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Activity of Epiroprim (Ro 11-8958), a Dihydrofolate Reductase Inhibitor, Alone and in Combination with Dapsone against *Toxoplasma gondii*

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We examined the effect of epiroprim (Ro 11-8958), a dihydrofolate reductase inhibitor, alone and in combination with dapsone, against *Toxoplasma gondii*. In vitro, the anti-*T. gondii* effects of epiroprim and dapsone were observed at nanogram-per-milliliter levels when a 72-h uracil assay and an infection rate of one parasite per 120 macrophages were used. In combination, these drugs exerted a synergistic effect that, however, was only parasitostatic. In a model of acute infection, mice were infected intraperitoneally with 10^4 parasites of the RH strain of *T. gondii* and were treated for 14 days by gavage (therapy divided into two daily dosages), starting 24 h after infection. Used alone, dapsone and epiroprim, each at a dose of 50 mg/kg of body weight per day, protected 10 and 0% of the mice, respectively. When these drugs were administered simultaneously, a 100% survival rate was observed. Pyrimethamine-sulfadiazine (4 and 250 mg/kg/day, respectively) protected 100% of the mice. A 3-week therapy of chronically infected mice with either epiroprim (50 mg/kg/day), dapsone (50 mg/kg/day), or pyrimethamine (15 mg/kg/day) reduced the numbers of *T. gondii* cysts and the inflammation in their brains. A combination of epiroprim and dapsone, both at 50 mg/kg/day, further reduced the number of brain cysts in comparison with the number after the corresponding monotherapies. Epiroprim may have a role in the prophylaxis or therapy of human toxoplasmosis, especially when combined with other drugs active against *T. gondii*, such as dapsone.

The most frequent cause of focal brain disease in patients with AIDS is *Toxoplasma* encephalitis (16). In the absence of specific therapy *Toxoplasma* encephalitis invariably worsens. The current therapy of choice for most forms of toxoplasmosis, including encephalitis in patients with AIDS, is the synergistic combination of pyrimethamine and sulfadiazine (11). Patients with AIDS suffering from *Toxoplasma* encephalitis are treated life-long with pyrimethamine plus sulfadiazine in order to avoid progression of the disease (14, 17). This combination, however, while being very effective, produces a number of adverse effects mainly linked to the toxicity of sulfadiazine. In such cases therapy with the combination is discontinued and an alternative regimen is given. Possible alternative therapies which have been proposed or used include high-dose pyrimethamine alone, clindamycin plus pyrimethamine, azithromycin or clarithromycin with pyrimethamine, doxycycline, and atovaquone (1–3, 7, 8, 10, 13). There is a general consensus that better-tolerated and effective drugs or combinations of drugs are urgently needed for the treatment and prophylaxis of *Toxoplasma* infections, particularly *Toxoplasma* encephalitis, in immunocompromised patients. Epiroprim is a dihydrofolate reductase inhibitor and an analog of trimethoprim with improved pharmacokinetic properties and an improved antimicrobial spectrum (22). In the studies described here we assessed the effects of epiroprim and dapsone, alone and in combination, against *Toxoplasma gondii* by using in vitro and in vivo models of infection.

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MATERIALS AND METHODS

Drugs. Epiroprim (formerly Ro 11-8958; Fig. 1), sulfadiazine, and pyrimethamine were synthesized at F. Hoffmann-La Roche Ltd. Dapsone was obtained from Sigma Chemical Co. (St. Louis, Mo.). For in vitro studies, the drugs were dissolved in dimethyl sulfoxide or ethanol and were diluted in sterile distilled water and then in culture medium. For in vivo studies, the drugs were suspended in sterile distilled water with ethanol (5%) and Tween 20 (0.1%).

Animals. Female Swiss-Webster mice (weight 25 ± 1 g; BRL, Füllinsdorf, Switzerland) were used.

Parasites. The virulent RH strain of *T. gondii*, maintained as described previously (6), was used for in vitro studies and in the model of lethal acute toxoplasmosis. To establish chronic toxoplasmosis, tissue cysts were obtained from the brains of mice infected with the Me49 strain as described previously (4).

