Effect of Therapeutic Chemical Agents In Vitro and on Experimental Meningoencephalitis Due to Naegleria fowleri

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Naegleria fowleri is a ubiquitous, pathogenic free-living amoeba; it is the most virulent Naegleria species and causes primary amoebic meningoencephalitis (PAME) in laboratory animals and humans. Although amphotericin B is currently the only agent available for the treatment of PAME, it is a very toxic antibiotic and may cause many adverse effects on other organs. In order to find other potentially therapeutic agents for N. fowleri infection, the present study was undertaken to evaluate the in vitro and in vivo efficacies of miltefosine and chlorpromazine against pathogenic N. fowleri. The result showed that the growth of the amoeba was effectively inhibited by treatment with amphotericin B, miltefosine, and chlorpromazine. When N. fowleri trophozoites were treated with amphotericin B, miltefosine, and chlorpromazine, the MICs of the drug were 0.78, 25, and 12.5 µg/ml, respectively, on day 2. In experimental meningoencephalitis of mice that is caused by N. fowleri, the survival rates of mice treated with amphotericin B, miltefosine, and chlorpromazine were 40, 55, and 75%, respectively, during 1 month. The average mean time to death for the amphotericin B, miltefosine, and chlorpromazine treatments was 17.9 days. In this study, the effect of drugs was found to be optimal when 20 mg/kg was administered three times on days 3, 7, and 11. Finally, chlorpromazine had the best therapeutic activity against N. fowleri in vitro and in vivo. Therefore, it may be a more useful therapeutic agent for the treatment of PAME than amphotericin B.

Until now, amphotericin B is the only agent with established clinical efficacy in PAME, and successes have been reported in some patients treated with amphotericin B alone or in combination with other drugs (1, 2, 14, 17, 23, 29). However, amphotericin B is a very toxic agent and has some side effects such as organ damage (9, 16, 18). Moreover, not all patients treated with amphotericin B have survived PAME (24).

In the present study, we investigated effects of amphotericin B, miltefosine, and chlorpromazine in vitro to identify potentially effective drugs for N. fowleri infection. Furthermore, we showed that miltefosine and chlorpromazine had better therapeutic effects in in vivo experiments than did amphotericin B.

MATERIALS AND METHODS

Culture of N. fowleri. N. fowleri trophozoites (Carter NF69 strain, ATCC 30215) were cultured under axenic conditions in Nelson’s medium at 37°C (31). Before N. fowleri trophozoites were used, their pathogenicity or cytotoxicity was tested in mice or target cells, respectively (12).

Pharmaceutical agents. Amphotericin B and chlorpromazine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Miltefosine was purchased from Cayman Chemical (Ann Arbor, MI). For in vitro study, amphotericin B (250 µg/ml), miltefosine (10 mg/ml), and chlorpromazine (10 mg/ml) were dissolved in saline. All drug solutions were sterilized by filtration through a 0.22-µm-pore-size filter (Millipore, Molsheim, France).

Determination of MIC of drugs against N. fowleri. Serial twofold dilutions of the drugs were prepared in Nelson’s medium. Miltefosine and chlorpromazine at concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µg/ml were applied directly in sterile 96-well culture plates (Nunc A/S, Roskilde, Denmark). Amphotericin B at concentrations of 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, and 25 µg/ml was used. N. fowleri trophozoites at a density of 10^5/ml was added at volume of 100 µl to each well, and the plates were then sealed and incubated at 37°C. The amoebae were treated with each drug at a volume of 100 µl for 6 days. Control...
wells received 100 μl of Nelson’s medium. Cell growth was determined on days 2, 4, and 6. Morphological features of N. fowleri trophozoites were observed by a light microscope, and amoebae were counted using a hemacytometer by trypan blue staining. All tests were performed in triplicate and repeated at least three times.

**LDH release assay after drug treatment.** To evaluate the amoebicidal activities (cytotoxicity against N. fowleri) of three drugs, a lactate dehydrogenase (LDH) release assay was performed according to a previous study (12). N. fowleri trophozoites were cultured as a monolayer in Nelson’s medium using 96-well culture plates at 37°C. The details of experiments were as follows: 10^6 of N. fowleri trophozoites only or 10^6 of N. fowleri trophozoites treated with each drug whose concentration ranging from 1.56 to 100 μg/ml (mitelofosine and chlorpromazine) or 0.39 to 25 μg/ml (amphotericin B). The optical density of the supernatant containing LDH released from N. fowleri was measured on days 2, 4, and 6. For the LDH release assay, 50 μl of reacted supernatant in each well was transferred onto 96-well assay plates (Nunc A/S). After 50 μl of the reconstituted assay buffer in CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) was added, the plate was incubated for 30 min at room temperature, and then 50 μl of stop solution was added. The reactions were read at 490 nm with a enzyme-linked immunosorbent assay reader. In the in vitro cytotoxicity was calculated as follows: cytotoxicity (%) = [(sample release – spontaneous release)/(maximum release – spontaneous release)] × 100.

