Chemotherapeutic compounds and Acanthamoebae from eye infections

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SYNOPSIS The amoebicidal and amoebistatic action *in vitro* of 24 compounds was tested on two strains of Acanthamoeba, *A. polyphaga* and *A. castellanii*, isolated from eye infections in this country. For comparison, the Ryan strain of *A. castellanii* and *Naegleria gruberi* L-1 were also examined.

Nine compounds showed sufficient activity to merit further consideration, ie, acriflavine, proflavine, hydroxystilbamidine isethionate, paromomycin, miconazole, amphotericin, neomycin, polymyxin, and the last two combined in Neosporin.

The recent discovery of progressive destructive corneal ulceration associated with *Acanthamoeba polyphaga* and *A. castellanii* in this country (Nagington *et al*, 1974) and *A. polyphaga* in the USA (Jones, 1973) drew attention to the need for effective chemotherapy.

Most of the reports on amoebicidal agents have been concerned with the treatment of *Naegleria* infections because of the predominance of *Naegleria* sp in primary amoebic meningoencephalitis (Carter, 1972), and only a few authors have discussed Acanthamoeba sensitivity (Culbertson *et al*, 1965; Červa, 1969; Casemore, 1970) because the incidence of human infections is so very low and there has been little need for therapeutic information.

The serious nature of ocular invasion by Acanthamoebae, which resulted in loss of the eye in four of the five patients, made it essential to obtain some information, even if preliminary, of compounds that might be of value.

The readiness with which Acanthamoebae encyst also drew attention to the need to find compounds with cysticidal activity to ensure that cessation of treatment is not followed by re-emergence of active trophozoites.

A strain of *Naegleria* was included to see if the two genera were likely to behave in the same way.

Material and Methods

AMOEBAE

A. polyphaga Elliott and *A. castellanii* Gresham were

isolated in this laboratory (Nagington *et al*, 1974) and definitive identification was made by F. C. Page and D. C. Warhurst (personal communication).

A. castellanii Ryan was originally isolated by Pereira *et al* (1966), identified by Warhurst and Armstrong (1968), and provided by W. P. Stamm as a well characterized strain for comparison.

Naegleria gruberi L-1 provided by D. C. Warhurst was isolated originally from an encephalitis infection in Bristol (Apley *et al*, 1970).

The chemotherapeutic compounds were selected on the basis of previously published work or known amoebicidal or fungicidal properties.

Methods

Initial tests were made by the generally accepted technique of adding the compound to an actively growing culture of amoebae and then observing the results microscopically (Červa, 1969; Casemore, 1970; Jamieson, 1975).

This was discontinued because it did not resemble the clinical conditions, in which treatment was likely to be tolerated for only a limited time and during which encystment was probable, after which excystment could occur. Some compounds which are known to give toxic residues, eg, hydroxystilbamidine, gave exaggerated titres with the original method whereas others gave apparently poor results because of their mainly amoebistatic action, eg, amphotericin.

The method finally selected was based on parallel tests in order to compare the activity of each compound on a suspension of active trophozoites for

amoebicidal action with the action on a suspension of cysts for cysticidal action.

Amoebicidal tests

An inoculum of *Acanthamoebae* was obtained by culture in Stamm's medium (SB) (dextrose 0.06 g, sodium chloride 0.05 g, yeast extract 0.02 g per 100 ml distilled water to which was added a thick suspension of an overnight culture of a strain of *Escherichia coli* heated at 60°C for 60 min (Stamm, W. P., personal communication, 1974)), or Casemore's (1970) proteose-peptone glucose medium, for 4-5 days at room temperature or confluent HEP2 tissue culture, maintained in Eagle's Basal Medium with 0.1% bicarbonate and without added serum, at 37°C. *Naegleria* were grown only in SB at both temperatures. 0.1 ml of suspension was adjusted to contain about 5×10^3 amoebae with very few cysts and added to 0.9 ml SB, and then 0.1 ml of fresh 10-fold dilutions of test compound was added.

Solutions were freshly prepared according to the manufacturers' instructions and control tests were made of all diluents. A maximum final concentration of 10^3 µg/ml was selected as often a higher dosage can be tolerated by the eye than is possible systemically.

Cysticidal tests

Cysts were obtained from old cultures with negligible trophozoites after being held for weeks at 4°C and diluted to give an inoculum of 5×10^3 cysts in 0.1 ml.

