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Phenothiazine Compounds Inhibit In Vitro Growth of Pathogenic Free-Living Amoebae

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The phenothiazine compounds trifluoperazine dihydrochloride and chlorpromazine hydrochloride have in vitro activity against the pathogenic free-living amoebae *Naegleria fowleri*, *Acanthamoeba culbertsoni*, and *Acanthamoeba polyphaga*. Drug concentrations of 10 μM were amoebastatic; concentrations of 50 μM were either amoebastatic or amoebicidal. Concentrations of 100 μM were generally amoebicidal. The mechanism of drug action is unclear. It may reflect sensitivity of amoeba calcium regulatory protein to the phenothiazine compounds or may be due to the lipophilic action of the drugs on the amoeba plasma membrane. Accumulation of these drugs in the central nervous system makes them potentially useful chemotherapeutic agents in humans for treatment of amoebic meningoencephalitis caused by *N. fowleri* and *Acanthamoeba* spp.

The free-living amoebae *Naegleria fowleri* and *Acanthamoeba* spp. cause human disease (13). *N. fowleri* is responsible for primary amoebic meningoencephalitis (PAM), a fulminating disease characterized by extensive brain tissue destruction after penetration of amoebae to the brain from the nasal passages. *Acanthamoeba culbertsoni* has been identified in brain sections of humans, where it is responsible for granulomatous amoebic meningoencephalitis in compromised hosts (18, 20). Another *Acanthamoeba* species, *A. polyphaga*, has been associated with ocular infections (14, 15, 17).

Amphotericin B is the drug of choice in treating *Naegleria* infections (4, 9, 16, 26). The amoebae exhibit in vitro sensitivity to the drug (10, 24). In the few cases when treatment of victims of *Naegleria* meningoencephalitis was initiated early after diagnosis, amphotericin B was probably instrumental in effecting recovery (1, 2, 25). The rapid onset of *Naegleria* PAM, coupled with difficulty in diagnosis, however, has often resulted in delayed start of appropriate chemotherapy, with poor prognosis for recovery (13). No particular drug therapy has been demonstrably successful in infections involving *Acanthamoeba* spp., although several have shown promise (7, 21, 23).

In studying the amoeba-to-flagellate transformation of the nonpathogenic *Naegleria gruberi*, trifluoperazine was found to have an inhibitory effect on flagellation (24a) as well as on trophic growth of the amoebae. These results prompted the present study of in vitro testing of the antipsychotic phenothiazine agents trifluoperazine dihydrochloride (TFP) and chlorpromazine hydrochloride (CPZ) on the pathogenic amoebae *N. fowleri* and *Acanthamoeba* spp. These compounds inhibit the growth of these pathogenic amoebae.

MATERIALS AND METHODS

Most of the drug testing on *N. fowleri* was carried out with the Carter 1966 isolate from Australia (5). Other *Naegleria* sp. strains from widely different geographical locations employed in drug testing included HB-3 (Czechoslovakia), NY (New York State), 6088 (California), and 0359 (Belgium). All *Naegleria* strains were isolated from PAM victims. *A. culbertsoni* A-1 and *A. polyphaga* Texas 14 were used as representative of pathogenic acanthamoebae.

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Growth. Amoebae were grown axenically for all testing, and sterile techniques were employed throughout. *Naegleria* medium consisted of 0.25% yeast extract (Difco Laboratories), 0.25% Difco Proteose Peptone, and 0.5% liver concentrate (Oxoid Ltd.), prepared in dilute saline (22) (pH 6). Newborn calf serum (GIBCO Laboratories) was added to give 10%. *Acanthamoeba* spp. were grown in 2% Difco Proteose Peptone–0.5% glucose prepared in dilute saline (22) (pH 7.2). Amoebae were cultured in 125-ml screw-cap Erlenmeyer flasks at 37°C.

Drug treatment. TFP (Stelazine) was a gift from Smith Kline & French Laboratories; a second sample of the drug purchased from Sigma Chemical Co. was found to have the same activity as the Smith Kline & French batch. CPZ was purchased from Sigma. The drugs were prepared as 0.01 M stock solutions in Tris buffer at pH 7.1. Portions of the drugs were added to *Naegleria* or *Acanthamoeba* growth media to give concentrations of 10 and 50 μM. Drug concentrations of 100 μM were also used, but not as a routine part of the experimental design. Equivalents in weight units of the molar concentrations of the two drugs employed in this study are: TFP, 10 μM (4,800 ng/ml), 50 μM (24,000 ng/ml), 100 μM (48,000 ng/ml); CPZ, 10 μM (3,550 ng/ml), 50 μM (17,800 ng/ml), 100 μM (35,500 ng/ml). Logarithmic-phase cultures of amoebae served as inocula for growth flasks, and growth was followed for 5 and 8 days for *Naegleria* and *Acanthamoeba* sp., respectively. Cell counts were made with a Coulter Counter (model Z_F).