In vitro studies. (i) [^{3}H]uracil assay. Two assays were used. In the first assay, the 24-h [^{3}H]uracil assay was performed as described previously (5). Briefly, 3×10^5 adherent murine resident macrophage monolayers were challenged with a suspension of 2.5×10^5 tachyzoites of *T. gondii* in medium 199 (Seromed, Mannheim, Germany) containing 3% heat-inactivated (60 min, 56°C) fetal calf serum for 1 h. The cell monolayers were then washed, treated with suspensions of antimicrobial agents, pulsed with 2.5 μl of [5,6-^{3}H]uracil (Amersham), and reincubated for 24 h. The incorporation of [^{3}H]uracil into acid-precipitable material was then assessed as

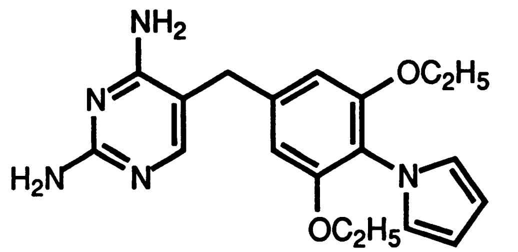

FIG. 1. Structural formula of epiroprim (Ro 11-8958).

a measure of intracellular parasitic multiplication (21). The protocol for the second assay was basically the same as that described above, but with modifications in the parasite inoculum (2.5×10^3), incubation period (72 h), and time of pulsing (8 h) prior to terminating the experiments. The potential toxicities of the antimicrobial agents to the host cells were assessed by the trypan blue dye exclusion test.

(ii) Light microscopy. Light microscopy was performed basically as described previously (5). Two protocols were used, with cell and parasite concentrations being similar to those used for the [^{3}H]uracil assay, and eight-chamber slides (Lab-Tek; Miles Scientific, Division of Miles Laboratories, Inc., Naperville, Ill.) were used. The percentage of infected cells and the number of intracellular parasites were determined by microscopic examination (5). The morphologies of the parasites in treated monolayers were compared with those of untreated control parasites.

In vivo studies. (i) Lethal acute toxoplasmosis. Mice were infected intraperitoneally with 10^4 tachyzoites of *T. gondii* RH in 0.5 ml of sterile 0.9% NaCl. Therapy was given by gavage twice daily (every 12 h), starting 24 h after infection and continuing for 14 days. Mortality was recorded daily for 30 days, and the cure rates of the survivors were assessed by subinoculation of brain material as described previously (3).

(ii) Chronic toxoplasmosis. Mice were infected with 10 cysts of the Me49 strain and were used at 2 months after challenge. Treatment was given by gavage once daily for 21 days, after which the mice were sacrificed and the brains were used for cyst counts and histological examination (4). Eight coronal sections were examined per brain, and the examinations were done in a blinded manner. Quantitation of the inflammatory response was done by using semiquantitative scores, as follows: 0, absence of cysts and absence of inflammation and/or necrosis; 1, presence of cysts, slight to moderate inflammation, but no foci of necrosis; 2, presence of cysts, moderate inflammation, and few foci of necrosis; 3, presence of cysts and a high level of inflammation and/or necrosis.

Levels of epiroprim in mouse serum. Groups of three normal fasting (1 h) mice were given a single dose of 100 mg of epiroprim per kg of body weight by gavage; blood samples were collected 30, 75, 120, and 240 min later. The concentrations of epiroprim in serum were assessed by a microbiological assay (24).

Statistical analysis. Data are expressed as means ± standard errors of the mean unless stated otherwise. For in vitro studies the 50 and 90% inhibitory concentrations (IC_{50}s and IC_{90}s, respectively) and the 90% confidence limits were calculated by probit analysis (15). For other analyses, the Fisher exact or Mann-Whitney U test was used. A *P* value of <0.05 was considered significant.

