Remarkable In Vitro and In Vivo Activities of the Hydroxynaphthoquinone 566C80 against Tachyzoites and Tissue Cysts of *Toxoplasma gondii*

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Compound 566C80, 2-[trans-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone, was studied for its in vitro and in vivo activities against *Toxoplasma gondii*. Replication within human foreskin fibroblasts of tachyzoites of seven different strains, five of them isolated from AIDS patients, was inhibited by concentrations as low as 4.8×10^{-9} M. In vivo, a dose of 100 mg/kg of body weight per day, administered by gavage for 10 days, protected 100% of mice against death due to infection with five different strains of *T. gondii*, including the highly virulent RH strain. A dose of 50 mg/kg/day protected at least 80% of mice infected with the same inoculum, and a dose as low as 9.3 mg/kg/day protected 40 to 60% of mice. Treatment with 50 mg/kg/day for 30 days completely eradicated parasites from mice infected with four of five strains of *T. gondii*. 566C80 was active in vitro against the cyst stage of *T. gondii* at concentrations of 50 to 100 μg/ml. In vivo activity against this form of *T. gondii* was examined in mice infected for 6 weeks with strain ME49 and then treated orally with 100 mg of 566C80 per kg per day for 8 weeks. Treated mice sacrificed at 2-week intervals revealed a steady decline in the numbers of cysts in their brains compared with untreated controls. In addition, mortality as well as clinical signs of brain infection was absent from treated mice, whereas control mice had a high mortality rate and showed clinical signs of central nervous system infection. These results reveal remarkable in vitro and in vivo activities of 566C80 against *T. gondii*.

In addition to causing debilitating disease in congenitally infected infants and children, *Toxoplasma gondii* has emerged as a major opportunistic pathogen of immunocompromised individuals, particularly those infected with the human immunodeficiency virus (17, 18, 21). The treatment of choice for toxoplasmosis has been the synergistic combination of pyrimethamine and a sulfonamide (3). Prolonged treatment with pyrimethamine, a potent inhibitor of the enzyme dihydrofolate reductase, may result in bone marrow depression, a side effect of particular importance in immunocompromised individuals (19). When pyrimethamine is administered with a sulfonamide, the combination is associated with untoward effects in at least 60% of patients with AIDS, often leading to discontinuation of at least the sulfonamide component (12). Moreover, because pyrimethamine is potentially teratogenic, its use during the early months of gestation is not recommended. Alternative drugs and treatment schemes have been shown to be effective in animal models of toxoplasmosis (1, 2, 4), but their usefulness in treating the disease in humans and their efficacy compared with that of the pyrimethamine-sulfonamide combination remains to be confirmed in clinical trials. Thus, there is a critical need for the development and evaluation of new drugs or new drug combinations for the prevention and treatment of congenital toxoplasmosis, ocular toxoplasmosis in older children and adults, and toxoplasmosis in immunocompromised patients. Certain hydroxynaphthoquinones have previously been shown to have some activity in experimental toxoplasmosis, but they are not of sufficient promise to warrant development (11). Hydroxynaphthoquinone 566C80 was originally synthesized at Wellcome Research

Laboratories, Beckenham, United Kingdom, and is now in development as an antimalarial agent (10). Since there were already some in-house data showing that this hydroxynaphthoquinone also had activity against *T. gondii* (10a), we decided to investigate this activity in detail. We report here results which reveal the remarkable in vitro and in vivo activities of this compound against both the tachyzoite and the tissue cyst stages of *T. gondii*.

MATERIALS AND METHODS

Mice. Outbreed Swiss Webster (Simonsen Laboratories, Gilroy, Calif.) and inbreed CBA/Ca (Bantin and Kingman Laboratories, Newark, Calif.) females, weighing 20 g at the beginning of each experiment, were used. Food and water were available to the animals at all times.

T. gondii. Strains RH (22), ME49 (24), POE, HART, VEL, CAST, SO, and MO were used. With the exception of strains RH and ME49, each strain was isolated in our laboratory from patients with AIDS. Following isolation, these strains have been maintained as chronic infections in mice. To obtain tachyzoites of each strain except RH for in vitro and in vivo experiments, brains of chronically infected mice were inoculated into the peritoneal cavities of normal mice pretreated with antiserum to gamma interferon, as previously described (25). Tachyzoites were harvested, filtered to remove contaminating peritoneal cells (5), counted in a hemacytometer, and used for both in vivo and in vitro experiments.