Viability testing. Determination of amoebicidal action of a drug concentration was based on viability testing. Inocula from growth flasks were transferred to fresh growth media in screw-cap test tubes, with ca. 50-fold dilution of the drug. Tubes were incubated at 37°C and checked for growth over 10 to 14 days. Absence of growth at that time was taken as a sign of amoebicidal action of that particular drug concentration.

Tissue culture. Rat glioma cells, C_6 strain (ATCC CCL107), were inoculated into petri dishes (35 by 100 mm) with HEPES(*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered medium 199, with either 5 or 10% newborn calf serum (GIBCO). Twenty-four hours after

seeding the plates with C_6 cells (2×10^5 to 3×10^5 cells per dish), medium was replaced with fresh medium 199 containing drug concentrations, and the amoeba inoculum (ca. 500 amoebae per dish) was added. Cultures were kept at 37°C and observed for several days.

RESULTS

Concentrations of TFP of 10 μM were amoebastatic for both *Naegleria* sp. and *A. culbertsoni* for ca. 2 days, but only slightly inhibitory for *A. polyphaga*. Concentrations of TFP of 50 μM were amoebastatic or amoebicidal for *Naegleria* and *Acanthamoeba* sp. (Fig. 1). The 50 μM level appeared to be a threshold; some cultures recovered from exposure to this concentration, whereas others contained only dead cells, as determined by viability testing in growth media. Except for *N. fowleri* in CPZ, drug concentrations of 100 μM were amoebicidal. The effects of the various drug concentrations on amoeba growth are summarized in Table 1 as percent inhibition of growth. Comparison of several pathogenic strains of *N. fowleri* in 10 μM TFP indicated varying sensitivity. NY was least sensitive (<90% inhibition); 0359 and HB-3 were equally inhibited (90 to 95% inhibition); 6088 was most sensitive (>95% inhibition).

It is likely that some breakdown of the drugs was occurring in the growth vessels during the course of the experiments. Introduction of additional portions of, for example, TFP at 24-h intervals to the growth flask maintained the amoebastatic effect at a level greater than the same total drug concentration added at the start of the experiment. This type of continued growth inhibition was more evident in flasks initially exposed to low (10 μM) than high (50 μM) drug concentrations. Possibly the immediate effects of high drug concentrations on the amoebae took a longer time to overcome before growth resumed. In general, as the numbers of amoebae per milliliter increased, additional portions of drug added to the growth flask proved less effective as an amoebastatic agent.

TABLE 1. Percent inhibition of axenic amoeba growth in TFP or CPZ determined at days 2 to 3

Organism	Concn (μM)	% Inhibition	
		TFP	CPZ
N. fowleri Carter 1966	10	90	25
	50	95	60
	100	Amoebicidal	75
A. culbertsoni A-1	10	80	50
	50	99; amoebicidal	Amoebicidal
	100	Amoebicidal	Amoebicidal
A. polyphaga Texas	10	20	20
	50	99; amoebicidal	90
	100	Amoebicidal	Amoebicidal

The addition of 10 or 20 μM TFP or CPZ to rat glioma cell cultures inoculated with amoebae had a limited protective effect on cell monolayers through inhibition of increase in numbers of amoebae (Fig. 2). Drug concentrations higher than 20 μM exercised stronger inhibition of amoeba growth for a longer period of time but also led to destruction of the tissue culture cell monolayer.