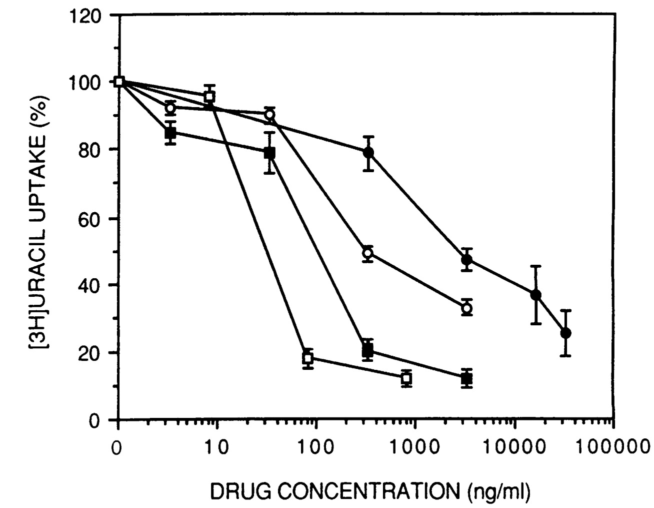

FIG. 2. Effects of various concentrations of drugs on the growth of *T. gondii* as measured by the uptake of [^{3}H]uracil by a 72-h assay. Pyrimethamine (□), epiroprim (■), dapsone (○), and sulfadiazine (●) were tested. Each datum point is the average ± standard error of the mean of two independent experiments, each based on the [^{3}H] uracil content in eight wells. The 100% values are those determined by the labeling of infected cultures incubated without drugs.

RESULTS

In vitro studies. Initial studies by the 24-h [^{3}H]uracil assay suggested that epiroprim had a significant effect on the intracellular multiplication of *T. gondii* at concentrations of about 25 μg/ml. However, studies aimed at assessing the potential toxicity for the host cells showed that this effect of epiroprim was accompanied by a toxic effect on the host cells (data not shown). We then switched to a modified 72-h in vitro assay in which we used a lower parasite inoculum (20). All compounds tested showed an inhibitory effect on the incorporation of [^{3}H]uracil by the intracellular parasites (Fig. 2). This allowed the calculation of their respective IC_{50}s and IC_{90}s (Table 1). No toxicities of the compounds to the host cells were observed at the concentrations tested, as assessed by the trypan blue dye exclusion test (data not shown).

To assess any potential synergistic effect of epiroprim with the other drugs tested, we performed experiments in which noninhibitory concentrations (when used alone) of all drugs were used. These experiments showed that equal concentrations of epiroprim (66 ng/ml) and dapsone (66 ng/ml) acted synergistically to inhibit intracellular *T. gondii* in comparison with the results for untreated infected controls ($P < 0.02$). Pyrimethamine (8.3 ng/ml) in combination with sulfadiazine (333 ng/ml) exerted the strongest inhibitory effect after 72 h of incubation (Fig. 3).

Light microscopy studies showed that epiroprim, sulfadiazine, dapsone, and pyrimethamine reduced the number of

TABLE 1. IC_{50}s and IC_{90}s by [^{3}H]uracil incorporation assay

Drug	IC_{50} (ng/ml) (95% fiducial range)	IC_{90} (ng/ml)
Pyrimethamine	34 (22.6–51)	100
Epiroprim	105 (5–210)	620
Dapsone	300 (60–1,500)	34,000
Sulfadiazine	2,500 (91–68,625)	90,000

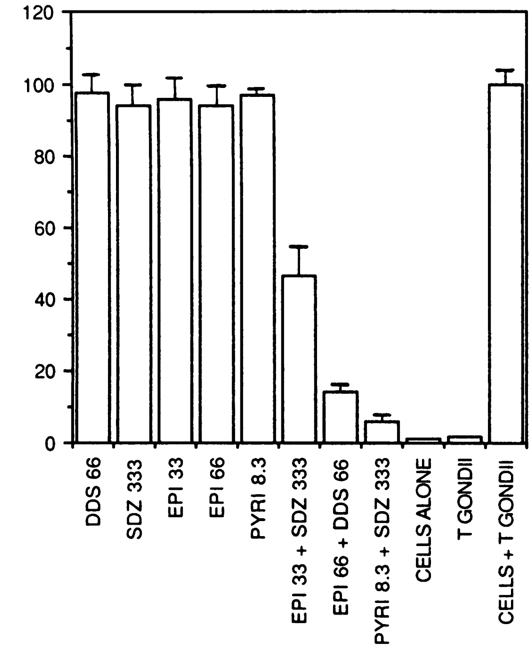

FIG. 3. Synergistic effect of epiroprim and dapsone by a 72-h [^{3}H]uracil assay. Dapsone (DDS), sulfadiazine (SDZ), pyrimethamine (PYRI), and epiroprim (EPI) were tested. Drug concentrations are given in nanograms per milliliter.

infected cells and the number of intracellular parasites. However, those studies, in which drug concentrations were the same as those used in the [^{3}H]uracil assay, suggested that epiroprim, sulfadiazine, and dapsone have parasitostatic rather than parasiticidal effects because parasite morphology was not distorted, whereas pyrimethamine alone produced a marked distortion of parasite morphology, suggesting a cidal effect.