566C80. The compound 566C80, 2-[trans-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone, with the empirical formula $C_{22}H_{19}O_3Cl$ and with a molecular weight of 366.83 (Fig. 1), was originally synthesized by The Wellcome Foundation Ltd., Beckenham, as an antimalarial agent
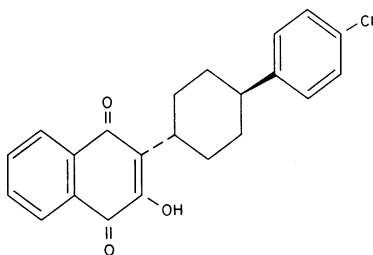
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FIG. 1. Structural formula of 566C80 {2-[trans-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone}.

(14). It was used either as a powder for in vitro experiments or as a suspension in 1% (vol/vol) Celacol for in vivo experiments.

In vitro activity. The in vitro activity of 566C80 was examined by using intracellular tachyzoites and isolated tissue cysts of *T. gondii*. For experiments with tachyzoites, 566C80 powder was dissolved to a concentration of 4.8 × 10^{-3} M in 2.1 ml of absolute ethanol and then further diluted 1:10, also in absolute ethanol. Subsequent $\log_{10}$ dilutions were prepared in RPMI 1640 tissue culture medium containing 10% human plasma obtained from a donor with no serological evidence of infection with *T. gondii*. Control medium contained the same concentrations of ethanol as did the medium that contained 566C80. The effect of the drug on the replication of intracellular *T. gondii* was evaluated by [³H]uracil incorporation, as previously described (16). Briefly, human foreskin fibroblasts (HFF) were grown almost to confluence and incubated for 1 h at 37°C with tachyzoites at a ratio of two parasites per cell. Thereafter, the monolayers were washed, medium containing the desired concentrations of 566C80 was added, and the cultures were incubated for various periods of time at 37°C in an atmosphere containing 5% CO_2. Cultures were pulsed for 8 h with [³H]uracil before being harvested and counted in a liquid scintillation counter. All determinations were performed in triplicate. To confirm results obtained with [³H]uracil incorporation, selected strains were also examined by staining the HFF cell monolayers with Diff-Quick (American Scientific Products, McGaw Park, Ill.). The monolayers were examined under light microscopy for intracellular replication of tachyzoites (20).

We have recently developed an in vitro method for testing drugs against the tissue cyst form of *T. gondii* (15a). Briefly, cysts were isolated from brains of CBA/Ca mice chronically infected with strain ME49 and were resuspended in RPMI 1640 tissue culture medium containing 10% inactivated fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B (Fungizone) per ml, and various concentrations of 566C80. In previous studies (unpublished), we have not noted any significant anti-*T. gondii* effect of amphotericin B. Thereafter, the cyst suspensions were incubated at 37°C for 24 and 72 h. Activity against tissue cysts was evaluated by intraperitoneal (i.p.) inoculation of treated or control cysts into two mice per group. The mice were pretreated with antiserum to gamma interferon to increase their susceptibility to the infection (25). Mice were monitored to determine time of death, total mortality, and the presence of tachyzoites in their peritoneal cavities. Surviving mice were tested for the presence of antibodies to *T. gondii* by the Sabin-Feldman dye test (DT) (23) and also for the presence of tissue cysts in their brains. Controls were cysts treated with medium without 566C80. Clindamycin, an

antibiotic which is active in murine models of toxoplasmosis (2), was also used.

In vivo activity. Mice were inoculated i.p. with 3 × 10^3 tachyzoites of strain SO, VEL, POE, RH, or CAST, and 24 or more hours thereafter they were treated with different concentrations of 566C80 administered as a single oral dose daily for 10 or 30 days. Controls were treated similarly but with the diluent only. Mice were checked twice daily for death, and the presence of parasites in dead animals was determined by microscopic examination of peritoneal fluid. Survivors were bled for DT antibody determination. In addition, survivors of each experimental group were euthanized with CO_2, and the presence of residual infection with *T. gondii* was determined by the subinoculation of triturated portions of spleen and liver (2) and by microscopic examination of brains for cysts of the parasite. Each individual brain was ground with mortar and pestle and suspended in 1 ml of phosphate-buffered saline, pH 7.2. Five aliquots of 20 μl each were examined by light microscopy, and the results were reported as the mean number of cysts ± standard deviation. Mice surviving the subinoculation of spleen and liver suspensions were bled, and their sera were examined for the presence of DT antibodies.

