Efficacy of DL-α-Difluoromethylornithine in a Rat Model of Pneumocystis carinii Pneumonia

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Pneumocystis carinii pneumonia is often the terminal event for patients with the acquired immunodeficiency syndrome. Eflornithine (DL-α-difluoromethylornithine [DFMO]; Ornidyli; Merrell Dow Research Institute, Cincinnati, Ohio) has been used successfully against this protozoan disease in limited clinical trials, although not all patients respond to therapy. In contrast, results of the only reported experiments with DFMO in an animal model were negative. We retested DFMO against P. carinii in an immunosuppressed rat model by inclusion of 3% DFMO in the drinking water, a dose rate about twice that used previously. A combination of trimethoprim and sulfamethoxazole, a proven anti-P. carinii agent, was used as a positive control. After 3 weeks of anti-P. carinii pneumonia therapy, the surviving rats were sacrificed and the degree of parasitosis was judged by examination of lung sections stained with silver methenamine to reveal cysts. In three separate experiments, DFMO showed definite anti-P. carinii pneumonia activity; the parasitosis of DFMO-treated animals was significantly less than that of control animals (P < 0.001 for all experiments). DFMO was not as active as trimethoprim-sulfamethoxazole, however. Several other experimental therapies were tested, including dapsone and two additional antiprotozoal agents: suramin and diminazene aceturate (Berenil; Farbwerke Hoechst, Frankfurt, Federal Republic of Germany). Diminazene aceturate, a veterinary drug related to the standard anti-P. carinii pneumonia agent pentamidine, was very active (P < 10^{-19}). Suramin and dapsone were weakly active. The combinations suramin-diminazene aceturate and suramin-DFMO were tested, but they were antagonistic rather than synergistic.

Pneumocystis carinii is a ubiquitous lung-dwelling protozoan. In healthy individuals it acts as a commensal organism but produces P. carinii pneumonia in immunocompromised hosts (13, 17, 20). P. carinii pneumonia is one of the most important opportunistic infections accompanying the acquired immunodeficiency syndrome (AIDS) (5, 10). The standard treatment for AIDS-associated P. carinii pneumonia is either a combination of trimethoprim and sulfamethoxazole (TMP-SMZ) or pentamidine. Both have marked anti-P. carinii activity, but they are often not efficacious, especially after the initial episode, and are associated with significant toxicity in patients with AIDS (9, 14).

DL-α-Difluoromethylornithine (DFMO; eflornithine; Ornidyli; Merrell Dow Research Institute, Cincinnati, Ohio) inhibits the first step in the synthesis of polyamines, which are small cationic molecules that are required for the growth processes of all cells (15). DFMO is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase (18), yet it is a nontoxic agent that is active against several protozoal pathogens, most notably African trypanosomes (19). DFMO is also highly active against experimental infections of the sporozoan parasite Eimeria tenella (8). Since P. carinii is also thought to be a sporozoan, DFMO was used on a compassionate basis for the treatment of P. carinii pneumonia associated with AIDS against which other agents had failed. Definite clinical activity was observed initially (6), and additional extensive clinical experience has supported the early observations (16, 19). In contrast, results of the only reported trials of DFMO against P. carinii in animals were negative (11), although results of in vitro studies of P. carinii in tissue culture showed DFMO to be active against the parasite (3). A reevaluation of the efficacy of DFMO in the animal model was therefore desirable. A positive result would confirm the activity against this parasite and would support the results of clinical studies. In addition, the demonstration of activity in an animal model would facilitate experiments designed to optimize DFMO dosage and would allow the testing of other antipolyamine agents. An animal model would also open the possibility of studying the interaction of DFMO with other drugs. Since our laboratory has had experience with DFMO in animal models of African trypanosome infections, we retested this compound in a rat model of P. carinii pneumonia.

In addition to reexamining DFMO, we tested several other drugs and drug combinations. Since both pentamidine and DFMO are active against African trypanosomiasis, we tested two additional antitrypanosome drugs for their efficacy against P. carinii pneumonia: diminazene aceturate (Berenil) and suramin. Diminazene aceturate is a veterinary drug that is related to pentamidine. Suramin has anti-human immunodeficiency virus activity in tissue culture and has been found to act synergistically with DFMO against trypanosomes (1). Suramin was tested against P. carinii pneumonia alone and in combination with diminazene aceturate and DFMO.

