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8-Aminoquinolines from Walter Reed Army Institute for Research for Treatment and Prophylaxis of *Pneumocystis* Pneumonia in Rat Models

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Three 8-aminoquinolines from the Walter Reed Army Institute for Research (WRAIR), WR6026, WR238605, and WR242511, strongly inhibited *Pneumocystis carinii* growth in vitro at 1 μg/ml. This activity was similar to that of primaquine. In rat therapy models, the WRAIR compounds affected *Pneumocystis* pneumonia at doses as low as 0.25 mg/kg (WR242511) or 0.5 mg/kg (WR6026 and WR238605). At these doses, primaquine alone was ineffective as therapy. In a rat prophylaxis model, all three WRAIR 8-aminoquinolines were extremely effective at daily doses of 0.57 mg/kg, showing activity greater than that of primaquine at this dosage and comparable to that of trimethoprim-sulfamethoxazole at 50/250 mg/kg.

In the United States, *Pneumocystis carinii* causes pneumonia in 60 to 80% of individuals infected with the human immunodeficiency virus. Traditional therapeutic and prophylactic regimens, including trimethroprim-sulfamethoxazole and pentamidine, can cause severe adverse reactions in this group of patients. The search for alternative drugs that are effective against *P. carinii* led to the discovery that the drug combination clindamycin-primaquine is effective both in vitro and in animal models for both therapy and prophylaxis (11). The combination proved effective in animals, although neither drug was effective alone. The regimen has been carried on to clinical therapy trials (6, 12, 13) and appears to be very effective for the treatment of *P. carinii* pneumonia. Results of clinical studies carried out by E. Toma in Montreal, Quebec, Canada, in which clindamycin-primaquine was compared with trimethoprim-sulfamethoxazole have not yet been published. Studies of other 8-aminoquinolines related to primaquine were undertaken to find agents with enhanced effectiveness, less toxicity than primaquine, or both.

Many 8-aminoquinolines have been synthesized and tested for activity against protozoal infections. The Walter Reed Army Institute for Research (WRAIR) has actively pursued the development of 8-aminoquinolines for antimalarial and antileishmanial activities (8). Three WRAIR compounds were selected that were more potent antimalarial agents than primaquine and for which pharmacologic and toxicologic data were available (3). Compound WR6026 has entered phase II testing in humans for leishmaniasis, and WR238605 is being considered for phase I and II testing in humans as an antimalarial agent. In animals, WR242511 has been more active against malaria than WR6026 or WR238605 has, but it has a greater potential for producing methemoglobinemia. The advanced state of development of the drugs made them appealing as candidates for testing against *P. carinii*.

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MATERIALS AND METHODS

The in vitro culture system for evaluating compounds for anti-*P. carinii* activity has been described previously (2). Briefly, 24-well tissue culture plates (Corning) containing confluent monolayers of WI-38 human embryonic lung fibroblasts in minimum essential medium (Sigma) with 10% fetal bovine serum (Whittaker M.A. Bioproducts) were inoculated with approximately 7×10^5 *P. carinii* trophozoites per well. The drugs to be tested were added to the wells so that for each drug concentration there were four wells to be examined at each of five time points. Wells with no drugs and wells with trimethoprim-sulfamethoxazole at 50/250 μg/ml were included as controls. Plates were incubated at 35°C in an atmosphere of 5% O_2 and 10% CO_2, with the balance being N_2. On days 1, 3, 5, 7, and 10, 10-μl samples were removed from the wells, placed on glass slides in 1-cm^2 areas, stained with Giemsa, and examined microscopically at ×1,000. *P. carinii* trophozoites and cysts as well as tissue culture cells were counted in 10 randomly selected fields of unknown slides by two individuals. For each time point, there were eight evaluations (four wells × two examiners) from which means and standard errors were calculated and plotted to provide growth curves.