To determine whether 566C80 has in vivo activity against tissue cysts of *T. gondii*, CBA/Ca mice were infected i.p. with 20 cysts of the ME49 strain. CBA/Ca mice infected with this strain develop a chronic progressive toxoplasmic encephalitis and, unless treated, begin to die approximately 6 weeks after the infection. Large numbers of toxoplasma cysts are present in their brains after 3 weeks of infection. Six weeks after infection, the mice were treated by gavage with 100 mg of 566C80 per kg of body weight per day; treatment was continued for 8 weeks. Control mice were treated with diluent only. At 2-week intervals after the initiation of treatment, two or three mice were killed, the entire brains were removed, and the numbers of cysts in the brain suspensions were determined as described above.

Serum levels of 566C80 were determined by treating mice by gavage with 100 mg/kg/day for 4 days. Groups of three mice each were killed at 3, 6, 12, 24, and 48 h after the last treatment, and then the sera were collected, immediately frozen, and shipped in dry ice to the Wellcome Foundation, where levels of 566C80 in serum were determined by a technique based on gas chromatography. Results are expressed in micrograms per milliliter.

RESULTS

Activity against intracellular replication of *T. gondii* in vitro. The capacity of 566C80 to inhibit intracellular replication of *T. gondii* within HFF was different for the different strains of the parasite (Fig. 2). For tachyzoites of the RH strain, a concentration as high as 4.8 × 10^{-6} M was not sufficient to inhibit their replication completely (Fig. 2A). In contrast, intracellular replication of organisms of strains VEL and HART was completely inhibited by a concentration of 4.8 × 10^{-9} M (Fig. 2B and C). For strains ME49 and POE, the lowest inhibitory concentration was 4.8 × 10^{-7} M (Fig. 2D and E); strain CAST was inhibited by 4.8 × 10^{-8} M but not by 4.8 × 10^{-9} M 566C80 (Fig. 2F). Results obtained by the measurement of [³H]uracil incorporation were confirmed by the results obtained when the cell monolayers were stained and replication of the organisms was determined by light microscopy (data not shown). Microscopic examination of the cell cultures also revealed that 4.8 × 10^{-6} M 566C80 was toxic for the HFF. Approximately 100% of
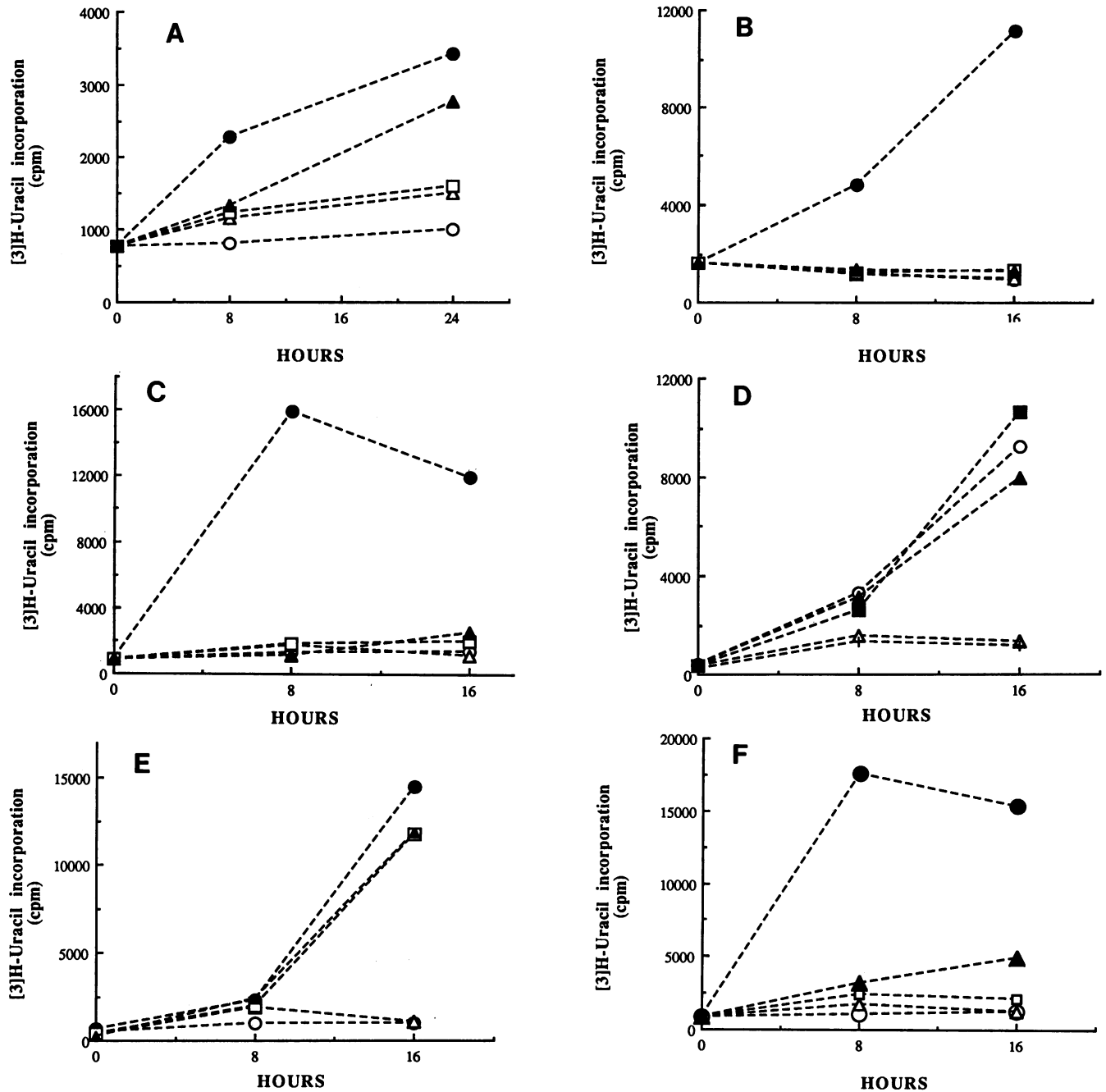
FIG. 2. In vitro activity of 566C80 on intracellular replication of tachyzoites of strains RH (A), VEL (B), HART (C), ME49 (D), POE (E), and CAST (F) of *T. gondii*. (A through C, E, and F) Symbols: $\bigcirc$, 4.8×10^{-6} M; $\triangle$, 4.8×10^{-7} M; $\square$, 4.8×10^{-8} M; $\blacktriangle$, 4.8×10^{-9} M; $\bullet$, no drug. (D) Symbols: $\triangle$, 4.8×10^{-6} M; $+$, 4.8×10^{-7} M; $\bigcirc$, 4.8×10^{-8} M; $\blacktriangle$, 4.8×10^{-9} M; $\blacksquare$, no drug.