All tubes were incubated at 37°C for 48 hours, examined for active amoebae by microscopy, and

then amoebae and cysts were deposited by centrifugation for 10 min at 1500 rev/min, supernatant was discarded, and a drop containing most of the deposit was placed on a 1.5% Noble agar plate. A drop of living *E. coli* suspension was placed adjacent and overlapping the first drop to provide food and further to dilute residual activity of the compound under test.

Plates were sealed with tape and incubated at room temperature, and the whole inoculated area was searched by $\times 10$ objective for living amoebae during the next 5-7 days. A fairly rigorous set of readings was selected and they were defined as follows:

Minimal inhibitory concentration (MIC): Lowest concentration of test compound which produced obvious reduction in number of trophozoites when inspected at 48 hours.

Amoebicidal concentration (AC): Lowest concentration after exposure to which no living trophozoites could be found after 7 days, ie, 100% amoebicidal dose.

Cysticidal concentration (CC): Lowest concentration after exposure to which a suspension of cysts gave no recoverable trophozoites, ie, 100% cysticidal dose.

Minimal cytotoxic dose: Lowest concentration to produce obvious cytotoxic change in HEP2 tissue cultures not containing amoebae.

Results

Repeated tests showed a working correlation but the degree of constancy was less than with bacterial

Compound	Minimum Cytotoxic Dose	A. polyphaga Elliott		A. castellanii				N. gruberi L-1	
		MIC	CC	Gresham		Ryan		MIC	CC
				MIC	CC	MIC	CC		
Acriflavine hydrochloride May and Baker	10	1	10	0.1	10^3	10	10^3	1	$>10^3$
Paromomycin sulphate (Humatin) Parke Davis	$>10^3$	1	10^3	1	10^3	10	10	10^3	10^3
Hydroxystilbamidine isethionate May and Baker	10^3	1	10^3	1	10^3	10	10	10^3	$>10^3$
Proflavine hemisulphate May and Baker	10	10^3	10^3	10	10^3	10	10^3	10^3	$>10^3$
Miconazole nitrate Ortho	10	10	10^3	10^3	10^3	10^3	10^3	10	10^3
Amphotericin B (Fungizone) Squibb	10^3	1	10^3	1	$>10^3$	10	10^3	0.1	10
Polymyxin B Burroughs Wellcome	10^3	10	$>10^3$	10	10^3	1	$>10^3$	1	$>10^3$
Neosporin Burroughs Wellcome	10^3	1	10^3	1	$>10^3$	1	$>10^3$	10	10^3
Neomycin sulphate Burroughs Wellcome	$>10^3$	1	$>10^3$	10	2×10^3	1	$>10^3$	10	4×10^3
Clotrimazole (Canesten) Bayer	10^3	0.1	$>10^3$	1	$>10^3$	1	$>10^3$	10^3	$>10^3$

Table I The 10 most active compounds

Concentrations in µg/ml. All tests in broth at 37° for 48 h.

assays. For this reason we have restricted ourselves to 10-fold dilution differences.

The 10 compounds with appreciable activity are shown in approximate order of activity in table I. For simplicity only the MIC and CC are given, the amoebicidal concentration being intermediate. Table I is based on exposure to the test compound in SB for 48 hours as above. When the 48 hours' exposure was carried out in HEP2 tissue culture there was generally less inhibitory action which was, however, only markedly less with paromomycin and clotrimazole.

The first nine compounds appear worth further investigation, having an AC activity at 10^3 $\mu\text{g/ml}$ or better. Clotrimazole appears to have a very poor AC and CC action despite the very low MIC, and this may help to explain the observations of Jamieson (1975) who found very poor *in vivo* protection of mice against *N. fowleri*.

The nine compounds can be arbitrarily grouped according to activity (table II) because the response of the three *Acanthamoeba* strains to them is fairly comparable.

It will be seen that the outstanding difference is the high degree of susceptibility of *Acanthamoeba* to acriflavine and proflavine, which was less marked with *N. gruberi* L-1. The well marked sensitivity of *Naegleria* to amphotericin (Carter, 1969) was evident but it was not found to have comparable AC and CC action on the *Acanthamoeba*.

Several compounds appeared to have a relatively specific anti-amoebic activity when judged by an MIC at least 100-fold less than the HEP2 cytotoxic dose. These were paromomycin, amphotericin, polymyxin, and neomycin. Hydroxystilbamidine appeared specifically active only against the *Acanthamoeba*.

