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Combination of PS-15, Epiroprim, or Pyrimethamine with Dapsone in Prophylaxis of *Toxoplasma gondii* and *Pneumocystis carinii* Dual Infection in a Rat Model

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In a rat model of dual infection, we studied such dihydrofolate reductase (DHFR) inhibitors as PS-15 (25 mg/kg of body weight), epiroprim (100 mg/kg), and pyrimethamine (3 mg/kg) alone or in combination with various doses of dapsone (50, 25, or 5 mg/kg) for the prevention of pneumocystosis and toxoplasmosis. Rats latently infected with *Pneumocystis carinii* were immunosuppressed by corticosteroids for 7 weeks, and the drugs were administered from the initiation of the corticosteroid treatment. At week 5, the rats were inoculated intraperitoneally with the RH strain of *Toxoplasma gondii*. Infections were monitored by the counting of *P. carinii* cysts in lung homogenates and the titration of *T. gondii* in organs by quantitative culture and an indirect immunofluorescence assay. Fourteen of the 15 untreated rats died after *T. gondii* challenge, with *P. carinii* infection in the lungs and *T. gondii* infection in the lungs, liver, spleen, and brain. Of the three tested DHFR inhibitors, only PS-15 exhibited anti-*P. carinii* activity; none prevented toxoplasmosis in 100% of the rats. After the DHFR inhibitors were combined with dapsone (50 or 25 mg/kg), both pneumocystosis and toxoplasmosis were completely prevented. On the basis of these results, PS-15 and epiroprim combined with dapsone are candidates for use for the prevention of both pneumocystosis and toxoplasmosis.

Despite the marked reductions in the incidence of pneumocystosis and toxoplasmosis in human immunodeficiency virus (HIV)-infected patients following primary prophylaxis and maintenance therapy (14), both infections are still highly prevalent, especially in Europe, where *Toxoplasma gondii* seroprevalence is high (65 to 70% of the general population); the arsenal of drugs remains limited. Case control studies (5) and controlled clinical trials (10) have demonstrated the high incidence of toxoplasmosis in HIV-infected patients latently infected with *T. gondii* who receive an anti-*Pneumocystis carinii* selective prophylaxis. Trimethoprim-sulfamethoxazole (TMP-SMX) is the first-choice drug, but side effects lead to a high frequency of discontinuation and so hamper its prescription (25). A substantial number of HIV-infected patients cannot tolerate long-term use of this combination, although the mechanisms of drug intolerance are poorly understood in this population (1, 4, 12, 23). The respective responsibilities of TMP and SMX in the occurrence of side effects are unclear, although SMX seems to predominate as a cause of side effects.

There is clearly a need to develop new, effective combinations against both infections. Several experimental studies performed in vitro and in vivo have shown that PS-15, a new biguanide, and epiroprim (EPI), an analog of TMP, were effective against both *P. carinii* (7, 8, 15, 26) and *T. gondii* (6, 20) and that they might be good candidates for alternative therapy or prophylaxis. However, these results were obtained in experimental models of single infection and did not take account of the frequent association of these infections in AIDS patients. We have previously developed a rat model of dual infection with *P. carinii* and *T. gondii* (2) in which TMP combined with SMX and pyrimethamine (PYR) combined with dapsone (DAP) were shown to be effective prophylaxis against both pneumocystosis and toxoplasmosis. The aim of the present study was to determine whether other dihydrofolate reductase (DHFR) inhibitors, such as PS-15, EPI, and PYR, might also act against pneumocystosis and toxoplasmosis in this model of dual infection. We decided to evaluate these drugs both alone and in combination with DAP, because clinical evaluation of DAP has demonstrated its efficacy against pneumocystosis and improved tolerance of DAP by HIV-infected patients compared with that of TMP-SMX (17).

(This work has previously been presented in part [1a, 2a].)

MATERIALS AND METHODS

This study was carried out in accordance with prevailing regulations regarding the care and use of laboratory animals in the European Communities (Journal Officiel des Communautées Européennes, 18 Décembre 1986, L358).