the cells cultured with this concentration exhibited cytoplasmic vacuolization and changed from spindle shaped to round. No visible signs of toxicity was noted with a concentration of 4.8×10^{-7} M.

Activity against isolated tissue cysts. Mice injected with cysts that had been incubated with medium alone or with medium containing 1 μg of 566C80 per ml (2.8×10^{-6} M) or 100 μg of clindamycin per ml all died between days 9 and 13 of the infection (Table 1). Tachyzoites were present in the peritoneal fluid of each of these mice. In contrast, none of

the mice inoculated with cysts that had been incubated with 100 μg of 566C80 per ml (2.8×10^{-4} M) died, developed antibodies to *T. gondii*, or had demonstrable tissue cysts in their brains.

Activity against acute infection in vivo. Preliminary experiments revealed that a concentration of 100 mg/kg/day administered for 10 days consistently protected 100% of mice against death due to i.p. infection with 3×10^3 tachyzoites of the RH strain when treatment was initiated 24 h after infection. A dose response is shown in Fig. 3. One hundred,

TABLE 1. In vitro activity of 566C80 against isolated cysts of *T. gondii*

Treatment drug and amt (μg)	Duration of treatment[a] (h)	Presence of tachyzoites in peritoneal fluid	Time to death (days)	Result for survivors[b]	
				DT titer	Test for cysts in brain
None	24	Yes	10[c]		
	72	Yes	10		
Clindamycin					
1	24	Yes	9		
	72	Yes	9		
100	24	Yes	9		
	72	Yes	9		
566C80					
1	24	Yes	10		
	72	Yes	13		
5	24	Yes	11		
	72	ND[d]	—[e]	Neg./Pos.	Neg./Pos.
50	24	ND	—	Pos.	Pos.
	72	ND	—	Neg.	Neg.
100	24	ND	—	Neg.	Neg.
	72	ND	—	Neg.	Neg.

[a] Duration of incubation of the cysts with the drug.

[b] Pos., Both inoculated mice tested positive; Neg., both mice tested negative; Neg./Pos., one mouse tested negative and the other tested positive.

[c] Day when all mice inoculated with treated or control cysts were dead.

[d] ND, Not done.

[e] —, Mice survived inoculation.