Inocula for cultures and for animals were prepared from infected rat lungs. Samples of lungs known to be infected, as determined by examination of Giemsa-stained impression smears, were ground in a Ten-Broeck homogenizer in minimum essential medium, and centrifuged slowly (250 × *g*) to settle the lung pieces, and the supernatant was decanted. A 10-μl sample of supernatant was stained with Giemsa as well as with viability stain (5) and examined microscopically. The number of trophozoites per milliliter was determined, and the inoculum was adjusted as necessary.

Animal models. Animal models were used both at Indiana University (IU) and the University of North Carolina (UNC). Since the models differed in several aspects, each model is described below.

(i) Animal models at IU. For the transtracheally inoculated rat model for treatment or prophylaxis (1), virus-free female rats (weight, 120 to 140 g; Harlan-Sprague-Dawley, barrier 202, in Indianapolis, Ind.) which were also free of *P. carinii*

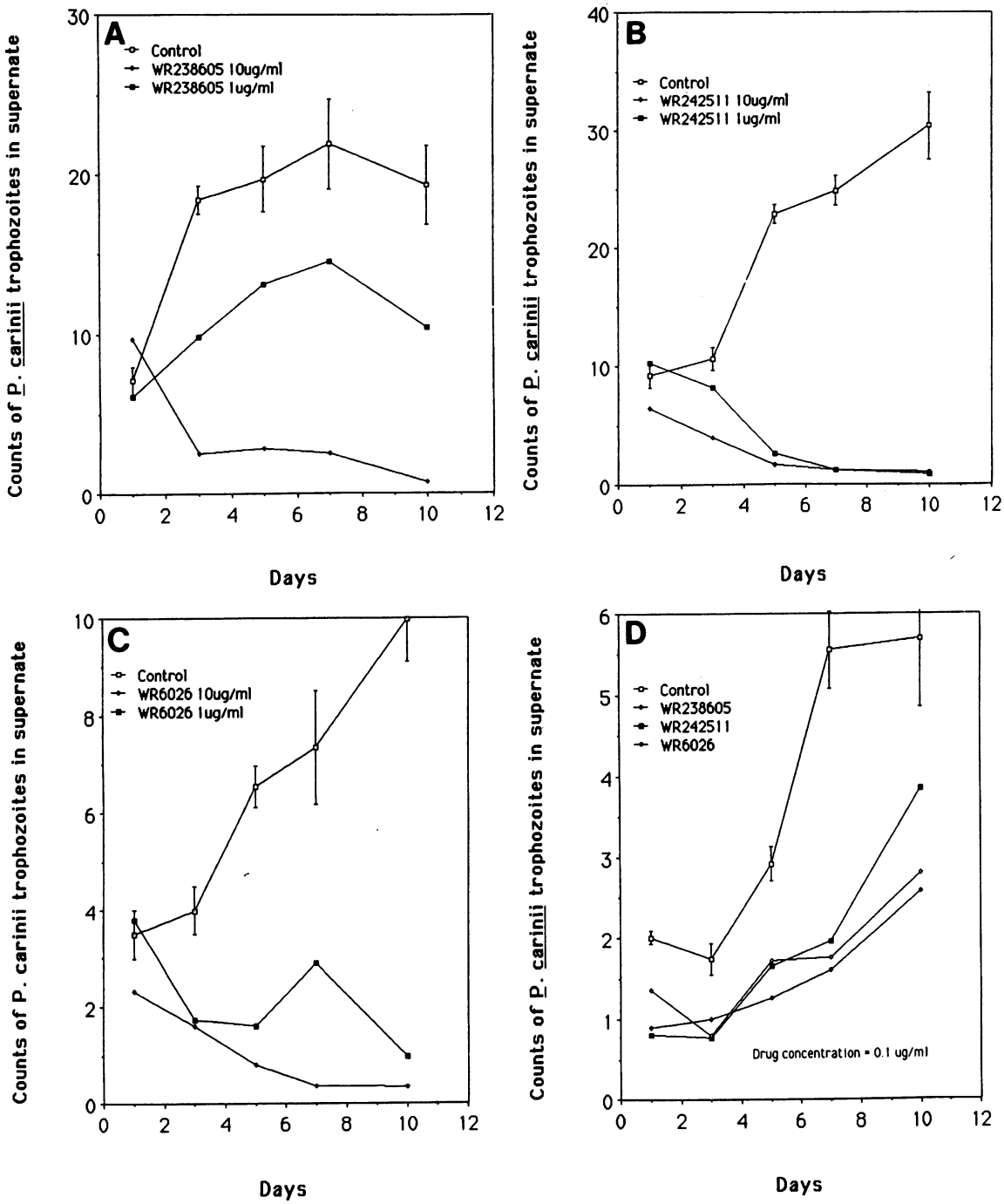

FIG. 1. Inhibition of *P. carinii* by the three WRAIR compounds. Data are from experiments 48 (A), 49 (B), 50 (C), and 89 (D).

were used. The animals received standard chow containing 23% protein and also received tetracycline (0.5 mg/ml) in the drinking water. Before being inoculated transtracheally, rats were immunosuppressed for 4 days either with dexamethasone at 1.2 μg/ml in drinking water or with cortisone acetate injected subcutaneously at 250 mg/kg twice per week, as specified for each study. The choice of immunosuppressive agent was determined by the drugs being tested in the study. Earlier studies showed that animals would not drink water containing dexamethasone along with clindamycin-primaquine (unpublished data from IU). For transtracheal inoculation, after 4 to 7 days of immunosuppression, rats were anesthetized intramuscularly with 0.2 ml of a "cocktail" containing ketamine hydrochloride (80.0 mg/ml), atropine (0.38 mg/ml), and acepromazine (1.76 mg/ml); and an incision of approximately 1 cm was made over the trachea, which was then exposed by blunt dissection. The inoculum of 10^6 trophozoites in 0.2 ml was injected followed by injection of 0.4 ml of air; the wound was closed with a single clip. Animals were continued on immunosuppressive agents.