Experimental design. The animal protocol that we used has been described in detail elsewhere (2). *P. carinii* infection was induced in Wistar rats weighing about 200 g (Janvier Breeding Laboratories, Le Genest St Isle, France) by subjecting them to an immunosuppressive regimen (25 mg of cortisone acetate [Hydrocortisone] [Hoechst-Roussel, Paris, France] injected subcutaneously twice weekly) and a low-protein-level (8%) diet (Usine Alimentation Rationelle, Villemoisson, France).

In each set of experiments, a group of rats was used to assess the development of *P. carinii* infection. Five rats were examined at the beginning of the study and after 5 weeks and 7 weeks of immunosuppression.

Rats, for purposes of prophylaxis, were treated with DHFR inhibitors and DAP from the initiation of the corticosteroid treatment. Other rats received no therapy and were the control group; they were inoculated intraperitoneally with 10^7 tachyzoites of the virulent RH strain of *T. gondii* after 5 weeks of immunosuppression. All rats were sacrificed after 7 weeks of corticosteroid treatment.

Assessment of *P. carinii* and *T. gondii* infections. The procedures have been reported in detail in our earlier study (2). Briefly, *P. carinii* cysts were counted in lung tissue after enzymatic digestion and toluidine blue O staining (11). The number of cysts per gram of lung was expressed as a mean log value ± 1 standard deviation. *T. gondii* infection was assessed by determining parasite burdens in the brain, liver, lungs, spleen, and pleural fluid, with a tissue culture method and an indirect immunofluorescence assay being used as previously described (22).

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TABLE 1. Prophylactic activity of PYR alone or combined with DAP against toxoplasmosis and pneumocystosis[a]

Drug(s) and amt(s)	Outcome after *T. gondii* inoculation	Day	No. of rats	Pleural fluid vol (ml)	*T. gondii* count (log_{10})					*P. carinii* count (log_{10}) in lungs
					Pleural fluid	Lung	Brain	Spleen	Liver	
PYR (3 mg/kg)	Sacrifice	14	5[b]	0		1.5 ± 1.2	0	0.3 ± 0.7	0.9 ± 1.8	6.5 ± 0.5
DAP (50 mg/kg)	Sacrifice	14	5[c]	0.1 ± 0.2	0	0.8 ± 1.6[d]	1.2 ± 1.6	1.6 ± 1.2	4.9 ± 1.2[d]	2.4 ± 0.5[d]
PYR (3 mg/kg) and DAP (50 mg/kg)	Sacrifice	14	10	0		0[d]	0	0	0	2.3 ± 0.7[d]
PYR (3 mg/kg) and DAP (25 mg/kg)	Death	7	2	0		1.5 ± 2.1	0.7 ± 1.0	0	0	2
	Sacrifice	14	8	0		0[d]	0	0	0	2.4 ± 0.4[d]
PYR (3 mg/kg) and DAP (5 mg/kg)	Death	4.3 ± 2.3	3	0		0	0	0	0	3.8 ± 0.8
	Sacrifice	14	12	0.6 ± 2.3	0	0[d]	0	0	0.6 ± 1.4	4.0 ± 1.1[d,e]

[a] Drugs were given per os 5 days per week for 5 weeks and daily after *T. gondii* inoculation (day 0 began at week 5) until death or sacrifice.
[b] One rat with high levels of *T. gondii* and *P. carinii* died at day 4.
[c] One rat died at day 12. The *P. carinii* cyst level was log 2.7/g of lung, and the *T. gondii* burden was log 5.3/g of liver.
[d] $P < 0.001$ versus values for the PYR-treated group. (For statistical analysis by Bonferroni's adjusted *t* test, the values for the dead and sacrificed rats were pooled.)
[e] $P < 0.001$ versus values for the other three treated groups (DAP alone and DAP at two different doses with PYR).

When the absence of parasitic foci was recorded, the *T. gondii* burden was indicated as zero. The parasitic burden for each organ was expressed as a mean log value ± 1 standard deviation.

Drugs. PYR and DAP were purchased from Rhône-Poulenc-Rorer, Antony, France. DAP was an iron-free pure formulation. PS-15 was supplied by D. P. Jacobus (Jacobus Pharmaceutical Co. Inc., Princeton, N.J.) EPI was supplied by R. L. Then and P. G. Hartman (F. Hoffmann-La Roche, Ltd., Basel, Switzerland). All compounds were prepared in 1% carboxymethyl cellulose in sterile water, sonicated, and administered by oral gavage.