**Experimental PAME in mice.** The N. fowleri trophozoites cultured for 3 days in Nelson’s medium at 37°C were harvested and inoculated into mice. N. fowleri trophozoites (10^6) were inoculated intranasally into 7-week-old BALB/c female mice (KIST, Daejeon, Republic of Korea) under secobarbital anesthetic. Intranasal inoculation of N. fowleri trophozoites showed 100% mortality of mice. All of the following experiments were carried out with 20 mice per group. When infected mice died or death was apparent, an autopsy or a biopsy was performed. In the brains of mice, PAME was observed grossly, and trophozoites were recovered from tissue cultivation.

**Analysis of BUN level in mice sera.** The blood urea nitrogen (BUN) level from mouse sera after treatment with each drug was measured in order to check renal toxicity. The analyses were performed at the Department of Clinical Pathology, Ajou University Hospital, with the chemical analyzer, Hitachi 747. Blood samples were collected once a day for 3 days from mice treated with each drug at 5, 10, 20, and 50 mg/kg or with saline as a negative control. Histopathological examination of the kidneys and livers was performed for the toxicity evaluation after treatment with the scheduled dosage of drugs.

**Dosage of drugs for treatment of N. fowleri-infected mice.** Drug treatments began intraperitoneally at day 3 after N. fowleri inoculation and were repeated on days 7 and 11. The control group received 100 μl of sterile saline solution. The treatment groups received 100 μl each of drug in concentrations of 20 mg/kg (miltefosine and chlorpromazine) or 10 mg/kg (amphotericin B).

**Survival rate and MTD of mice for evaluation of drug effect.** Mice were held for 1 month after inoculation of N. fowleri, and the cumulative percentage of death was recorded on a daily basis. The mean time to death (MTD) was also determined for each treatment group. In order to verify the cause of death, brain tissue from dead mice was cultured at 37°C in Nelson’s medium, containing penicillin (500 U/ml) and streptomycin (500 μg/ml), with 25-cm² culture flasks, and trophozoites were observed microscopically.

Histopathological examination of mice tissues. The organs of dead or live mice were histopathologically examined to confirm whether amoebae existed or not. The mouse organs (liver, kidney, and brain) were fixed in 10% buffered neutral formalin and then carefully dissected. Each fragment of tissues was included in paraffin. Paraffin-embedded tissues on glass slides were stained with hematoxylin and eosin and examined with a light microscope. If possible, a complete cross-section of each organ was evaluated (liver, kidney, and brain).

**Statistical analysis.** Statistical differences between groups or samples were determined by using a Student two-sample t test. The difference was considered significant when P values were < 0.05.

**RESULTS**

Effects of drugs against N. fowleri in vitro. The amoeba growth curves of N. fowleri during incubation at 37°C were calculated by counting the trophozoites in each well. After 6 days of incubation, the control group treated with Nelson’s medium produced 2.7 × 10^6 amoeba/b well, which was a 274-fold increase compared to the start value (P < 0.01) (Fig. 1A). When drug was added, the growth of N. fowleri was inhibited (Fig. 1A), and the lowest concentrations of drugs that completely inhibited amoebic growth (MIC<sub>100</sub>) on days 2, 4, and 6 are shown in Table 1. Amphotericin B at 0.39 μg/ml completely suppressed the amoeba proliferation (100%) on day 2. The MIC<sub>100</sub> of amphotericin B was 0.78 μg/ml on days 2, 4, and 6, respectively. Miltefosine also effectively inhibited the growth during the experimental period, with an MIC<sub>100</sub> of 25 μg/ml. Under the same conditions, chlorpromazine showed 100% growth inhibition at a concentration of 12.5 μg/ml on days 2, 4, and 6 (Table 1).

To further investigate the amoebicidal activities of each drug, the cytotoxicity against N. fowleri was examined by LDH assay (Fig. 1B). Amphotericin B showed 99.5% cytotoxicity at a concentration of 25 μg/ml on day 6 and >50% cytotoxicity at concentrations of >1.56 μg/ml on day 4. Miltefosine showed 79.3% cytotoxicity at a concentration of 50 μg/ml on day 6, whereas the result on day 2 showed <30% cytotoxicity in all concentrations (P < 0.01). Chlorpromazine showed 64.4% cytotoxicity at a concentration of 25 μg/ml on day 6 and 69.1% cytotoxicity at a concentration of 100 μg/ml on day 4 (Fig. 1B).