75, 40, and 40% of mice inoculated i.p. with 3×10^3 RH tachyzoites survived when treated with concentrations of 75, 37.5, 18.7, and 9.3 mg/kg/day, respectively. A concentration as low as 4.6 mg/kg/day afforded some protection. Further experiments revealed that 100% of mice inoculated with RH tachyzoites as described above survived the infection when treated with 100 mg of 566C80 per kg per day for 10 days if treatment was initiated 24 h after infection. When treatment was initiated later, the percent survival decreased but a significant delay in the time to death occurred (Table 2). Thus, 100% of infected and untreated mice were dead by day 8 after infection. Infected mice whose treatment began 96 and 72 h after infection had 100% mortality rates on days 10 and 13, respectively. One hundred and 40% of mice whose treatment started 24 or 48 h after infection survived up to day 35, when the experiment was terminated (Table 2).

Activity against different strains of *T. gondii* was determined by using an i.p. inoculum of 3×10^3 tachyzoites. Initiation of treatment was begun 24 h after infection, with 10, 20, or 50 mg/kg/day administered as a single daily dose for 30 days. As noted in the in vitro experiments, the results obtained in vivo also revealed a striking variation in the activity of 566C80 against the different strains of *T. gondii* (Fig. 4). For example, 100% of mice infected with strain SO and treated with 10 mg/kg/day survived (Fig. 4A), whereas none of the mice infected with strain VEL survived when treated with the same dose (only 60% survived when treated with 50 mg/kg/day [Fig. 4B]). Results with the other strains were between those observed with strains SO and VEL (Fig. 4C through E). For each strain, untreated controls were all dead between days 5 and 8 of infection. One day after treatment was discontinued, two or three surviving mice were euthanized and the presence of residual infection was determined by microscopic examination of suspensions of brains and by subinoculation of suspensions of triturated
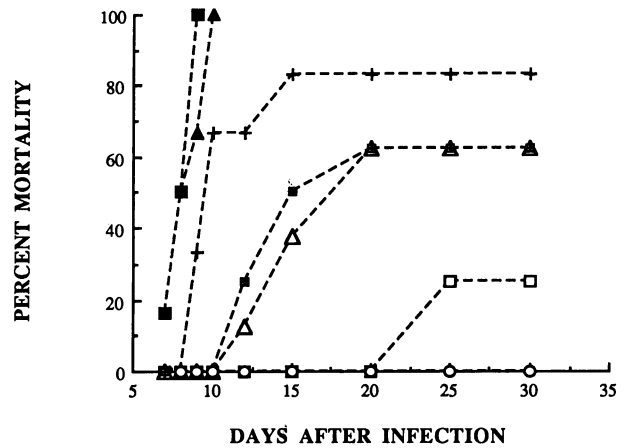

DAYS AFTER INFECTION

FIG. 3. Effect of different concentrations of 566C80 in mice infected with strain RH of *T. gondii*. There were five to eight animals in each group. Symbols: ○, 75 mg/kg; □, 37.5 mg/kg; △, 18.7 mg/kg; ⊞, 9.3 mg/kg; +, 4.6 mg/kg; ▲, 2.3 mg/kg; ■, control.

spleens and livers into normal mice. The results are shown in Table 3. *T. gondii* cysts were not observed by microscopy in the brains of mice treated with any of the three doses of 566C80, suggesting that colonization and cyst formation in the brains were prevented by 566C80. The presence of parasites, however, was demonstrated by subinoculation of spleen-liver suspensions of all survivors that had been treated with 10 or 20 mg of 566C80 per kg. On the other hand, treatment with 50 mg/kg was sufficient to completely eradicate parasites of four of the five strains. Thus, all mice subinoculated with spleen-liver suspensions of survivors that had been infected with strain RH, MO, SO, or CAST did not have tachyzoites in their peritoneal cavities and had negative DT titers. One of three mice inoculated with spleen-liver suspensions of survivors that had been infected with strain VEL died of toxoplasmosis. The remaining two mice did not have parasites in their peritoneal cavities and had negative DT titers.

Activity against *T. gondii* cysts in vivo. In spite of the wide mouse-to-mouse variation in the number of *T. gondii* cysts in the brain, treated mice had considerably fewer cysts in their brains than the controls (Fig. 5). Moreover, the mortality rate among the control mice was continuous; by 13 weeks of infection, all were dead from the infection. No deaths occurred in the treated mice up to 14 weeks of infection (8 weeks of treatment), when the experiment was terminated.