TABLE 1. Treatment at IU of *P. carinii* pneumonia in rats with 8-aminoquinolines from WRAIR

Drug	Dose (mg/kg/day)	Giemsa stain infectivity score[a]	No. infected/ total no.	Silver stain infectivity score[a]	No. infected/ total no.
WR6026	2	2.2 ± 0.4[b]	10/10	1.8 ± 0.3	10/10
WR238605	2	1.8 ± 0.3[c]	10/10	1.8 ± 0.3	8/9
WR242511	2	0.2 ± 0.1[c]	4/10	0.5 ± 0.4	10/10
Untreated control		4.2 ± 0.1	7/7	3.7 ± 0.2	7/7
Trimethoprim-sulfamethoxazole	50/250	0.5 ± 0.4[c]	4/6	0.9 ± 0.7	5/6

[a] Results are expressed as means ± standard errors. The scores are described in the text.
[b] $P < 0.01$ compared with untreated controls.
[c] $P < 0.001$ compared with untreated controls.

For the therapy model, treatment was begun 4 weeks later and was continued for 2 to 3 weeks. For the prophylaxis model, drug administration was begun after transtracheal inoculation and was continued for 6 to 7 weeks. Animals were monitored daily and were weighed weekly. Drug doses were given by gavage or were added to drinking water and assessed by monitoring water consumption. At the time of sacrifice, animals were anesthetized with ketamine cocktail (0.2 ml intramuscularly) and were exsanguinated by cardiac puncture. Lungs were removed and used to prepare impression smears and sections, which were stained with Giemsa and rapid methenamine silver nitrate stains. Stained preparations were examined as unknowns by three individuals, and scores of infection were determined by the following scheme, which is approximately logarithmic and is based on the numbers of organisms per ×1,000 field: >100, 5; 11 to 100, 4; 1 to 10, 3; 2 to 9 per 10 fields, 2; ≤1 in 10 fields, 1; 0 in 50 fields, 0. Standard errors of mean scores were calculated for each group of animals. *P* values were calculated and are given when appropriate. Ten rats were given each drug dose, and both untreated controls and positive controls treated with trimethoprim-sulfamethoxazole (50/250 mg/kg) were included.

