Effect of clotrimazole on *Naegleria fowleri*

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SYNOPSIS The sensitivity of 18 strains of *Naegleria fowleri* to clotrimazole (Bay b5097) was tested. They showed minimal inhibitory concentrations in the range 0.03-0.125 µg/ml, and minimal amoebicidal concentrations in the range 0.125-0.25 µg/ml. Mice inoculated with *N. fowleri* were not protected from infection by doses of 100 mg clotrimazole/kg per day given for five days after inoculation. Mice had serum levels of up to 6 µg/ml in the first 32 hours after inoculation. Therefore clotrimazole appears to be ineffective in protecting against infection with *N. fowleri*.

Few drugs have *in vitro* activity against *Naegleria fowleri*, the amoebic flagellate which causes primary amoebic meningo-encephalitis in man. Carter (1969) showed that amphotericin B immobilized *N. fowleri* at a concentration of 0.6 µg/ml and inhibited growth at 0.075 µg/ml. Mice inoculated intranasally with *N. fowleri* were protected from infection by intraperitoneal doses of 7.5 mg/kg per day of amphotericin B. This drug has been used to treat successfully at least one patient with primary amoebic meningo-encephalitis (Anderson and Jamieson, 1972a). However, amphotericin B is nephrotoxic in 80% of patients (Goodman and Gilman, 1970).

Amphotericin B is primarily an antifungal agent. Clotrimazole (Bay b5097), already known to have antifungal and trichomonocidal activity (Woutters, 1971; Weuta, 1972), has shown very promising amoebicidal activity *in vitro* (Jamieson and Anderson, 1974). *In vitro* studies of clotrimazole have been extended, and the effectiveness of this drug in protecting mice from infection with *N. fowleri* has been evaluated.

Material and Methods

IN VITRO STUDIES

A stock 0.1% solution of clotrimazole, which is poorly soluble in water, was prepared with dimethylsulphoxide.

This was diluted with water and Chandler Fulton 'A' (CFA), the medium for axenic growth (Anderson and Jamieson, 1972b), to give a concentration of 10 µg clotrimazole/ml. All further dilutions were

Strain	Source	Country
NHI	Human	New Zealand
Morgan	"	Australia
Northcott	"	"
McMahon	"	"
Oram	"	"
Vitek	"	Czechoslovakia
0359	"	Belgium
0360	"	"
Q838	"	"
HB-1	"	USA
MW4U	Tap water	Australia
PA14	" "	"
PA34	" "	"
PA90	" "	"
PA105	" "	"
PA117	" "	"
K1	" "	"
K71	" "	"

Table I Sources of strains of *Naegleria fowleri*

made with CFA. The sources of strains used are shown in table I.

Studies of the survival of 18 strains of *N. fowleri* in CFA containing clotrimazole were done as follows: 100 mm³ of a culture containing a known concentration of trophozoites was inoculated into tubes with 1 ml of the CFA-clotrimazole in doubling dilutions. The tubes were incubated at 37°C for five days. Then motile cells were counted using a Spencer bright-line haemocytometer. A control of CFA without clotrimazole, inoculated as above, was used. A control of CFA with dimethylsulphoxide was not included because *N. fowleri* is able to grow in the presence of 0.7% of this substance (unpublished observation). The highest concentration of clotrimazole in CFA used was 10 µg/ml; this contained only 0.01% dimethylsulphoxide.

IN VIVO STUDIES

Mouse Protection

Mice were dosed orally with a suspension of clotrimazole in 1% methyl cellulose mucilage. (Some mice died with very severe distension of the gastrointestinal tract in a trial study using a solution of clotrimazole in polyethylene glycol 400.)

The dose used was 100 mg/kg per day with mice weighing approximately 15 g; 0.75 mg clotrimazole in 0.25 ml methyl cellulose was given morning and evening with an oral dosing needle.

Three groups of mice were used: 10 uninfected and 10 infected control mice were given methyl cellulose, and 10 infected test mice were given clotrimazole in methyl cellulose.