Prophylactic treatment. Twenty-five rats (five rats per group) were treated for purposes of prophylaxis with DAP (50 mg/kg of body weight) or with any one of such DHFR inhibitors as PYR (3 mg/kg), PS-15 (25 or 50 mg/kg), and EPI (100 mg/kg).

Seventy rats were treated for purposes of prophylaxis with DHFR inhibitors combined with DAP (50, 25, or 5 mg/kg). Such combinations included PYR and DAP (35 rats), PS-15 and DAP (20 rats), and EPI and DAP (15 rats).

The different doses were selected with previous studies of treatment for infections with *P. carinii*, *T. gondii*, or both pathogens being taken into account as references (2, 6, 15, 26). Drugs were administered from the beginning of corticosteroid administration for 5 days a week for 5 weeks and then for every day after *T. gondii* inoculation until death or sacrifice. The following study was done in three experiments, and data were pooled.

Statistical analysis. *P. carinii* cysts and *T. gondii* parasitic burdens were analyzed by one-way analysis of variance, and pairs of groups were compared with Bonferroni's adjusted *t* test being used. Differences in values were considered significant if the *P* value was ≤0.05 or 0.05 divided by the number of tests made. Data were expressed as means ± 1 standard deviation.

RESULTS

Efficacies in the various experiments, which included untreated controls and a reference treatment (PYR and DAP), were evaluated. Since *P. carinii* and *T. gondii* titers were similar for the control and reference-treated rats from each single experiment, data from the three experiments were pooled in order to facilitate presentation and interpretation of the results.

Pneumocystosis development. At the beginning of the study, the mean count of *P. carinii* cysts was log 3.3 ± 0.3 per g of lung, reflecting the usual latent infection observed in *P. carinii*-exposed rats. After 5 and 7 weeks of corticosteroid treatment, the mean values were, respectively, 6.6 ± 0.6 and 7.2 ± 0.3 cysts per g.

***P. carinii* and *T. gondii* infections in immunosuppressed rats.** Fourteen of the 15 rats that were challenged with *T. gondii* after 5 weeks of immunosuppression died 4.0 ± 0.5 days after inoculation. (One rat survived and was sacrificed at day 14 post-*T. gondii* infection. The *P. carinii* cyst level was log 7.2 per g of lung, and the *T. gondii* burden was log 1.7 per g of lung.) The mean pleural fluid effusion was 5.0 ± 1.5 ml, with a *T. gondii* count of log 3.4 ± 1.4. *T. gondii* was found in the organs of all of the rats, with a mean parasitic burden of log 5.0 ± 0.9 in the lungs, 2.7 ± 1.4 in the brain, 4.9 ± 0.9 in the spleen, and 5.8 ± 1.4 in the liver being found. The mean numbers of *P. carinii* were not significantly different from cyst counts in immunosuppressed rats which had not been challenged with *T. gondii* (log 6.6 ± 0.5 in the lungs).

Efficacy of prophylactic regimens in coinfected immunosuppressed rats. Results for PYR (3 mg/kg) and DAP (50 mg/kg) alone and for combinations of PYR (3 mg/kg) and DAP (50, 25, and 5 mg/kg) are shown in Table 1. When PYR was administered alone, it was not effective against pneumocystosis, but *T. gondii* parasitic burdens were reduced compared with those for untreated controls. DAP alone was effective in preventing pneumocystosis, with parasitic burdens being maintained at the baseline value, but was only partially effective against *T. gondii*, producing a significant reduction of infection in pleural fluid and tissues. DAP and PYR combinations were effective against both infections, except at the lower dose of DAP (5 mg/kg), which did not completely prevent pneumocystosis ($P < 0.001$ versus values for the other combinations).

Results for PS-15 alone at two doses (25 and 50 mg/kg) and combined with DAP (50, 25, and 5 mg/kg) are shown in Table 2. PS-15 alone was fully effective against pneumocystosis but did not prevent toxoplasmosis, as 9 of 10 treated rats died within 5 days after *T. gondii* inoculation. A combination of PS-15 and DAP was highly effective against both infections when the DAP dose was 50 or 25 mg/kg. The administration of PS-15 combined with DAP at 5 mg/kg only reduced *T. gondii* parasitic burdens, but pneumocystosis was completely prevented.