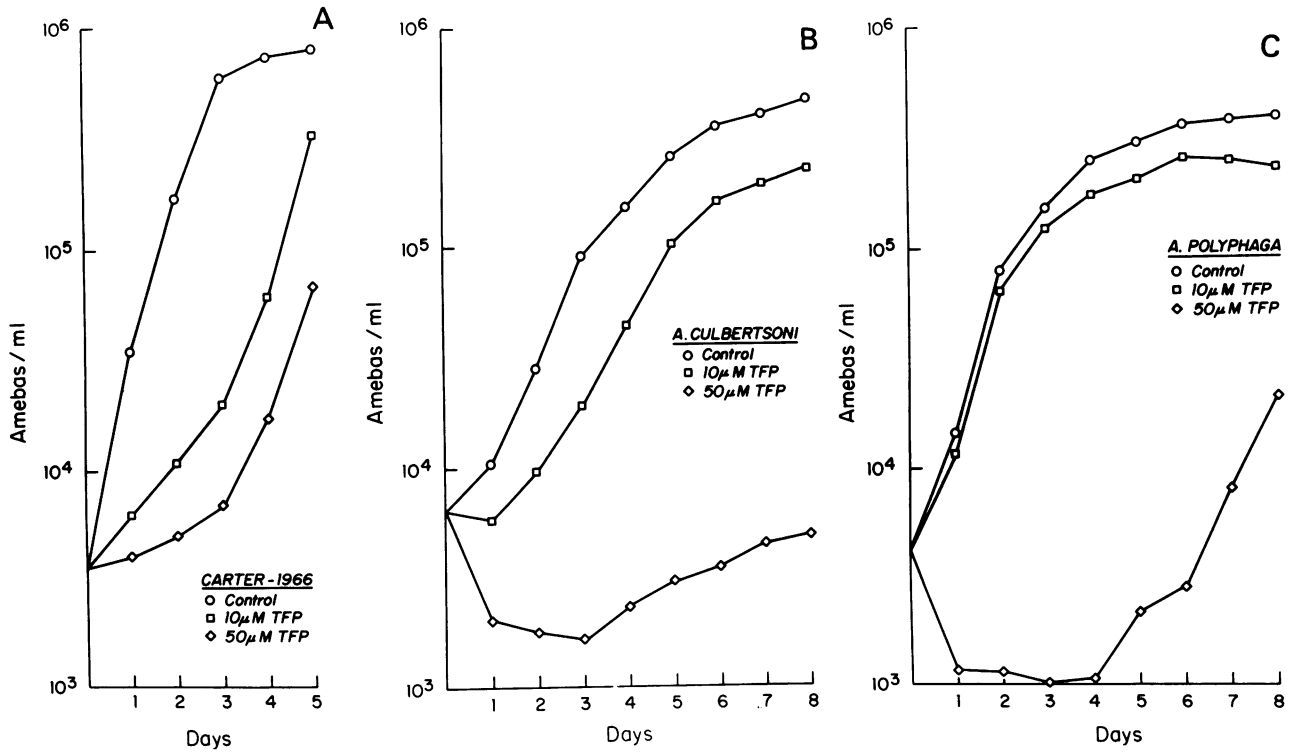

FIG. 1. Growth curves for amoebae in TFP. (A) *N. fowleri* Carter 1966. The amoebastatic drug effect on growth is seen at both concentrations of TFP used. After a delay of ca. 2 days, amoeba growth increased in both concentrations of TFP. (B) Drug response of *A. culbertsoni*. At 10 μM, the drug was amoebastatic. A TFP concentration of 50 μM was strongly amoebastatic in some experiments and amoebicidal in others. (C) Response of *A. polyphaga*. Slight growth inhibition is seen at 10 μM TFP; 50 μM TFP produced amoebastatic or amoebicidal effects in different experiments.