TABLE 2. Activity of 566C80 against *T. gondii* when drug was administered at different times after infection[a]

Treatment start time (h)[b]	% Mortality on day after infection												
	7	8	9	10	11	12	13	14	15	20	25	30	35
24	0	0	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	20	20	20	20	20	20	40	40	60	60
72	0	0	40	60	60	80	100						
96	20	60	60	100									
—[c]	40	100											

[a] Infection was i.p. with 3×10^3 RH tachyzoites.

[b] Treatment was 100 mg of 566C80 per kg per day, started at the indicated times after infection and continued for 10 days. Five mice were used in each set of treatments.
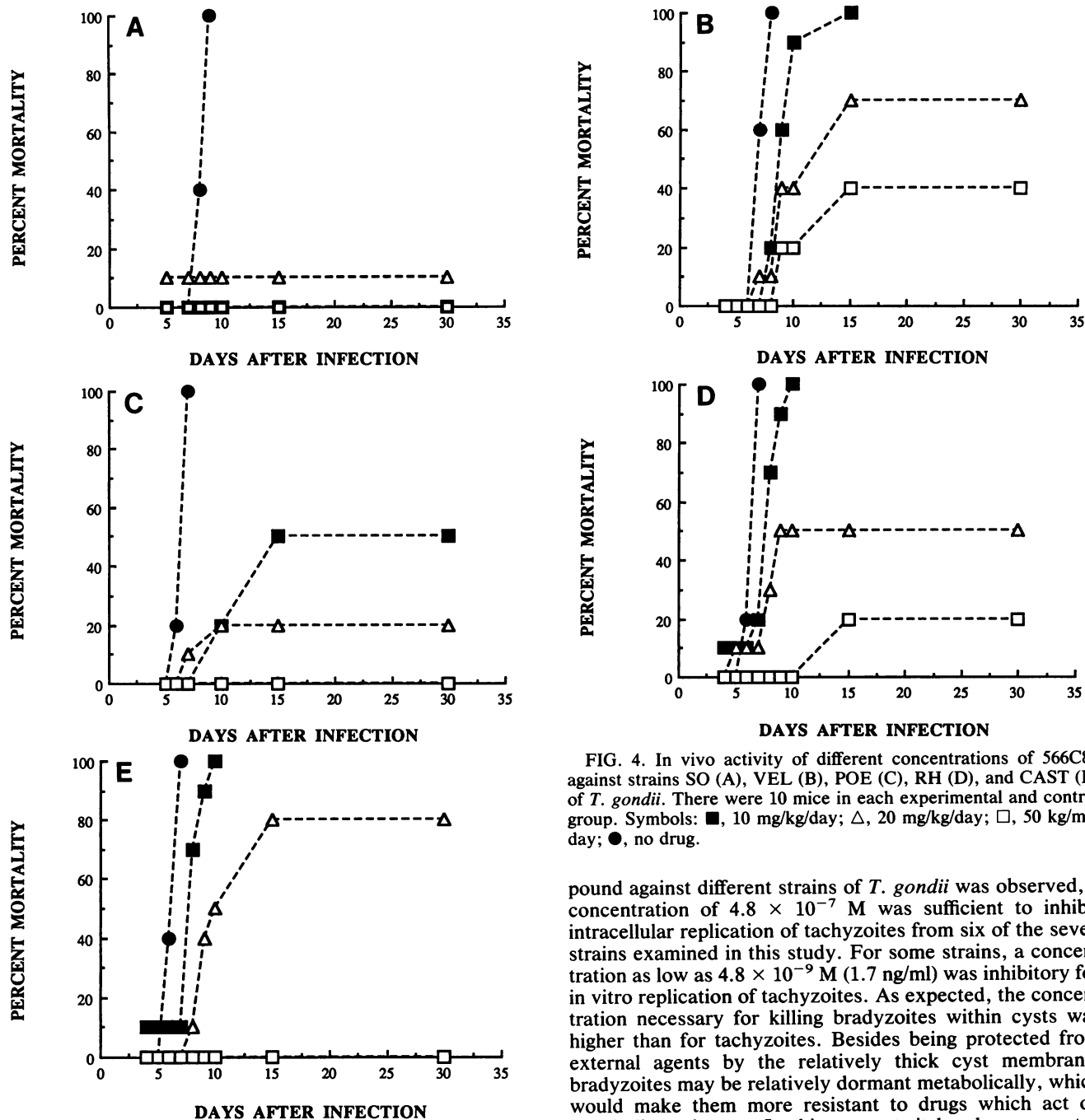
[c] —, No treatment.