Results for EPI (100 mg/kg) alone and combined with DAP (50, 25, and 5 mg/kg) are shown in Table 3. EPI alone was not effective against pneumocystosis but provided mild protection against *T. gondii*, as two of five rats died of widespread toxoplasmosis. The combinations of EPI (100 mg/kg) and DAP (5, 25, or 50 mg/kg) were fully effective against both infections.

DISCUSSION

Both *P. carinii* and *T. gondii* possess the necessary enzymes for de novo folate synthesis (18). Inhibitors of folic acid synthesis are the main drugs to have been evaluated for *P. carinii* and *T. gondii* infection, especially DHFR and dihydropteroate synthetase inhibitors. This model of dual infection was used because of several potential advantages. First, the development of both infections in immunosuppressed animals better

TABLE 2. Prophylactic activity of PS-15 alone or combined with DAP against toxoplasmosis and pneumocystosis[a]

Drug(s) and amt(s)	Outcome after *T. gondii* inoculation	Day	No. of rats	Pleural fluid vol (ml)	*T. gondii* count (log_{10})					*P. carinii* count (log_{10}) in lungs
					Pleural fluid	Lung	Brain	Spleen	Liver	
PS-15 (25 mg/kg)	Death	5.2 ± 1.0	5[b]	3.4 ± 1.7	1.4 ± 2.0	1.4 ± 2.7	1.1 ± 1.4	4.9 ± 0.8	5.3 ± 0.8	2.4 ± 0.8
PS-15 (50 mg/kg)	Death	4.4 ± 1.5	5	2.6 ± 1.5	1.7 ± 2.1	4.0 ± 0.8	1.7 ± 1.3	4.3 ± 1.1	5.7 ± 1.2	2.4 ± 0.6
PS-15 (25 mg/kg) and DAP (50 mg/kg)	Sacrifice	14	5	0		0[c]	0	0[c]	0[c]	2.3 ± 0.7
PS-15 (25 mg/kg) and DAP (25 mg/kg)	Sacrifice	14	5	0		0[c]	0	0[c]	0[c]	2.5 ± 0.7
PS-15 (25 mg/kg) and DAP (5 mg/kg)	Death	8.5 ± 3.3	4	2.6 ± 2.4	0[c]	0	2.1 ± 1.6	3.2 ± 2.1	3.7 ± 2.5	2.2 ± 0.4
	Sacrifice	14	6	0		0[c]	0.7 ± 1.2	0.2 ± 0.6[c]	1.2 ± 1.9[c]	2.8 ± 0.4

[a] Drugs were given per os 5 days per week for 5 weeks and daily after *T. gondii* inoculation (at week 5) until death or sacrifice.
[b] One rat survived and was sacrificed at day 14. The *T. gondii* burden in the liver was found to be log 3.6/g, and the *P. carinii* cyst level was log 3.1/g of lung.
[c] $P < 0.001$ versus values for PS-15-treated groups. (For statistical analysis by Bonferroni's adjusted *t* test, the values for dead and sacrificed rats were pooled.)

mimics the clinical setting in which the development of the two opportunistic infections is often found. Second, it avoids the variations in the pharmacokinetic behaviors of the drugs after they are administered to different animal species (rats for *P. carinii* and mice for *T. gondii*). Third, it takes account of the possible mutual interference in the development of the two infections. In the present study, we found evidence that the activities of two new folate inhibitors, PS-15 and EPI, were markedly reinforced when these drugs were given in combination with DAP.

Indeed, we have confirmed in the rat model of dual infection that the combination of PYR and DAP could be considered as a reference therapy. This combination has been shown previously to be effective for the treatment of either pneumocystosis (26) or toxoplasmosis (9) or both types of infection in the rat (2). The complementary experiments described here also demonstrated that the minimum effective dose of DAP could be reduced to 25 mg/kg without a loss of activity against either pathogen.