MATERIALS AND METHODS

Induction of P. carinii pneumonia. Female Sprague-Dawley-derived rats (weight, approximately 200 g; Taconic Farms, Germantown, N.Y.) were used for all experiments. Two immunosuppression protocols were employed in three separate experiments. In protocol 1 (first experiment), rats were given dexamethasone (Sigma Chemical Co., St. Louis, Mo.) in their drinking water (2 mg/liter) for 3 weeks prior to the initiation of experimental anti-P. carinii pneumonia therapies. In immunosuppression protocol 2, rats were given
twice weekly injections of 25 mg of cortisone (Cortone; Merck & Co. Inc., Rahway, N.J.) per kg for 5 weeks prior to anti-P. carinii pneumonia therapy. All experimental and control animals were maintained on the immunosuppressive protocols for the duration of the experiment. Oxytetracycline (Terramycin; Pfizer Inc., New York, N.Y.) was always included in the drinking water (500 mg/liter) of the animals to suppress bacterial infections. In experiment 3, the rats were also protected from fungal infection by twice weekly subcutaneous injections of amphotericin B (Fungizone; E. R. Squibb & Sons, Princeton, N.J.) at 20 mg/kg for the duration of the experiment. During the first 4 weeks of immunosuppression, seed animals (animals placed on an immunosuppressive regimen at least 4 weeks earlier) were rotated through the cages of newly immunosuppressed animals to ensure heavy exposure to this apparently airborne parasite.

**Experimental and control therapies.** In each experiment, animals were randomly assigned to a specific treatment group. One group was always a control induced to develop P. carinii pneumonia but was not treated otherwise. Once begun, all anti-P. carinii pneumonia therapies were continued until the animals died or were sacrificed. A positive drug control with the standard anti-P. carinii agent TMP-SMZ was included for comparative purposes. Treatment with TMP-SMZ was by the addition of 25 ml of a cherry-flavored pediatric suspension (Sepral; Burroughs Wellcome Co., Research Triangle Park, N.C.) to each liter of drinking water, yielding a final concentration of 200 mg of TMP and 1 g of SMZ per liter. The TMP-SMZ-containing water bottles were shaken twice a day to resuspend the drugs. DFMO, which was supplied by the Merrell Dow Research Institute, was always administered as a 3% solution in the drinking water. Since previous experience revealed that rats drink less when 3% DFMO is included in the drinking water, we added 320 mg of sodium saccharine and 8 g of glucose (individual Sweet and Low packets; Cumberland Packing Corp., New York, N.Y.) to each liter of 3% DFMO for the second and third experiments. This caused the DFMO-treated rats to drink about the same volume of water as control rats. The mean water consumption rates of the three groups of rats, were as follows: for water with TMP-SMZ, 207 ml kg \(^{-1}\) day \(^{-1}\); for water with DFMO, 250 ml kg \(^{-1}\) day \(^{-1}\); for controls, 226 ml kg \(^{-1}\) day \(^{-1}\). This yielded TMP and SMZ doses of 41 mg kg \(^{-1}\) day \(^{-1}\) and 207 mg kg \(^{-1}\) day \(^{-1}\), respectively, and a DFMO dose of 7.5 g kg \(^{-1}\) day \(^{-1}\).

Dapsone (Jacobus Pharmaceutical Co., Princeton, N.J.) was added to food pellets that were ground and moistened. The resultant paste was placed into a small bowl in the rat cages and was changed daily to prevent microbial growth in the bowl. The weight of food consumed was measured, and enough ground dapsone tablet was mixed into the food to yield a daily dose of 125 mg/kg. Diminazene aceturate (Berenil; Farbwerke Hoechst, Frankfurt, Federal Republic of Germany) was dissolved in 0.85% saline (2 mg/ml) and was given subcutaneously twice weekly at 20 mg/kg. Suramin (FBA Pharmaceuticals, New York, N.Y.) was dissolved in 0.85% saline (2 mg/ml) and was administered intravenously via a tail vein at 20 mg/kg. Drugs given in combination were used at the same dose as when used singly.

**Evaluation of P. carinii pneumonia.** Parasitosis was judged by the examination of lung sections stained with silver methenamine (12), which causes the cyst walls of P. carinii to appear to be a distinct silver-grey. The left lung was removed; and samples of the upper, middle, and lower regions were fixed, sectioned, and stained. A scale of 0 to 5 was used to score the degree of P. carinii pneumonia. A score of 0 indicated that no organisms were seen in the lung sections; 1 indicated that one parasite or a very few were seen in isolated alveoli; 2 indicated that parasites were easily found in a significant portion of the alveoli; 4 indicated that many organisms were found in about half the alveoli; and 5 indicated that over half the alveoli were filled with P. carinii. At least 25 fields from stained sections of each lung region were examined. The slides were coded and read blind to avoid bias.

An effort was made to evaluate the parasitosis of all animals that died after they were assigned to experimental or control groups, whether death was spontaneous or by sacrifice. This was not possible for some animals that died spontaneously because of cannibalism. The loss of such animals from the data base caused there to be a variation in the size of the treatment groups.

