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Efficacy of a Hydroxynaphthoquinone, 566C80, in Experimental *Pneumocystis carinii* Pneumonitis

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The efficacy of a new class of drugs for *Pneumocystis carinii* pneumonitis was demonstrated. 566C80, a hydroxynaphthoquinone, administered orally in a dose of ≥100 mg/kg of body weight per day prophylactically prevented *P. carinii* pneumonitis in 90% or more of rats, while all untreated control animals developed pneumonitis. When 566C80 (100 mg/kg per day) was administered for 3 weeks after *P. carinii* pneumonitis was established, therapy was totally effective and all of the untreated controls had progressive *P. carinii* pneumonitis. A dose of 566C80 of between 25 and 50 mg/kg per day protected 50% of the rats from *P. carinii* pneumonitis, and a dose of between 50 and 100 mg/kg per day cured 50% of those treated for *P. carinii* pneumonitis. Both prophylaxis and treatment with 566C80 were at least as effective as with trimethoprim-sulfamethoxazole. Animals maintained on immunosuppression after completion of treatment remained free of *P. carinii*, suggesting a killing effect. Clearance of *P. carinii* was associated with levels of 60 μg or more of 566C80 per ml of plasma. This hydroxynaphthoquinone offers promise as an anti-*P. carinii* drug.

Until recently, *Pneumocystis carinii* pneumonitis was regarded as a relatively rare parasitic infection seen occasionally in infants and in immunocompromised adults. It could be controlled readily either with pentamidine isethionate or with trimethoprim-sulfamethoxazole (TMP-SMZ), the latter being preferred because of its oral bioavailability and its low level of adverse reactions. With the advent of acquired immune deficiency syndrome in the 1980s, the magnitude of the *P. carinii* pneumonitis problem has increased enormously. First, about 70% of acquired immune deficiency syndrome patients develop *P. carinii* pneumonitis, which is fatal if untreated. Second, for reasons that are still not clear, the frequency of serious adverse reactions with TMP-SMZ has increased to around 60% in patients with acquired immune deficiency syndrome, similar to that seen with pentamidine. Clearly, there is an urgent need for new drugs which are both highly efficacious and free from serious adverse reactions for the prophylaxis and therapy of *P. carinii* pneumonitis. We report here the efficacy of a new compound in the murine model of *P. carinii* pneumonitis which comes from a class of drugs strikingly different from those of other anti-*P. carinii* agents. The compound 2-[trans-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80 [Wellcome Research Laboratories, Beckenham, United Kingdom]), already in clinical development as an antimalarial agent, is effective by the oral route in both the prevention and treatment of *P. carinii* pneumonitis in the rat without obvious toxicity.

In 1946, it was noted that certain 2-hydroxy-3-alkylnaphthoquinones inhibited the respiratory processes of *Plasmodium* species (13). Subsequently, these findings were substantiated by Fieser and his collaborators (3), though no drug suitable for human use was discovered. A variety of naphthoquinones were also found to have activity against other protozoa, including trypanosomes (1, 2, 10, 12), *Theileria parva* (6, 11), *Toxoplasma* spp. (6), and certain *Eimeria* and *Plasmodium* species (6). Hydroxynaphthoquinones block protozoal respiratory chain electron transport at complex III, probably by functioning as analogs of ubiquinone (4).

There are no well-developed in vitro culture systems to measure the intrinsic susceptibility of *P. carinii* to drugs. The corticosteroid-treated rat model has been found to be remarkably similar to the human infection. This animal system is based on the premise that *P. carinii* pneumonitis can be provoked in over 90% of latently infected rats given dexamethasone and tetracycline for 4 to 6 weeks. Drugs found to be effective against *P. carinii* pneumonitis in these animals (9) have been shown subsequently to be equally effective in humans (8).

The studies to be described here represent a collaborative effort of investigators at St. Jude Children's Research Hospital in Memphis, Tenn., and the Wellcome Research Laboratories in Beckenham, United Kingdom. The hydroxynaphthoquinone 566C80 was under development at Wellcome as an antimalarial drug, and studies were in progress at St. Jude to identify new compounds effective against *P. carinii*. One of the Beckenham investigators (W.E.G.) suggested that 566C80 be tested in the St. Jude laboratory for efficacy, since *P. carinii* has been found to be susceptible to certain drugs with antiprotozoal activity. After demonstration of efficacy in the initial animal studies at St. Jude, similar studies were done at Wellcome for confirmation. Subsequently, these studies were expanded in both laboratories. We are presenting our data from the two laboratories separately, where appropriate, to demonstrate confirmation through similarity of results of independent experiments on separate continents.

