* we have found were higher than those reported by Derouin and Chastang (3). On the other hand, the results are in good agreement with the in vitro results reported by others, with regard to the parental drug (2, 5, 8). It is interesting that the *IC₅₀* we have found correspond to the plasma drug concentrations which are effective in vivo (20).

This study shows that hydroxylated metabolites of sulfadiazine possess antimicrobial activity against *T. gondii*. The extent of hydroxylation depends on the type of species, age, and dose (11, 12, 17-19). The presence of antimicrobially active metabolites may have consequences for the efficacy of treatment when these metabolites are present in high concentrations or when effective concentrations in plasma are reached. Therefore, extrapolation from animal studies to the human situation concerning the pharmacokinetics and efficacy of sulfadiazine should be done with care.

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REFERENCES