(ii) Animal model at UNC. Male Sprague-Dawley rats (weight, 150 to 200 g) not certified to be virus-free were obtained from Hilltop Laboratories (Scottsdale, Pa.). Animals received a low-protein diet (8%), drinking water containing tetracycline (0.5 mg/ml), and for immunosuppression, cortisone acetate at 250 mg/kg injected subcutaneously twice weekly. The regimen was continued for 8 weeks, with fluid intake being monitored daily and animals weighed weekly. After 6 weeks, animals were placed in eight groups of 10 animals each. Test compounds were given in the drinking water. At the end of 2 weeks of treatment with the test compounds, all animals were sacrificed by exsanguination following anesthesia. The lungs were inflated in situ with 10% Formalin and fixed for histologic examination. The lung tissue was sectioned and stained with the Grocott methenamine silver stain. Two scorers determined the extent of lung involvement as follows: <10 cysts per two fully examined sections, 0.5; scattered cysts, <5% of lung involved, 1; scattered cysts with limited focal involvement 10 to 25% of lung involved, 2; scattered cysts with numerous intense areas of focal involvement and 26 to 50% of lung involved, 3; cysts throughout the tissue with numerous very intense focal areas and 75% of lung involved, 4.

In all studies described here, 8-aminoquinolines provided by WRAIR to investigators at both IU and UNC were used.

RESULTS

In vitro cultures showed that all three compounds inhibited *P. carinii* at 10 and 1 μg/ml; they were still somewhat inhibitory at 0.1 μg/ml (Fig. 1). In one experiment, compound 242511 appeared to be very effective at 0.1 μg/ml. This level of activity was similar to that observed previously for primaquine (11).

In vivo therapy studies with all three compounds were performed at doses of 2.0 mg/kg, because this was the dose of primaquine that was effective in combination with clindamycin for therapy in rats (11). In all studies scores for both Giemsa- and silver-stained slides are shown because different features are emphasized. Giemsa stain shows the higher scores for trophozoites, and silver stain may detect rare cysts.

In the first therapy study, which was done at IU, we used

TABLE 2. Treatment at IU of *P. carinii* pneumonia with 8-aminoquinolines from WRAIR alone and with clindamycin

Drug	Dose (mg/kg/day)	Giemsa stain infectivity score[a]	No. infected/ total no.	Silver stain infectivity score[a]	No. infected/ total no.
WR6026	2	0.2 ± 0.1[b]	5/10	0.4 ± 0.2	6/10
WR6026 + clindamycin	2/225	0.3 ± 0.2[b]	4/10	0.9 ± 0.3	9/10
WR238605	4	0.06 ± 0.1[b]	3/10	0	0/10
WR238605 + clindamycin	4/225	0.1 ± 0.1[b]	4/10	0.1 ± 0.1	4/10
WR242511	2	0.03 ± 0.1[b]	1/10	0	0/10
WR242511 + clindamycin	2/225	0.1 ± 0.1[b]	2/10	0.1 ± 0.1	4/10
Primaquine + clindamycin	2/225	0.3 ± 0.2[b]	3/10	0.5 ± 0.1	7/10
Untreated		3.9 ± 0.2	10/10	3.4 ± 0.2	10/10
Trimethoprim-sulfamethoxazole	50/250	0.03 ± 0.1[b]	1/10	0.3 ± 0.2	3/10

[a] Results are expressed as means ± standard errors. The scores are described in the text.
[b] $P < 0.001$ compared with untreated controls.