MATERIALS AND METHODS

Experimental plan. The design of the experiments was based on the fact that treatment with a corticosteroid plus an antibiotic (to prevent bacterial infection) administered daily for 6 weeks or longer provokes *P. carinii* pneumonitis in 90% or more of the animals. The test drug was administered throughout the period of immunosuppression to determine

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TABLE 1. Extent of *P. carinii* after prophylaxis: histopathology of lung sections[a]

Site and drug group	Dose (mg/kg per day)[b]	No. of rats tested	No. of rats evaluated[c]	No. of rats with *P. carinii* pneumonitis				
				Extent of disease				Total (% of evaluated rats)
				None	1+	2+	3+	
Memphis								
Control	No drug	10	9	0	1	0	8	9 (100)
566C80	200 (r)	10	9	9	0	0	0	0 (0)
566C80	100 (r)	10	10	10	0	0	0	0 (0)
566C80[d]	100 (g)	10	8	8	0	0	0	0 (0)
566C80	100 (g)	10	9	8	1	0	0	1 (11)
566C80	50 (g)	10	9	7	0	1	1	2 (22)
566C80	25 (g)	10	8	1	2	1	4	7 (88)
566C80	10 (g)	10	10	1	1	0	8	9 (90)
TMP-SMZ	50/250 (r)	10	10	10	0	0	0	0 (0)
Beckenham								
Control	No drug	15	7	0	0	3	4	7 (100)
Control	Vehicle (g)	15	11	0	3	0	8	11 (100)
566C80	100 (g)	15	9	9	0	0	0	0 (0)
TMP-SMZ	50/250 (g)	15	11	11	0	0	0	0 (0)

[a] Lung sections stained with Grocott-Gomori stain.
[b] Mode of administration in parentheses. r, Rations; g, gavage.
[c] Excludes accidental deaths from gavage and loss from cannibalism.
[d] Initial screening study.

prophylactic efficacy in treated rats, which were compared with untreated controls. Assessment was based upon histological examination of the lungs for *P. carinii* pneumonitis. Also, therapeutic efficacy was determined by permitting the rats to develop *P. carinii* pneumonitis during immunosuppression and then intervening with the test drug. Untreated control animals as well as those receiving the test drug were maintained on the corticosteroid and the antibiotic throughout the experiments. To determine the extent of eradication of *P. carinii*, some animals were continued on the immunosuppressive drug for 4 weeks after the completion of a 3-week course of 566C80 and were then sacrificed for microscopic examination of the lungs. Concentrations of 566C80 in plasma were correlated with the absence and extent of infection.

Animals. Male Sprague-Dawley rats, not certified as virus-free, weighing approximately 200 g, were obtained from Hilltop Laboratory Animals, Inc., Scottdale, Pa., or Olac 1976 Ltd., Blackthorn, Bicester, Oxon, United Kingdom. The animals were caged in groups of five and were fed standard rations. All rats received dexamethasone and tetracycline in drinking water continuously throughout the experiments. The rats used for study of the prophylactic effects of the drug were sacrificed in a carbon dioxide chamber after 6 weeks of study, and those studied for the therapeutic effects were sacrificed after 8 to 8.5 weeks of immunosuppression, although some animals died earlier. At autopsy, the lower lobe of the right lung was removed and bisected. One portion was placed in Formalin and processed for sections with Grocott-Gomori methenamine silver nitrate stain. The other portion of lung was imprinted from its cut surface onto microscope slides and stained separately with toluidine blue O or cresyl echt violet. The histological sections were coded and read by W.T.H. (in the United States) or Adrian Brito-Babapulle or Christine Taylor (in the United Kingdom). The lung sections were scored as "none" if no *P. carinii* organisms were seen, 1+ if cysts were seen sparsely distributed with less than one organism per 25 high-power fields, 2+ if focal areas of *P. carinii* pneumonitis were seen surrounded by 10 to 25 high-power fields of normal lung, and 3+ if the lung was diffusely and extensively penetrated by organisms in almost all high-power fields. Ten or fifteen rats made up a group for both the drug-treated and the control groups.

Drugs. The drugs were administered orally (p.o.) by gavage or in food or drinking water.