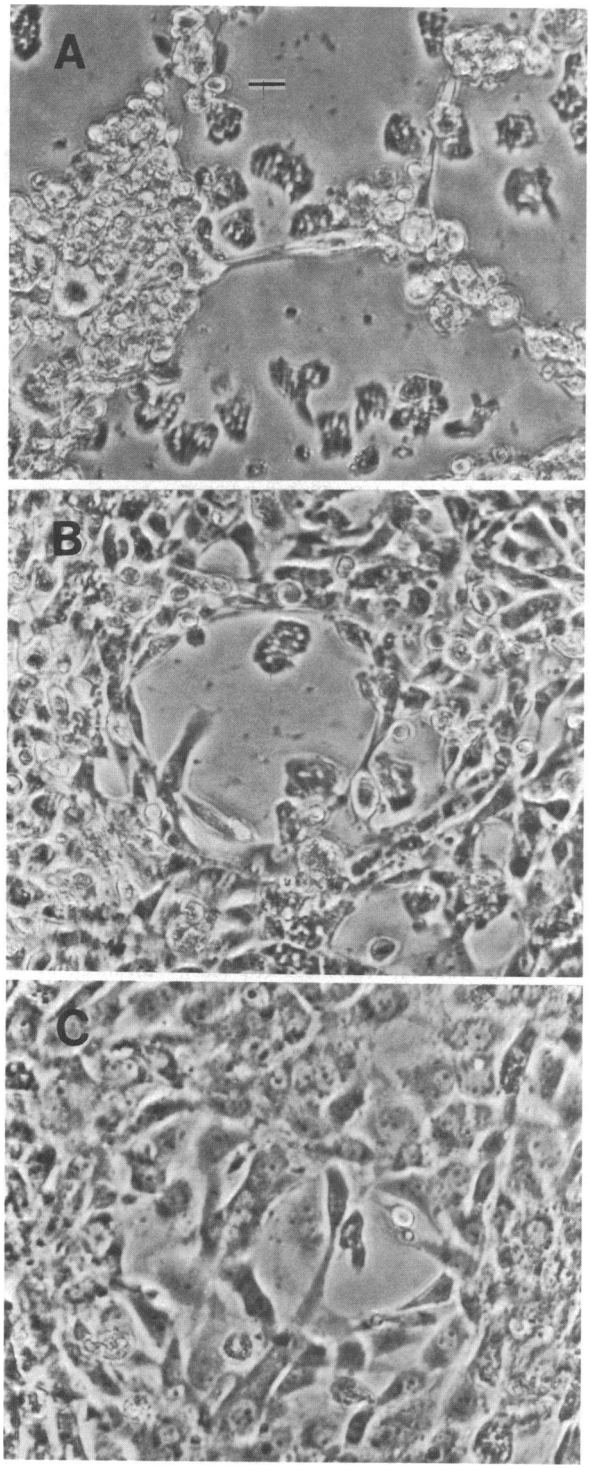

FIG. 2. *A. culbertsoni* A-1 growing on rat glioma (C_6) tissue culture monolayers on day 3 after inoculating amoebae. Phase-contrast micrographs were taken of the petri dish surface on the stage of an inverted microscope. (A) Control culture; C_6 cells without drug. Amoebae have fed extensively on glioma cells, leaving islands of residual monolayer surrounded by numerous amoebae. (B) Tissue culture medium containing 10 μM TFP. Amoebae are feeding on the cell layer, but the number of amoebae is less than in the control dish without drug. (C) Tissue culture medium containing 20 μM TFP. Amoebae are present, but damage to the monolayer caused by feeding is minimal at this point; it took 2 additional days for this dish to reach the condition of the control dish on day 3. Bar, 20 μm; ×215.

DISCUSSION

Phenothiazine compounds are effective in blocking cyclic AMP (cAMP) stimulation in cells (27, 28). Trifluoperazine, in particular, has been widely employed as an antagonist of calcium regulatory protein or calmodulin (6). In attempting to demonstrate a basis for amoeba growth inhibition, cyclic nucleotides (dibutyryl- and free acid-cAMP, at 0.1, 1, and 2 mM; dibutyryl guanosine-3:5′-cyclic monophosphoric acid at 1 mM) were added to *Naegleria* medium containing 10 μM TFP. Cholera toxin, known to promote cAMP production (12), was also added (1 μg/ml) to TFP-inhibited cultures. None of these agents reversed the growth inhibition of TFP, nor did the addition of 10 mM Ca^{2+} or Mg^{2+} as chlorides. Added cAMP appeared to enhance the TFP inhibitory effect in *Naegleria* cultures. Thus, although it is possible that TFP might be interfering with cAMP production in *Naegleria* sp., the connection has not been demonstrated. Trifluoperazine (and CPZ) may be acting at some other point under the influence of calcium regulatory protein (6) or, since the drugs are highly lipophilic (3, 11), may affect the amoeba plasma membrane. We note that De Carneri (8) tested chlorpromazine on bacterized *N. fowleri* and *Acanthamoeba* spp. and reported amoebicidal activity at concentrations of >2,000 μg/ml. These concentrations are in excess of those that we found to be effective amoebastatic and amoebicidal levels in axenic cultures.

The in vitro amoebastatic and amoebicidal effects of the phenothiazine drugs employed in this study against *Naegleria* and *Acanthamoeba* spp. represent a promising lead in finding an effective and safe chemotherapeutic agent with in vivo activity. The levels of TFP and CPZ used in the present study are unphysiologically high. For CPZ, in vivo toxicity in humans is associated with drug levels of 750 to 1,000 ng/ml, whereas our lowest effective dose is about 3,500 ng/ml. Average oral doses range from 2 to 10 mg of TFP and 25 to 50 mg of CPZ (11); extreme levels are 60 and 200 mg for TFP and CPZ, respectively (3). The level of CPZ in the brain can be up to 10 times that in the blood, although precise correlation between oral dosage and plasma level is lacking (3). The accumulation of phenothiazine compounds in the central nervous system, coupled with their ability to inhibit amoeba growth, warrants testing of the drugs in animal model systems (19).

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