FIG. 4. In vivo activity of different concentrations of 566C80 against strains SO (A), VEL (B), POE (C), RH (D), and CAST (E) of *T. gondii*. There were 10 mice in each experimental and control group. Symbols: ■, 10 mg/kg/day; △, 20 mg/kg/day; □, 50 kg/mg/day; ●, no drug.

Levels of 566C80 in serum. Levels of 566C80 in serum are shown in Fig. 6. At 3 and 24 h after the last dosing, the concentrations of 566C80 in the sera of the three mice killed at each time were 34.6 ± 4.1 and 10.6 ± 4.9 μg/ml, respectively (mean ± standard deviation). There was considerable mouse-to-mouse variation.

DISCUSSION

The results above indicate that compound 566C80 has remarkable in vitro as well as in vivo activity against *T. gondii*. Although some variation of the activity of the compound against different strains of *T. gondii* was observed, a concentration of 4.8 × 10⁻⁷ M was sufficient to inhibit intracellular replication of tachyzoites from six of the seven strains examined in this study. For some strains, a concentration as low as 4.8 × 10⁻⁹ M (1.7 ng/ml) was inhibitory for in vitro replication of tachyzoites. As expected, the concentration necessary for killing bradyzoites within cysts was higher than for tachyzoites. Besides being protected from external agents by the relatively thick cyst membrane, bradyzoites may be relatively dormant metabolically, which would make them more resistant to drugs which act on metabolic pathways. In this context, it has been reported that morphological changes observed by light and electron microscopy occurred in replicating organisms within cysts, within the cyst wall, and in bradyzoites of cysts obtained from chronically infected mice treated with sulfamethoxy-pyrazine-pyrimethamine (28). The degree of damage was observed to be proportional to the intensity of metabolism ("metabolism" was not defined). Small cysts with dividing, "metabolically active" bradyzoites showed damage, whereas large, "metabolically dormant" cysts were not affected (28). The mechanism of action of 566C80 against toxoplasmas has not been determined yet. However, antiprotozoal hydroxynaphthoquinones appear to interfere with the electron transport pathway by acting as analogs of ubiquinone (11).

TABLE 3. Residual infection in mice infected with different strains of *T. gondii* that survived treatment with 566C80

Strain of T. gondii	Result with 566C80 treatment at (mg/kg/day)[a]:					
	10		20		50	
	No. of cysts[b]	Subinoc- ulation[c]	No. of cysts	Subinoc- ulation	No. of cysts	Subinoc- ulation
RH	ND[d]	ND	0	Pos.[e]	0	Neg.[f]
MO	0	Pos.	0	Pos.	0	Neg.
SO	0	Pos.	0	Pos.	0	Neg.
VEL	ND	ND	0	Pos.	0	Pos.
CAST	ND	ND	0	Pos.	0	Neg.

[a] Each set of treatments was for 30 days.
[b] Number of cysts in brains.
[c] Results of subinoculation of suspensions of spleen and liver (see text).
[d] ND, Not done.
[e] Pos., *T. gondii* was demonstrated in at least one of the two or three mice inoculated with the spleen-liver suspensions.
[f] Neg., *T. gondii* was not demonstrated in any of the three mice inoculated; serological results (DT) were also negative in all subinoculated mice.

FIG. 6. Levels of 566C80 in serum. Mice were treated with 100 mg of 566C80 per kg per day for 4 days and were then killed at the indicated times after the last dosing.