Under light ether anaesthesia the mice were infected by intranasal inoculation of 50 mm³ of a culture of strain Q1230 containing 1600 trophozoites/mm³. Clotrimazole treatment was started immediately after inoculation and continued twice daily for five days. The mice were fed and watered *ad libitum* and observed for 16 days thereafter. After this time the remaining infected mice were killed and their brains were removed and bisected. One half brain and a portion of liver were cultured, and the other was examined histologically along with liver, kidney, spleen, and suprarenal tissue.

Bioassay of Clotrimazole Levels in Mouse Serum

A stock 1% solution of clotrimazole in chloroform was prepared and stored in the dark at 4°C. It was diluted with a 0.01 M phosphate buffer (pH 7.2) to give standard solutions containing 0.02, 0.1, 0.5, 2, and 10 µg clotrimazole/ml. *Candida pseudotropicalis* was used as the test organism because it required a minimal inhibitory concentration (MIC) of below 0.01 µg/ml (Holt and Newman, 1972). Assay plates were prepared using Sabouraud agar (pH 7.2). A 10 ml base layer in a 9 cm Petri dish was covered with a 4 ml seed layer containing 0.02 ml of an overnight culture of *C. pseudotropicalis* in Sabouraud broth without cycloheximide.

Eighty-five mice were used: 17 controls received methyl cellulose alone and 68 received clotrimazole in methyl cellulose. The dose was 0.25 ml containing 0.75 mg clotrimazole (as used in the protection study). The first dose was given at 9 am. Four mice receiving clotrimazole and one control mouse were killed hourly for 8 hours, when the next dose was given to the remainder. Five mice were killed at 9 am the following day, the third dose was given to the remainder, and then five mice were killed hourly for the next 8 hours.

Blood was obtained by cardiac puncture under ether anaesthesia, and the serum was used to deter-

mine the degree of inhibition of *C. pseudotropicalis* in the assay plates.

Antibiotic assay discs (Whatman AA, 13 mm) were used; one (test) soaked with 0.1 ml serum and one (standard) soaked with 0.1 ml of 10 µg/ml solution of clotrimazole were used on each plate. The plates were incubated overnight at 37°C, and the zones of inhibition were measured with calipers fitted with a Vernier scale.

Ten sets of standard plates using the same batch of agar as in the test plates were set up with two discs per plate. As well as the five standard solutions, a control of phosphate buffer alone was used. These plates were incubated and measured as described above.

Results

IN VITRO STUDIES

The susceptibility of 18 strains of *N. fowleri* to clotrimazole is shown in table II. The MIC ranged from 0.03 to 0.215 µg/ml and the minimal amoebicidal concentrations (MAC) from 0.125 to 0.25 µg/ml.

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Strain	Inoculum ¹	MIC (µg/ml)	MAC (µg/ml)
NHI	1700	0.125	0.25
"	1380	—	0.15
"	260	0.06	0.125
Morgan	870	0.03	0.125
"	390	—	0.15
"	365	—	0.15
Vitek	560	0.06	0.125
"	370	—	0.15
"	300	—	0.15
McMahon	740	0.06	0.125
"	440	—	0.15
0360	310	—	0.15
"	300	0.06	0.25
Northcott	1000	0.06	0.25
"	375	—	0.15
HB-1	310	0.06	0.125
"	185	—	0.15
Oram	790	0.125	0.25
"	355	—	0.15
0359	600	0.06	0.25
"	410	—	0.15
MW4U	225	—	0.15
PA105	550	0.06	0.25
"	240	—	0.15
PA90	420	0.125	0.25
"	270	—	0.15
K71	1050	—	0.15
K1	260	—	0.15
PA117	720	0.06	0.25
"	300	—	0.15
PA34	650	0.06	0.25
"	350	—	0.15
PA14	420	0.125	0.25
"	410	—	0.15
Q838	830	0.125	0.25

Table II In vitro susceptibility of 18 strains of *Naegleria fowleri* to clotrimazole

¹Inoculum = no. of trophozoites/mm³

Inoculum	No. of Strains	MIC ($\mu\text{g/ml}$)	MAC ($\mu\text{g/ml}$)
185-250	2	—	0.15
251-350	9	0.06	0.16 (0.125-0.25)
351-450	10	0.125	0.17 (0.15-0.25)
451-550	1	0.06	0.25
551-650	3	0.06	0.21 (0.125-0.25)
651-750	2	0.06	0.19 (0.125-0.25)
751-850	2	0.125	0.25
851-950	1	0.03	0.125
950-1050	2	0.06	0.2 (0.15-0.25)
1400	1	—	0.15
1700	1	0.125	0.25

Table III Effect of inoculum size on in vitro susceptibility of *Naegleria fowleri* to clotrimazole

Inoculum = no. of trophozoites/mm³
Values in parentheses represent ranges

from 185 to 1700 trophozoites/mm³) on the MIC and MAC is shown in table III. The number of times each strain was tested at different inoculum sizes is too small to show any reliable trend.