PS-15 is a synthesized inhibitor of DHFR and has previously been shown to be active against *Plasmodium falciparum* and *P. carinii* both in vitro (3, 7) and in vivo (8, 15, 16). In the latter studies, two experimental models of pneumocystosis were used: the SCID mouse model involving intratracheal infection and the rat model involving latent infection with *P. carinii*. PS-15 was found to be effective alone and had an additive effect when low doses were combined with DAP. The high efficacy of PS-15 against *P. carinii* has been confirmed here. However, we found that PS-15 alone was not effective against *T. gondii*, whereas PS-15 combined with DAP appeared to be as effective as the reference regimen (DAP and PYR) for preventing toxoplasmosis.

EPI (Ro 11-8958) is an analog of TMP and is substituted at position 4 of the benzyl moiety (24). Its main characteristics are a marked activity against gram-positive cocci, *Nocardia* species, and anaerobes and a larger volume of distribution and longer half-life than TMP. Although the anti-*P. carinii* and anti-*T. gondii* activities of EPI were demonstrated in vitro and in vivo individually in different models (6, 7, 13, 19, 20, 26), the rat model of dual infection is the first model in which *T. gondii* burdens were quantified in parallel with the number of *P. carinii* cysts. We have shown that EPI administered alone exhibited only mild anti-*T. gondii* activity (delayed death) and did not prevent pneumocystosis. On the other hand, EPI plus DAP acted in synergy against both *P. carinii* and *T. gondii* (even at a low dose of DAP), preventing pneumocystosis and toxoplasmosis.

The combinations of PS-15 or EPI and DAP (50 or 25 mg/kg) were more effective than PS-15 or EPI alone against toxoplasmosis. These results were similar to those obtained with the reference therapy of PYR combined with DAP (same doses). EPI seems poorly effective against both *P. carinii* and *T. gondii* after being administered alone, but a synergism was observed after EPI was combined with DAP against *T. gondii*. The anti-*P. carinii* activity was similar to that of DAP alone, preventing any detection of a synergistic effect. Of the drugs tested here, EPI plus a low dose of DAP (5 mg/kg) was significantly better than the reference treatment of PYR plus DAP (5 mg/kg).

PS-15 has a unique activity against *P. carinii*, which contrasts

TABLE 3. Prophylactic activity of EPI alone or combined with DAP against toxoplasmosis and pneumocystosis[a]

Drug(s) and amt(s)	Outcome after *T. gondii* inoculation	Day	No. of rats	Pleural fluid vol (ml)	*T. gondii* count (log_{10})					*P. carinii* count (log_{10}) in lung
					Pleural fluid	Lung	Brain	Spleen	Liver	
EPI (100 mg/kg)	Death	9 ± 5.6	2	0		3.7 ± 0.1	3.5 ± 0.7	6.6	7 ± 0.1	5.8 ± 0.6
	Sacrifice	14	3	2.3 ± 3.2	0	0	0	0	3.0 ± 2.9	6.4 ± 1.1
EPI (100 mg/kg) and DAP (50 mg/kg)	Sacrifice	14	5	0		0	0	0	0[b]	2.1 ± 0.3[b]
EPI (100 mg/kg) and DAP (25 mg/kg)	Sacrifice	14	5	0		0	0	0	1.0 ± 1.6[b]	2.4 ± 0.5[b]
EPI (100 mg/kg) and DAP (5 mg/kg)	Sacrifice	14	5	0		0	0	0	0[b]	2.2 ± 0.4[b,c]

[a] Drugs were given per os 5 days per week for 5 weeks and daily after *T. gondii* inoculation (at week 5) until death or sacrifice.
[b] $P < 0.001$ versus values for the EPI-treated group. (For statistical analysis by Bonferroni's adjusted *t* test, the values for dead and sacrificed rats were pooled.)
[c] Not significant versus the values obtained for the other two treated groups C (the groups treated with the EPI-DAP combinations).

with the poor activity of other DHFR inhibitors against this parasite. This drug could also be better tolerated, as it is not structurally related to other DHFR inhibitors, such as TMP, PYR, or trimetrexate and piritrexim. Its potential value for preventing opportunistic infections in AIDS patients is also supported by its activity against mycobacteria (21).

We conclude that both PS-15 and EPI are attractive candidates for mixed prevention of toxoplasmosis and pneumocystosis only after being given in combination with DAP.

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