The results of the in vivo experiments revealed that all mice treated with 100 mg of 566C80 per kg per day survived the infection with each of the strains of *T. gondii*, including the highly virulent RH strain. This concentration of the drug yielded levels of 566C80 in serum as high as 42 μg/ml, which did not cause any obvious toxicity to the mice. This concentration was well tolerated; none of the mice had any signs of drug toxicity in spite of the fact that in some experiments they were treated with 100 mg/kg/day for 8 weeks. Thus, although the results of the in vitro experiments indicated cytotoxicity at a concentration of 4.8×10^{-6} M (1.7 μg/ml), the in vivo experiments did not reveal toxicity at a much higher level of the drug in serum. This lack of toxicity in vivo has been confirmed in volunteer studies in humans (11). Treatment with a concentration of 50 mg/kg/day for 30 days was sufficient to prevent the death of 100% of the mice
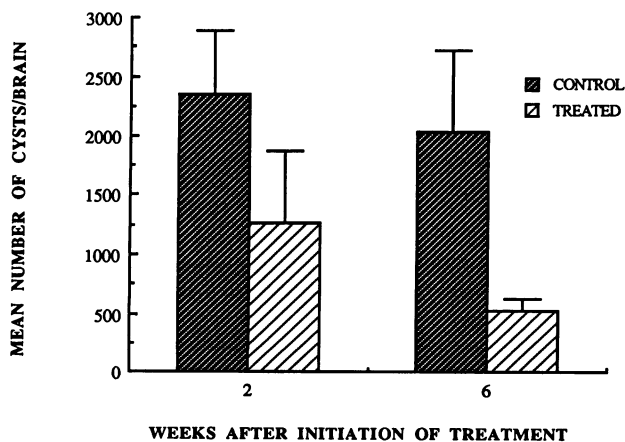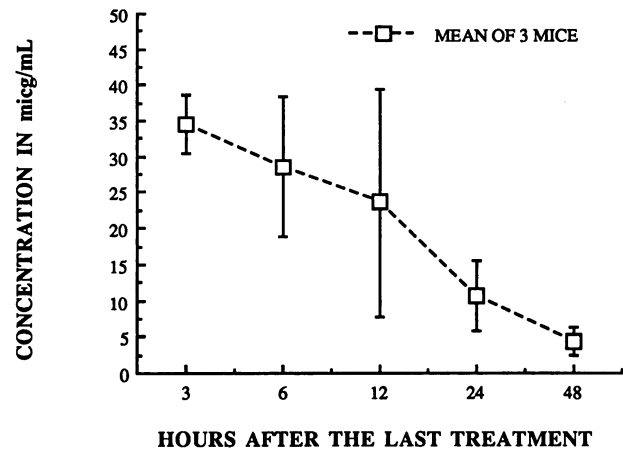

WEEKS AFTER INITIATION OF TREATMENT

FIG. 5. In vivo activity of 566C80 against tissue cysts of *T. gondii* in brains of mice. Oral treatment with 100 mg/kg/day was initiated 6 weeks after infection with strain ME49 and was continued for 8 weeks. Mice were sacrificed at the indicated times, and the numbers of cysts in their brains were determined as described in the text. All control mice were dead by 8 weeks after initiation of treatment. At that time treatment in the test mice was terminated. At 2 weeks, each bar represents the mean and standard deviation of two mice ($P < 0.05$, *t* test), and at 6 weeks, each bar represents the same for three mice ($P < 0.01$, *t* test).

infected with strains SO, POE, and CAST and at least 60% of those infected with strains VEL and RH. Of interest was the observation that cysts of the parasite were not observed in the brains of surviving mice by microscopy. Moreover, these mice were proven to be free of parasites by subinoculation experiments and by serology. Thus, eradication of *T. gondii* was achieved by treatment with a relatively low dose of 566C80, provided that it was initiated early during the infection. The protective activity of 566C80 against death due to the infection decreased when treatment was begun later than 24 h after infection. It must be noted, however, that although the experiment was conducted with tachyzoites of the highly virulent RH strain, some protection, represented by a delay in the time of death, was observed. In the experiment for determining activity against the cyst form in vivo, the mildly virulent strain ME49 was used. In this case, protection against death was achieved when the treatment was initiated as late as 6 weeks after the mice had been infected. Thus, mice begun on treatment with 100 mg of 566C80 per kg per day 6 weeks following infection and continued on treatment for 8 weeks did not die. In contrast, untreated controls began dying 6 weeks after infection, and all were dead by week 13. Our results concerning the levels of the drug in serum suggest that more frequent administration of 566C80 may be even more effective.

An important observation in this study was that treatment resulted in a steady decline in the number of *T. gondii* cysts in the brains of the chronically infected mice. There are reports in the literature suggesting that prolonged treatment with the pyrimethamine-sulfonamide combination may result in a reduction of the number of cysts in the brains of chronically infected mice (9, 26, 27). However, because of the variation in the reported observations, the general consensus is that tissue cysts of *T. gondii* are resistant to killing by the pyrimethamine-sulfonamide combination (6, 8) or by other drugs used either alone or in combinations. Although the opinions of scientists and clinicians regarding the anticyst activities of various drugs for the treatment of *T. gondii* may vary (6, 9, 26, 27), most agree that the cyst form is the most important of the stages of the life cycle of the parasite in regard to the pathogenesis of toxoplasmosis in immunocompromised individuals, particularly in regard to the development of toxoplasmic encephalitis (7, 8, 13). Therefore, any drug or drug combination with activity against tissue cysts of

T. gondii will be an important addition to our armamentarium of drugs for the treatment of this often devastating infection. Our results with 566C80 allow for consideration of its use in carefully designed clinical studies.

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