TABLE 3. Extent of disease by histologic score at UNC

Compound or drug	Dose (mg/kg/day)	Mean score	No. of animals with the following scores:				
			0.5	1	2	3	4
Saline		3.0 (3.3)[a]	0 (0)	0 (0)	1 (0)	8 (7)	1 (3)
Clindamycin	225	2.8 (2.5)[b]	0 (0)	0 (1)	5 (4)	2 (4)	3 (1)
WR238605	4.0	0.9 (0.8)[b,c]	4 (5)	5 (5)	1 (0)	0 (0)	0 (0)
WR238605 + clindamycin	4.0/225	0.5 (0.9)[b,d]	7 (2)	0 (5)	0 (0)	0 (0)	0 (0)
WR242511	2.0	0.6 (0.6)[b,d]	9 (8)	1 (2)	0 (0)	0 (0)	0 (0)
WR242511 + clindamycin	2.0/225	0.8 (0.9)[b,c]	5 (6)	2 (1)	1 (0)	0 (1)	0 (0)
WR6026	2.0	0.7 (0.8)[b,d]	6 (5)	4 (5)	0 (0)	0 (0)	0 (0)
WR6026 + clindamycin	2.0/225	1.0 (0.9)[b,d]	3 (2)	6 (8)	1 (0)	0 (0)	0 (0)

[a] Numbers in parentheses indicate the results of the second scorer.
[b] $P = 0.0001$ compared with saline controls.
[c] $P > 0.0005$ compared with controls treated with clindamycin only.
[d] $P = 0.0001$ compared with controls treated with clindamycin only.

immunosuppression with dexamethasone and drug treatment by gavage once per day. All three drugs were effective (Table 1). Animals that received clindamycin at 225 mg/kg in addition to the 8-aminoquinolines once per day by gavage had scores similar to those of the groups that received 8-aminoquinolines alone (data not shown).

A second study was performed at both IU and UNC by using their respective models. The data from the study performed at IU are shown in Table 2. Animals were immunosuppressed with cortisone acetate, and drug doses were given in drinking water. Again, all three drugs were effective.

In the comparable study at UNC, the same lots of drugs provided by WRAIR to IU were used. Table 3 shows the results of the study done at UNC by using their scoring system. The findings are comparable to those obtained at IU.

A third therapy study was performed at IU to establish the lowest effective doses of the compounds (Table 4). The protocol was similar to that used in the second study (Table 2); drug doses were given in the drinking water, and immunosuppression was with dexamethasone. The compounds were active at 0.25 mg/kg/day for WR242511 and 0.5 mg/kg/day for WR6026 and WR238605.

The three drugs were also tested at IU for their effectiveness in prophylaxis (Table 5). Drugs were given in the drinking water, and immunosuppression was with dexamethasone. All three compounds were effective at 0.57 mg/kg/day.

In a separate study, primaquine also was tested in a prophylaxis model of rats immunosuppressed with cortisone acetate given subcutaneously with and without clindamycin at 225 mg/kg/day in drinking water (Table 6). Primaquine showed minimal effectiveness when used alone at a dose of 0.57 mg/kg/day but was quite effective when used at this dose in combination with clindamycin.

DISCUSSION

Results of the studies done at IU and UNC establish the efficacy of the 8-aminoquinolines WR238605, WR242511,

TABLE 4. Establishment at IU of lowest effective dose for therapy of *P. carinii* pneumonia with 8-aminoquinolines from WRAIR

Compound	Dose (mg/kg/day)	Giemsa stain infectivity score[a]	No. infected/ total no.[b]	Silver stain infectivity score[a]	No. infected/ total no.[b]
WR6026					
	2.0	0.3 ± 0.1	3/5	0.4 ± 0.2	3/5
	1.0	1.1 ± 0.2	5/8	0.9 ± 0.4	5/8
	0.5	1.8 ± 0.6	7/7	2.1 ± 0.4	7/7
	0.25	4.7 ± 0.2	7/7	3.9 ± 0.1	7/7
WR238605					
	4.0	0.03 ± 0.1	1/7	0.3 ± 0.2	3/7
	2.0	0.9 ± 0.4	4/6	1.0 ± 0.4	5/6
	1.0	2.3 ± 0.9	5/5	2.4 ± 0.6	5/5
	0.5	2.6 ± 0.2	3/3	2.7 ± 0.1	3/3
WR242511					
	2.0	0.16 ± 0.2	1/2	0.5 ± 0.2	2/2
	1.0	0.5 ± 0.2	3/6	0.8 ± 0.3	4/6
	0.5	2.4 ± 0.8	4/5	2.2 ± 0.6	4/5
	0.25	1.8 ± 0.6	7/7	1.8 ± 0.5	7/7
	0.1	4.0 ± 0.3	8/8	3.6 ± 0.2	8/8
Trimethoprim-sulfamethoxazole	50/250	0.9 ± 0.6	3/5	0.4 ± 0.1	4/5
Untreated		4.2 ± 0.2	8/8	3.6 ± 0.2	8/8