**Statistical analysis of results.** In each experiment, the initial statistical procedure applied to the data was the Kruskal-Wallis nonparametric analysis of variance. This procedure was used to determine whether at least one treatment group mean rank score differed from that of a control group. If such was the case (P < 0.05), treatment mean rank scores were then compared on an individual basis with a control mean rank score by using a nonparametric (Dunnett analog) multiple comparison procedure (4). Differences between a treatment group mean rank score and a control group mean rank score were deemed significant when the calculated test statistic, Z, exceeded the critical value for that particular comparison, Z\(_{0.05/2p}\) (p is equal to the number of comparisons of treatment groups with the control group in an experiment).

<table>
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<tr>
<th>Table 1. Mean parasitosis scores for various P. carinii pneumonia treatments</th>
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<td><strong>Treatment</strong></td>
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<td>Control</td>
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<td>TMP-SMZ</td>
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<td>DFMO</td>
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<td>Diminazene aceturate</td>
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RESULTS

Table 1 presents the mean parasitosis scores, the standard errors of the means, and the sample sizes for treatment and control groups from all three experiments. Details from individual experiments and statistical analyses are presented below.

First experiment. A total of 60 rats were immunosuppressed with dexamethasone. Of these, 51 survived the 3 weeks until the scheduled time for the initiation of therapy. They were then divided into three treatment groups with the aid of a random number table. The first group was an untreated control, the second group was treated with TMP-SMZ, and the third group was treated with DFMO. The protocol for the experiment assigned five rats to be sacrificed from each treatment group at the end of each week of treatment in order to follow their responses to therapy. Spontaneous deaths occurred in all groups during the treatment period, and the results shown in Fig. 1 and Table 1 include all animals, whether they died spontaneously or were sacrificed. The time of death is indicated in Fig. 1 by the position of the symbol with respect to the x-axis; the intensity of parasitosis at death is indicated by the position of the symbol with respect to the y-axis. A pattern of decline in the parasitosis of the animals treated with TMP-SMZ was seen when they were compared with the controls. The pattern of effect of DFMO treatment was also seen. The mean parasitosis score of the animals treated with DFMO was significantly less than that of controls ($P = 4.5 \times 10^{-6}$), as was that of the TMP-SMZ-treated animals ($P = 2.8 \times 10^{-8}$). TMP-SMZ reduced the parasitosis more than DFMO did, however, as reflected in the mean parasitosis scores of 0.47 and 1.29, respectively.

Second experiment. A total of 120 rats was placed on the cortisone protocol, with anti-P. carinii pneumonia treatment begun at the start of the sixth week of immunosuppression. At that time 99 animals remained, and these animals were divided into three treatment groups, as described above. In this experiment no animals were sacrificed until the termination of the experiment 3 weeks after treatment was begun. There were spontaneous deaths (Fig. 2). The results were very similar to those of the first experiment, in that a response to DFMO and TMP-SMZ was seen when compared with the response of the controls. The mean score for DFMO-treated animals was significantly less than the control score ($P = 3.7 \times 10^{-4}$), as was the mean score for animals treated with TMP-SMZ ($P < 10^{-10}$). DFMO reduced the mean parasitosis score to 1.48, compared with 0.33 for TMP-SMZ.

Third experiment. The results for the third experiment are presented in Fig. 3. The immunosuppression method was the same as that used in the second experiment, but the animals were also given amphotericin B to suppress fungal growth. Examination of Fig. 3 makes the benefit of inclusion of amphotericin B apparent. A greater proportion of the animals survived to the conclusion of the experiment compared with the survival of animals in experiments 1 and 2. This was true even in the controls, even though the P. carinii pneumonia intensity was virtually the same as that in the previous experiments without amphotericin B. The effect of TMP-SMZ was seen more clearly in this third protocol: a parasitosis score of 0.07 versus 2.86 for the controls ($P < 10^{-10}$). The effect of DFMO was also more marked, although it was still less than that of TMP-SMZ: a parasitosis score of 0.71 ($P < 10^{-10}$).

In the third experiment we included a treatment of twice

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FIG. 1. Anti-P. carinii pneumonia (PCP) therapies are described in detail in the text. Circles represent individual animals. The position of a circle on the ordinate scale of P. carinii pneumonia intensity refers to the degree of lung involvement at death; a score of 0 indicates that no P. carinii cysts were found, and 5 indicates that half of the alveoli were filled with cysts. Further details of the scoring are given in the text. The position of a circle on the abscissa scale refers to the time of death with respect to the time of initiation of immunosuppression. The first value on the abscissa scale is the time of initiation of anti-P. carinii pneumonia therapy.
weekly injections of 20 mg of diminazene aceturate per kg and found this to be quite active in suppressing parasitosis. The diminazene aceturate-treated group mean parasitosis score of 0.20 was significantly different from that of the controls ($P < 10^{-10}$). Diminazene aceturate treatment was more effective than DFMO treatment and was almost as effective as TMP-SMZ treatment.