IN VIVO STUDIES

Mouse Protection

All the uninfected controls receiving methyl cellulose survived. The results for the infected mice are shown in table IV. Twenty-one days after infection there were three mice left in the treated, and two in the untreated group. These mice were killed and their brains were removed and bisected. One-half of the brain and a portion of liver were cultured; the other half was examined histologically. The results of culture are shown in table V. Only the mouse with the positive brain culture had amoebae in the histological sections.

Bioassay of Clotrimazole Levels in Mouse Serum

The zone diameters from the 10 sets of standard plates were averaged to give a standard curve from

Day	Deaths		Deaths	
	Methyl Cellulose	Culture ¹	Clotrimazole/ Methyl Cellulose	Culture ¹
5	2	+	2	+
6	5	+	3	+
15	1	+	1	+
17			1	+

Table IV In vivo activity of clotrimazole in mice infected with *Naegleria fowleri*

¹ + = Amoebae grown from suspension of mouse brain in water

Methyl Cellulose		Clotrimazole/Methyl Cellulose	
Brain	Liver	Brain	Liver
—	—	—	—
+	—	—	—
—	—	—	—

+ Amoebae present; — No amoebae present

Table V Culture of brain and liver of mice infected with *Naegleria fowleri*

which the serum level corresponding to a given zone diameter could be read. The standard disc on each test plate was used to locate a curve, parallel to the standard, which gave the serum level. The results from the four test mice killed each hour were averaged and are presented in the figure.

Figure Serum levels of clotrimazole in mice. $\nearrow$ = dose of 50 mg clotrimazole/kg given; — serum level for each mouse; • mean of levels. Where less than four values are shown, insufficient serum was obtained from some of the mice; the graph is drawn with dotted lines to indicate this.

Discussion

The *in vitro* studies showed that the MIC of clotrimazole for various strains of *N. fowleri* ranged from 0.03 to 0.125 $\mu\text{g/ml}$ and the MAC from 0.125 to 0.25 $\mu\text{g/ml}$. These concentrations should be attainable therapeutically, although actual cerebrospinal fluid and brain levels in humans are not known.

Plempel *et al* (1969) successfully treated *Candida albicans* meningitis in mice with doses twice daily of 50 mg/kg clotrimazole for five days after infection. Mice were not protected from infection by *N.*

fowleri with the same regimen. The serum levels achieved in mice in the first 32 hours after infection should have been sufficiently high to inhibit amoebic multiplication. A level of 6 µg/ml 3 hours after a single dose of 50 mg/kg is comparable with a level of 7 µg/ml 4 hours after a dose of 100 mg/kg (Plempel *et al*, 1969). In the well vascularized region of the nasal mucosa, where the amoebae are found in the first three days after inoculation (Carter, 1970), there would be ample exposure of the amoebae to blood-containing amoebicidal levels of clotrimazole.

Plempel and Bartmann (1972) showed that an effective level of clotrimazole in the blood from the fourth to the fifth days following infection cannot be achieved. This is because clotrimazole causes the induction or activation of the liver enzymes which catabolize it. In this paper it has been shown that adequate serum levels were achieved in mice during the first 32 hours of treatment. As pointed out, this is the period when the amoebae should be especially vulnerable.

Thus, although clotrimazole is very promising in its *in vitro* activity against *N. fowleri*, its efficacy in treating infections (even when given within minutes of inoculation) is negligible. In the clinical sphere, a patient would be unlikely to receive treatment until at least 72 hours after infection, by which time the amoebae would have begun to invade the meninges and brain (Carter, 1970).

In view of these results, clotrimazole cannot replace amphotericin B as the drug of choice in the treatment of primary amoebic meningo-encephalitis caused by *N. fowleri*.

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