Acute lethal toxoplasmosis. Administration of epiroprim alone at doses of up to 100 mg/kg/day for 14 days did not protect mice against lethal infection with *T. gondii* (Table 2). Dapsone alone at dosages of 25, 50, and 100 mg/kg/day protected 0, 10, and 100% of mice, respectively. No side effects or toxicities were observed in mice as a result of treatment with the high doses of dapsone (100 mg/kg/day). Complete protection against lethality was observed when ineffective doses of epiroprim and dapsone (50 mg/kg/day each) were given in combination to the infected mice ($P < 0.02$ in comparison with untreated controls). An enhancement of the cure rate was observed when the highest doses (100 mg/kg/day) of epiroprim and dapsone were given to the mice (Table 2). Pyrimethamine (4 mg/kg/day) in combination with sulfadiazine (250 mg/kg/day) protected 100% of the mice, and with a 100% cure rate.

Chronic toxoplasmosis. After 3 weeks of drug administration, mice were sacrificed and their brains were taken to count the number of cysts and for histological evaluation of inflammation. The cyst counts were as follows: dapsone (50 mg/kg/day), 509 ± 128 (mean ± standard deviation of cysts for five brains); epiroprim (50 mg/kg/day), 564 ± 73; pyrimethamine (15 mg/kg/day), 253 ± 42; dapsone plus epiroprim (each at 50 mg/kg/day), 222 ± 27; infected untreated controls, 838 ± 89. These results showed that administration of dapsone and epiroprim alone reduced the number of brain cysts ($P < 0.05$ in comparison with the numbers in controls). Administration of pyrimethamine alone and a combination of dapsone and epiroprim produced a marked reduction in the number of brain cysts ($P < 0.05$ in comparison with the results after treatment with dapsone and epiroprim alone). All treatments were able to reduce inflammation. However, inflammation was not further reduced after administration of the combination of dapsone and epiroprim in comparison with the inflammation after administration of any of the drugs alone (Fig. 4). The results of the scoring for the different groups of mice were as follows (means for five brains): uninfected untreated controls, 0; epiroprim, 1; dapsone, 1; pyrimethamine, 1; epiroprim plus dapsone, 1; infected untreated controls, 3.

Levels of epiroprim in serum. Administration of a single dose of 100 mg of epiroprim per kg produced a peak level in serum after 30 min of administration, and levels were still detectable 240 min after administration (Fig. 5).

TABLE 2. Effects of epiroprim and dapsone alone or in combination on acute toxoplasmosis in mice

Drug and dosage (mg/kg/day)[a]	Mean time (days) to death for 50% of mice	No. of survivors/no. of treated mice	% Survivors cured
Epiroprim			
25	8	0/10	
50	8	0/10	
100	8	0/10	
Dapsone			
25	7	0/10	
50	9	1/10	0
100		10/10	0
Epiroprim-dapsone			
25 and 25	10	0/10	
50 and 50		10/10	30
100 and 50		10/10	20
100 and 100		10/10	50
Pyrimethamine-sulfadiazine, 4 and 250		10/10	100
Controls	7	0/50	

[a] Treatment was for 14 days.

DISCUSSION

The results of our in vitro studies, using a 72-h assay, demonstrate the inhibitory effects of sulfadiazine, epiroprim, dapsone, and pyrimethamine on the intracellular multiplication of *T. gondii* when the drugs are used at very low concentrations. These observations are in agreement with previous reports of Pfefferkorn et al. (19) and Derouin and Chastang (9), who have demonstrated that sulfadiazine, dapsone, and pyrimethamine have inhibitory effects similar to those described here. In addition, our observations suggest a synergistic effect of epiroprim and dapsone on the intracellular growth of *T. gondii*, because ineffective concentrations of both drugs inhibited parasite multiplication when they were added simultaneously.