Dapsone, given at a daily dose of 125 mg/kg in the food, was included for comparative purposes, since there is interest in this drug as an anti-P. carinii pneumonia agent. In this trial dapsone showed significant activity when compared with that of the controls ($P < 10^{-10}$), but the mean parasitosis score was greater than that in groups treated with DFMO (1.6 versus 0.71).

Suramin was administered intravenously weekly at 20 mg/kg and had an effect when compared with controls; a mean score of 1.80 compared with the controls ($P < 10^{-10}$). The mean parasitosis score in groups treated with suramin was near that of groups treated with dapsone.

The combinations suramin-DFMO and suramin-diminazene aceturate appeared to be antagonistic rather than synergistic, as seen in Fig. 3 and by the fact that the mean scores of combination-treated groups were higher than those of each of the drugs given alone.

**DISCUSSION**

DFMO has anti-P. carinii pneumonia activity in the rat model. This conclusion differs from that of Hughes and Smith (11), who reported that DFMO is inactive when tested in the rat model. The conditions, however, were not the same. Calculations from their data indicate that they presented 30 ml of a 1.6% DFMO solution day$^{-1}$ for each 200-g rat, or 2.5 g of DFMO kg$^{-1}$ day$^{-1}$. Since they also indicated that the rats consumed 30 to 50 ml of water day$^{-1}$, it is likely that there were considerable periods when no water was available and thus there was no drug administration for these periods. Frequent drug administration may be important because the half-life of DFMO in rats is only 83 min (7). In our experiments, 3.0% DFMO was always available; and from the observed drinking rate, we calculated the dose to have been 7.5 g kg$^{-1}$ day$^{-1}$, or about 3 times the rate used by Hughes and Smith (11). The total drug administered was not so different since they treated the animals for 6 weeks rather than for 3 weeks (11). The results presented here demonstrate that judicious, relatively constant administration of a single polyamine synthesis inhibitor at an adequate dose rate produces a clinical response in the rat P. carinii pneumonia model.

Our in vivo findings support earlier positive in vitro results (2) and positive clinical results (6, 16, 19). However, the response of the rat model to DFMO treatment was less than the response to TMP-SMZ treatment. The less than complete response of the rat model to DFMO is convenient from an experimental point of view because it permits the ready detection of any improvement in activity that may be obtainable by using other antipolyamine agents or by modifying the dose or route of administration.

The activity of diminazene aceturate is especially interesting, and this lead should be pursued. Considering the relatively low activity of suramin and the toxicity as well as its antagonism with DFMO and diminazene aceturate, we consider this drug to have little promise for the treatment of P. carinii pneumonia. Our results with dapsone were not as encouraging as those reported by others (11).
FIG. 3. The anti-\( P. \text{carinii} \) pneumonia (PCP) therapies are described in detail in the text. Scales and symbols are as described in the legend to Fig. 1.
ACKNOWLEDGMENTS

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ADDITIONAL MATERIAL

Subsequent to acceptance of this paper, the results from a related study were published. Walzer et al. (P. D. Walzer, C. K. Kim, J. Foy, M. J. Linke, and M. T. Cushion, Antimicrob. Agents Chemother. 32:896–905, 1988) examined a series of antityrpanosome compounds, including DFMO and diminazene, for activity against P. carinii. The diminazene results were comparable and supportive, but the DFMO results were very different. Walzer et al. administered DFMO as a 4% solution in the drinking water but shifted at some point to a 2% solution because the rats developed diarrhea and deteriorated clinically. There was little if any effect on P. carinii pneumonia. We maintained a steady administration of 3% DFMO and observed very little diarrhea, a general improvement in the appearance of the animals compared with the steroid-treated controls, and a reduction of parasitosis. Recently, we began a dose-response study with DFMO. The results of the first experiment confirmed the positive response to 3% DFMO; however, neither 2 nor 4% was effective. These preliminary results agree with the earlier report of Hughes and Smith (11), who used less than 2% DFMO in the drinking water, and with those of Walzer et al., since they used 4% and then 2% (Walzer et al., Antimicrob. Agents Chemother. 32:896–905, 1988). At this time, we have no explanation for the lack of response to the higher dose, and additional experiments are being conducted.

LITERATURE CITED