(i) Dexamethasone-tetracycline. Dexamethasone sodium phosphate, injection USP (Elkins-Sinn, Inc., Cherry Hill, N.J., or Sigma Chemical Co. Ltd., Poole, United Kingdom) (2.0 mg), and tetracycline hydrochloride (Sumycin, E. R. Squibb & Sons, Princeton, N.J.; or Sigma) (500 mg) were added to 1 liter of drinking water. Each rat consumed 30 to 50 ml of the medicated water each day ad lib.

(ii) 566C80. 566C80 was formulated at Wellcome Research Laboratories as a suspension in methylcellulose. The doses by gavage were given once daily in a volume of 0.5 ml of suspension. In experiments in which the drug was administered in food, the daily allocation of rations was pulverized and mixed with 566C80 in powder form. After thorough mixing, water was added to make a thick paste, and the preparation was dried into pellets.

(iii) TMP-SMZ. TMP at 50 and SMZ at 250 mg/kg of body weight per day were administered in the drinking water. A suspension of fixed drug combination (Septra suspension; Burroughs Wellcome Co., Research Triangle Park, N.C., or Wellcome Foundation Ltd., Dartford, United Kingdom) was used. A daily dose was added to the volume of dexamethasone-tetracycline water consumed each day.

Administration of drugs. The drug preparations were prepared freshly every other day. The drinking water and bottles were changed daily or on alternate days. Medicated food pellets were administered within 5 days after preparation. The drugs administered by gavage were given either daily (in Beckenham) or 5 days a week (in Memphis) by metal feeding tube. The animals were weighed biweekly. The volume of water and weight of food consumed daily were measured and used to calculate the amount for subse-

TABLE 2. Extent of *P. carinii* after treatment: histopathology of lung sections[a]

Site and drug group	Dose (mg/kg per day)[b]	No. of rats tested	No. of rats evaluated	No. of rats with *P. carinii* pneumonitis				
				Extent of disease				Total (% of evaluated rats)
				None	1+	2+	3+	
Memphis								
Control	No drug[c]	10	10	0	1	3	6	10 (100)
Control	No drug	10	9	0	1	0	8	9 (100)
566C80	100 (g)	10	8	8	0	0	0	0 (0)
566C80	50 (g)	10	9	2	0	3	4	7 (78)
566C80	25 (g)	10	8	1	0	1	6	7 (88)
TMP-SMZ	50/250 (r)	10	10	8	1	1	0	2 (20)
Beckenham								
Control	No drug	5	5	0	1	3	1	5 (100)
566C80[d]	100 mg	5	5	4	1	0	0	1 (20)

[a] Lung sections stained with Grocott-Gomori stain.
[b] Mode of administration in parentheses. g, Gavage; r, rations. Rats in Memphis study received drugs for 3 weeks; rats in Beckenham study received drugs for 2 weeks.
[c] Five of ten rats sacrificed at 4 weeks of immunosuppression when therapeutic drugs were started.
[d] Started on 566C80 treatment after 6 weeks of dexamethasone administration.

quent delivery of daily drug doses. The amount of wastage of food and water was estimated to be about 15% daily.

Drug concentration. Plasma for determining 566C80 concentrations was collected by cardiac puncture at the time of sacrifice in the CO_2 chamber. 566C80 concentrations were measured at Wellcome Research Laboratories by a method (unpublished) based on gas chromatography.

RESULTS

An initial screening experiment tested 566C80 prophylactically along with other candidate compounds at a dosage of 100 mg/kg per day p.o. by gavage. The results suggested that 566C80 is totally effective in comparison with untreated controls and that it is as good as the benchmark, TMP-SMZ (Table 1). The experiment was repeated in Beckenham and yielded similar results.

A second prophylactic study confirmed the activity of 566C80 at 100 mg/kg per day p.o. by gavage and in addition showed a dose-related effect in which smaller doses were less effective (50 mg/kg) or essentially ineffective (25 or 10 mg/kg). When the drug was administered in the rations rather than by gavage, infection was totally prevented in rats given either 200 or 100 mg/kg per day (Table 1).