[a] Results are expressed as means ± standard errors. The scores are described in the text.
[b] Total is the number of rats for which evaluable slides were obtained; animals that died during the study were not always evaluable.

TABLE 5. Prophylaxis at IU of rats with *P. carinii* pneumonia with 8-aminoquinolines from WRAIR

Drug	Dose (mg/kg/day)	Giemsa stain infectivity score[a]	No. infected/ total no.	Silver stain infectivity score[a]	No. infected/ total no.
WR6026	0.57	0.09 ± 0.1[b]	3/10	0.4 ± 0.1	6/10
WR238605	0.57	0.4 ± 0.2[b]	4/10	0.4 ± 0.2	4/10
WR242511	0.57	0[b]	0/7[c]	0.1 ± 0.1	2/7[c]
Trimethoprim-sulfamethoxazole	50/250	0.07 ± 0.1[b]	2/10	0.1 ± 0.1	2/10
Untreated		4.0 ± 0.3	8/8[c]	3.3 ± 0.2	8/8

[a] Results are expressed as means ± standard errors. The scores are described in the text.
[b] $P < 0.001$ compared with untreated controls.
[c] Three rats in the group treated with WR242511 and two untreated rats died soon after the start of the experiment; neither drug therapy nor *P. carinii* infection appeared to be contributing factors.

and WR6026 for the treatment of *Pneumocystis* pneumonia. These three compounds were effective for treatment at doses of 1 mg/kg/day or less (1.7 to 2.4 μmol/kg/day), whereas primaquine was somewhat effective at 2 mg/kg/day (4.4 μmol/kg/day) and showed better activity at this dose when combined with clindamycin at a dose of 225 mg/kg/day.

Results of the second and third of the three therapy studies were comparable (Tables 2 and 4). In both of these studies, drugs were given in drinking water. In the first therapy study (Table 1), the drugs were less effective, although their effectiveness was clearly different from that of the control, possibly because the drugs were given by gavage in a single daily dose. In earlier experiments (unpublished data) we observed that clindamycin is effective in combination with primaquine when given continuously in water but is ineffective when given once daily by gavage.

The models for animal studies at IU and UNC differed in several significant ways. IU used smaller female rats, while UNC used older, heavier male rats. No study has specifically addressed the susceptibility of rats to either *P. carinii* infection or immunosuppression related to the age and sex of the animals, although it is known that sex hormones can affect the immune response (9). At IU, animals received a regular diet, while at UNC animals received a low-protein diet. Diarrhea was noted in the clindamycin-treated rats at UNC (Table 7), while none was detected at IU. The diarrhea noted at UNC might have been related to the low-protein diet, the different intestinal flora of the rats, or both. In clinical trials with the combination of clindamycin and primaquine, diarrhea has not been a significant problem.

Finally, although there were many differences in the animal models, including sex, body size, diet, source of *P. carinii*, and evaluation systems, both models yielded comparable evaluations of these drugs for therapy.

Compounds WR6026, WR238605, and WR242511 also were effective for prophylaxis against *Pneumocystis* pneumonia when given continuously at daily doses of 0.57 mg/kg (1.4, 0.98, and 1 μmol/kg, respectively) (Table 5). At that same dose, primaquine alone (1.25 μmol/kg) showed only a weak effect but was effective in combination with clindamycin (Table 6).