The reason why epiroprim, sulfadiazine, and dapsone have substantial effects on parasite multiplication only after a certain delay in incubation (72 h) is unclear. However, as pointed out by Pfefferkorn et al. (19), in the case of sulfadiazine and dapsone, the mechanism may depend on the assumption that the final effects of these drugs are to inhibit the synthesis of dihydrofolic acid in the parasites. Thus, assuming that the intracellular parasites may contain a large supply of dihydrofolic acid, inhibition of the synthesis of dihydrofolic acid would

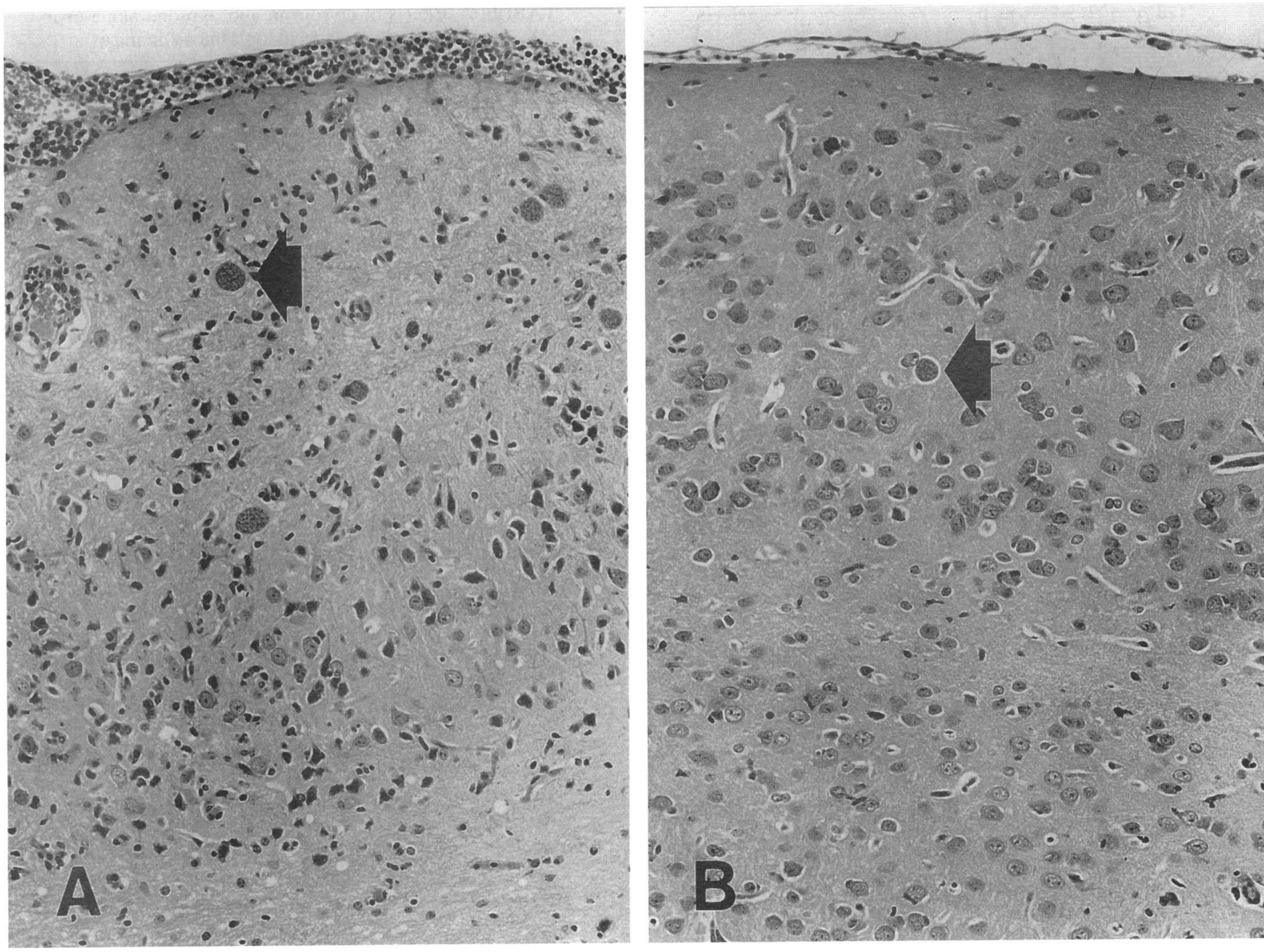

FIG. 4. (A) Section of a brain from an infected untreated mouse at 11 weeks after infection. A high number of inflammatory cells in the meninges and parenchyma as well as perivascular cuffing and numerous cysts (arrow) of *T. gondii* can be seen. (B) Significant reduction in the inflammatory response and the presence of only a few *T. gondii* cysts (arrow) in the brain of a mouse treated for 21 days with epiroprim (50 mg/kg/day) plus dapsone (50 mg/kg/day). Hematoxylin-eosin staining was used. Magnification, ×150.

not have an immediate effect on them. However, their supply would be depleted once several rounds of multiplication had taken place. Once the amount of available dihydrofolic acid is reduced, parasitic multiplication slows down and finally stops. A similar mechanism might account for the effect of epiroprim, which inhibits the reduction of dihydro- to tetrahydrofolic acid rather than the synthesis of dihydrofolic acid, which sulfadiazine and dapsone inhibit.

Epiroprim and dapsone were able to inhibit the intracellular growth of the parasites at concentrations that are far lower than those achievable in blood. However, only dapsone was effective in the model of acute lethal toxoplasmosis when it was administered alone. The results obtained with the combination of epiroprim and dapsone, therefore, suggest that epiroprim acts essentially to potentiate the effect of dapsone by providing a sequential blockade of the folic acid pathway. The data obtained with the chronic model of toxoplasmosis further point to a synergistic effect of the combination, as evidenced by the marked reduction in the number of *Toxoplasma* brain cysts.