Cytotoxicity was evident in the case of the disin-

fectant compounds acriflavine and proflavine although this may not preclude their topical use.

The 15 compounds tested that were found to have no appreciable activity are tabulated in table III.

Compound	Manufacturer
(Berenil) a diamidine diacetate trypanocide	Hoechst
Chloroquin sulphate	May and Baker
Clotrimazole (Canesten)	Bayer
Diododehydroxyquinoline	May and Baker
Iodoform	May and Baker
Mepacrine mesylate	May and Baker
Metronidazole (Flagyl)	May and Baker
Neosarsphenamine bromide	May and Baker
Nystatin	Squibb
Quinine sulphate	May and Baker
Rubidomycin (Cerubidin)	May and Baker
Sulphacetamide	May and Baker
Sulphadiazine	May and Baker
Trifluorothymidine	Sigma
5-fluorocytosine (Alcobon)	Roche

Table III Compounds with no consistent amoebicidal or cysticidal activity at 10^3 $\mu\text{g/ml}$ or less

Discussion

The results obtained are in general compatible with those of other authors but tend to be less optimistic due to the use of a limited 48-hour exposure to the compound under test and a more rigorous assessment by 100% amoebicidal or cysticidal action. Some of the more important differences and agreements with other authors' results are as follows:

Casemore (1970) did not find neomycin active at 100 $\mu\text{g/ml}$ whereas in our tests 1-10 $\mu\text{g/ml}$ inhibited all three *Acanthamoeba* strains, although 2×10^2 $\mu\text{g/ml}$ or greater was required for CC activity. Conversely, we could not confirm the activity of 5-fluorocytosine that he observed. This may well have been due to cytosine residues in our cultures. We agree with Casemore that the activity of sulphonamides *in vitro* is weak and inconstant, although Culbertson *et al* (1965) found that sulphadiazine was protective *in vivo* for mice inoculated with *A. castellanii* A1.

The use of acriflavine was suggested by Červa's (1969) observation on the *in vitro* efficacy of Gona-crine, a European trade name of Specia, France, for acriflavine.

Paromomycin is very promising *in vitro*, as Červa (1969) found, especially in the broth culture tests, but the clinical experience of Jones (1973) has been disappointing.

As regards the nine compounds with appreciable activity that seem worthy of further investigation, we hope that our results will act as a guide for the further work that needs to be done. *In vitro* activity is, how-

	<i>Acanthamoeba</i>	<i>Naegleria gruberi</i> L-1
A Cysticidal at 10^2 $\mu\text{g/ml}$ or better	Acriflavine	Amphotericin Miconazole
B Cysticidal at 10^3 $\mu\text{g/ml}$	Paromomycin ¹ Hydroxystilbamidine Proflavine Miconazole	Paromomycin Neosporin Neomycin
C Cysticidal at $>10^3$ $\mu\text{g/ml}$ but AC at 10^3 $\mu\text{g/ml}$ or better	Amphotericin Polymyxin Neomycin Neosporin	Acriflavine Proflavine Polymyxin Hydroxystilbamidine

Table II Grades of activity and comparison of action on *Acanthamoeba* and *Naegleria* strains

¹Paromomycin is markedly less active in tissue culture.

ever, not necessarily indicative of *in vivo* action, as, for example, Jamieson (1975) found with clotrimazole and *N. fowleri*, nor are animal experiments necessarily applicable to man.

It is of interest that the patient Elliott did not lose her eye, probably because the progress of the infection was halted by removal of the ulcerated cornea before a replacement graft, almost certainly before trophozoites had penetrated to the anterior chamber, and postoperative treatment included Neosporin drops.

In repeated experiments we have found that Neosporin, and its constituents neomycin and polymyxin, have 100% amoebicidal activity at concentrations around $10^3 \mu\text{g/ml}$. It seems plausible that the combination of surgical removal of the infected area of cornea, coupled with antibiotic therapy, may have enabled this infection to be cured. If so, this may be the first successful treatment.

Five confirmed *Acanthamoeba* eye infections in this country and the USA within about 18 months suggest that further infections are to be expected.

Early diagnosis is possible and should allow successful treatment, possibly by courses of more than one of these compounds, monitoring for eradication of infection by repeated stained films and cultures.

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