For prophylaxis the 8-aminoquinolines were used at doses of 0.57 mg/kg, because a previous study (11) showed that this dose of primaquine combined with clindamycin at 225 mg/kg effectively prevented the development of *P. carinii* pneumonia. In the clindamycin-primaquine prophylaxis study (Table 6), primaquine alone at 0.57 mg/kg reduced the infectivity score by about one unit (roughly equivalent to a 90% decrease in the numbers of organisms). At that same dose, the WRAIR 8-aminoquinolines were more effective than primaquine and possibly could be used in much lower doses or in less frequent dosing regimens. Combination with clindamycin might provide even better prophylactic regimens. In future studies, we hope to establish the lowest effective doses for prophylaxis and to suggest optimal dosing regimens.

Although synergy between clindamycin and primaquine has been demonstrated in both therapy and prophylaxis models of *Pneumocystis* pneumonia, no such synergy has yet been demonstrated between the newer WRAIR 8-aminoquinolines and clindamycin, because the animal therapy studies which used 8-aminoquinolines in combination with clindamycin showed that the 8-aminoquinolines alone had excellent activities at the doses used. The dose range study of the three 8-aminoquinolines established that the lowest active doses were 0.25 to 0.5 mg/kg for WR242511 and 0.5 to 1.0 mg/kg for WR238605 and WR6026. With these dose ranges established, clindamycin combination studies can be performed to assess synergy and the possibility of using even lower doses of these 8-aminoquinolines when they are given in combination with clindamycin.

Although mechanisms of action have not been established for lincosamines such as clindamycin or for 8-aminoquinolines against *P. carinii*, both classes of drugs have been used for 15 years or more for the treatment of bacterial infections and *Plasmodium vivax* malaria. Methemoglobinemia is a

TABLE 6. Prophylaxis at IU of rats with *P. carinii* pneumonia with primaquine or primaquine plus clindamycin

Drug[a]	Dose (mg/kg/day)	Giemsa stain infectivity score[b]	No. infected/ total no.	Silver stain infectivity score[b]	No. infected/ total no.
Clindamycin + primaquine	225/0.57	1.4 ± 0.4[c]	8/11	1.8 ± 0.3	11/11
Primaquine	0.57	3.3 ± 0.3	7/7	3.2 ± 0.2	7/7
Untreated		4.4 ± 0.3	5/5	3.9 ± 0.1	5/7

[a] Drugs were administered in drinking water; immunosuppression was with cortisone acetate administered at 250 mg/kg subcutaneously twice weekly.
[b] Results are expressed as means ± standard errors. The scores are described in the text.
[c] $P < 0.001$ compared with untreated controls.

TABLE 7. UNC toxicity score

Group (dose [mg/kg])	Toxicity score[a]	Effect
Untreated		None, untreated controls
Clindamycin (225)	1+	Severe diarrhea in wk 8 of immunosuppression
WR238605 (4)	0	None
WR238605 (4) + clindamycin (225)	2+	Three rats died, severe diarrhea in wk 8 of immunosuppression
WR242511 (2)	0	None
WR242511 (2) + clindamycin (225)	2+	Two rats died, severe diarrhea in wk 8 of immunosuppression
WR6026 (2)	0	None
WR6026 (2) + clindamycin (225)	1+	Severe diarrhea in wk 8 of immunosuppression

[a] The toxicities of the test compounds were evaluated and scored as follows: 0, no observed local, clinical, or histologic toxicity; 1+, all animals survived the test dose without severe distress and no histopathology was noted; 2+, all animals survived the test dose but were observed to have clinical side effects, some histopathology, or both.

frequent consequence of primaquine therapy, and hemolysis may occur in patients with glucose 6-phosphate dehydrogenase deficiency. The WRAIR compounds have less of a potential than primaquine to cause methemoglobinemia because they can be used at much lower doses than primaquine can.

The rat model has predicted the usefulness of a number of drugs for treatment and prophylaxis of *Pneumocystis* pneumonia (4, 7, 10). The successful treatment and prophylaxis studies reported here suggest that clinical evaluations of these interesting orally administered compounds should be considered.

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