Epiroprim appears to have a favorable pharmacokinetic profile in dogs (i.e., a larger volume of distribution and a longer half-life) in comparison with that of trimethoprim (22), and we extend these observations to mice, in which we were able to detect levels of epiroprim in serum even 6 h after administration of a single dose of 100 mg/kg. For its part, dapsone has a long half-life in humans, with levels detectable in blood even 24 h after a administration of a single oral dose (25). If pharmacokinetic studies confirm the expectation that epiroprim also has a long half-life in humans, perhaps a major clinical use of the combination dapsone-epiroprim would be the prophylaxis of *Toxoplasma* encephalitis in patients with AIDS, which would allow an intermittent dosing schedule. Furthermore, the synergistic activity of dapsone and epiroprim in vivo is relevant to the clinical situation, because dapsone has been used as part of a regimen for the prophylaxis of *T. gondii* encephalitis and *Pneumocystis carinii* pneumonia and for the therapy of *P. carinii* pneumonia (12, 18). Considering the fact that epiroprim-dapsone has also been found to be synergistic in a rat model of *P. carinii* pneumonia (23), this combination may well be a useful addition in the armamentarium for the prophylaxis of both *T. gondii* encephalitis and *P. carinii* pneumonia. However, such assumptions should await the results of further studies.

Taken together, results of our studies indicate that epiroprim may have a role in the prophylaxis or therapy of human toxoplasmosis, especially when it is combined with other drugs

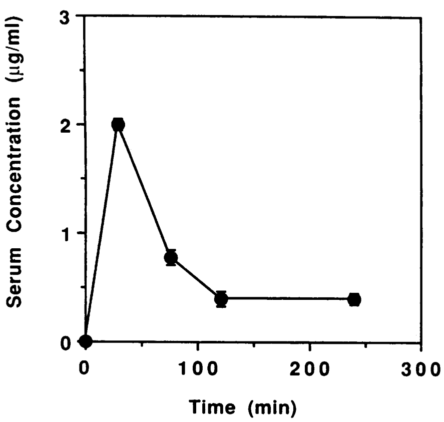

FIG. 5. Levels of epiroprim in the sera of mice after administration of a single 100-mg/kg oral dose.

active against *T. gondii*, such as dapsone. The results of future work on the ability of humans to tolerate this compound may influence its potential therapeutic use in humans.

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