Animals were allowed to develop pneumonitis, and 566C80 was then administered to determine its therapeutic effect. A Beckenham study in which rats were treated with 566C80 at 100 mg/kg per day p.o. by gavage beginning after 6 weeks of immunosuppression, when *P. carinii* pneumonitis was in an advanced stage, yielded an 80% cure rate (Table 2). Even better results were seen in the Memphis study, in which treatment was begun earlier, after 4 weeks of immunosuppression. All animals that received doses of 100 mg/kg per day were cured, as determined by histological examination, compared with an 80% cure rate with co-trimoxazole (TMP-SMZ) (Table 2). Lower doses of the drug (50 and 25 mg/kg per day) were less effective.

To gain some insight into the killing and static effects of 566C80, immunosuppressed rats were treated in Memphis for 3 weeks with the equivalent of 100 mg/kg per day p.o. of drug in the diet beginning at the time dexamethasone administration was started. Animals were then continued on dexamethasone for a further 4 weeks after the last dose of 566C80 before sacrifice. Of the 10 animals managed in this manner, none had histologically discernible *P. carinii* infection, suggesting that, in contrast to pentamidine and co-trimoxazole (7), 566C80 has a killing effect on *P. carinii*.

The concentrations in plasma obtained 2 to 3.5 h and 1 week after the last gavage dose of 566C80 of the Memphis experiments shown in Table 1 and measured as coded samples in Beckenham are shown in Table 3. Even though the parasites are sequestered in the lung, there is a correlation between concentration in plasma and level of infection (Fig. 1).

DISCUSSION

The results show that a dose of 566C80 of between 25 and 50 mg/kg per day p.o. will protect 50% of rats from *P. carinii* pneumonitis and that a dose of between 50 and 100 mg/kg per day p.o. will cure 50% of those already infected. The dose of 100 mg/kg per day is totally effective in the treatment and prevention of the pneumonitis. These doses are markedly higher (>100 times) than those required to protect mice from rodent-infecting strains of malaria (5). However, no gross evidence of toxicity, such as early deaths, excessive weight loss, bleeding, diarrhea, or neurological deficits, was observed during our experiments. The preclinical toxicological studies carried out with the compound at Beckenham before

TABLE 3. Concentrations of 566C80 in plasma

Dose of 566C80 (mg/kg per day)[a]	Interval between dose and plasma specimen	No. of rats studied	Concn of 566C80 in plasma (μg/ml)		
			Range	Mean	SD ± 1
None	NA[b]	3	0	0	0
10	2 h	4	17–31	24.3	4.9
25	2.5 h	6	7–75	51.7	22.3
50	3 h	6	63–94	71.2	10.8
100	3.5 h	5	61–88	73.6	8.9
100	1 wk	7	7–44	14.4	12.6

[a] All doses administered by gavage. Blood collected by cardiac puncture after CO_2 sacrifice.
[b] NA, Not applicable.

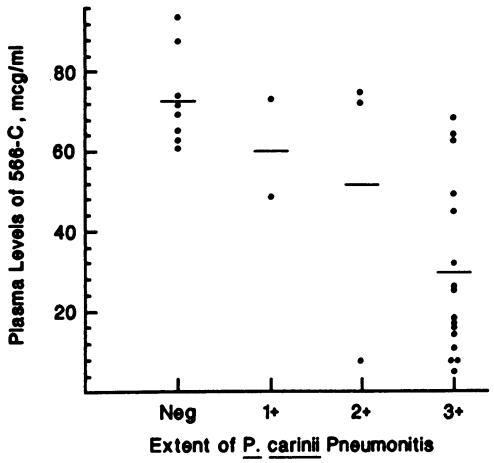

FIG. 1. Concentration of 566C80 in plasma taken at the time of autopsy matched with extent of *P. carinii* pneumonitis determined by histopathological examination of lungs. See Materials and Methods for designations for the extent of pneumonitis ("Neg" in the figure corresponds to "none" in the text and tables).

human volunteer studies suggest that as an antimalarial agent, 566C80 has a very large therapeutic window. Furthermore, we have preliminary evidence to suggest that as with other protozoa, this compound exerts its antimicrobial effects via a blockade of electron transport. Its mode of action is thus quite distinct from those of the antifols (TMP, pyrimethamine, trimetrexate, piritrexim, SMZ, and dapsone), which together with pentamidine (whose mode of action is unknown) dominate not only those therapies already in clinical use but also those in development. There is a strong case for evaluating 566C80 in humans with *P. carinii* pneumonitis as soon as possible, especially in patients with acquired immune deficiency syndrome. We do not know at this stage whether the high 566C80 concentration in plasma that is likely to be required will be attainable and safe.

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