**Determination of drug dosage and administration method for in vivo experiments.** To decide the dose, frequency, and intervals of drug administration, an in vivo preliminary study was performed. At first, the inoculum of 10<sup>5</sup> amoeba trophozoites produced 100% mortality in normal mice. At 3 days postinoculation of the N. fowleri, two groups (20 mice each) were intraperitoneally injected with chlorpromazine and miltefosine at doses of 10, 20, and 50 mg/kg, respectively. One group (20 mice) was intraperitoneally injected with amphotericin B at doses of 5, 10, and 20 mg/kg, respectively. The most effective dose of the drugs was 20 mg/kg, except for amphotericin B (10 mg/kg). Also, the best effect was observed when the drugs were administered into mice three times at 4-day intervals (see Fig. 3).

**BUN levels in mice.** BUN levels in mouse sera were measured to investigate the toxicity of liver and kidney due to drug treatment (Table 2). When mice were treated with miltefosine or chlorpromazine at a dose of 50 mg/kg, the BUN levels were 82.4 and 57.5 mg/dl, respectively, which were out of the physiological range in normal mouse (15 to 40 mg/dl). However, BUN levels at doses of 10 and 20 mg/kg were within the physiological range in normal mouse. In the case of amphotericin B treatment, the BUN levels in mice were 27.4, 38.6, and 44.1 mg/dl at doses of 5, 10, and 20 mg/kg, respectively. The control mice treated with 100 μl of saline had BUN levels of 34.1 mg/dl (Table 2). Pathologically, the hepatocytes of mice treated with chlorpromazine at a dose of 20 mg/kg were of normal size and shape and showed no morphological changes (Fig. 2A and B); there was no degeneration of the renal tubules or necrosis in the cortex of the kidney, and the renal cortical tubules were of normal size and shape (Fig. 2C and D). In the case of amphotericin B or miltefosine treatment, we obtained microscopic results similar to those obtained with chlorpromazine (data not shown).

**Survival rate and MTD of N. fowleri-infected mice after drug treatment.** The mice were treated with each drug on days 3, 7, and 11 after N. fowleri inoculation. The survival rate of mice given amphotericin B three times was 40%, and the MTD was 17.3 days. However, the survival rate of mice given amphotericin B two times was 10% (Fig. 3A). The survival rates of mice
administered miltefosine two times and three times were 20 and 55%, respectively (Fig. 3B). The survival rate of mice administered chlorpromazine three times was stably maintained to 75% throughout experimental periods (1 month) (Fig. 3C). Otherwise, the survival rate of mice administered the drug two times was 35%. Thus, the average MTDs with amphotericin B, chlorpromazine, and miltefosine were 17.3, 17.2,

![Image](image-url)

**TABLE 1. MICs of the drugs tested for N. fowleri**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC&lt;sub&gt;100&lt;/sub&gt; (μg/ml)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Miltefosine</td>
<td>25.00</td>
<td>25.00</td>
<td>25.00</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>12.50</td>
<td>12.50</td>
<td>12.50</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC<sub>100</sub>, the lowest drug concentration that caused a 100% inhibition of growth. Complete inhibition of trophozoite growth in Nelson's medium was scored on days 2, 4, and 6.

**TABLE 2. BUN analysis in mouse serum after drug treatment**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Mean BUN&lt;sub&gt;a&lt;/sub&gt; level (mg/dl) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>5</td>
<td>27.4 ± 7.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>38.6 ± 6.73</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>44.1 ± 6.44</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>10</td>
<td>29.6 ± 8.28</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>34.6 ± 5.37</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>82.4 ± 5.29</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>10</td>
<td>36.3 ± 2.53</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>38.4 ± 6.86</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>57.5 ± 3.32</td>
</tr>
<tr>
<td>Control (saline)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>34.1 ± 2.90</td>
</tr>
</tbody>
</table>

<sup>a</sup> The reference value of BUN in mice ranges from 15 to 40 mg/dl. Values represent the means of three experiments.

<sup>b</sup> The control group was treated with 100 μl of saline.

FIG. 1. In vitro effects of three drugs against *N. fowleri*. (A) Growth curves of *N. fowleri* after treatment with each drug. *N. fowleri* trophozoites were cultured at a density of 10^4/ml in the absence (control) or presence of each drug. (B) The amoebicidal activity of three drugs against *N. fowleri* was evaluated by LDH release assay. Amoebae were treated with miltefosine and chlorpromazine at concentrations from 1.56 to 100 μg/ml and with amphotericin B at concentrations from 0.39 to 25 μg/ml. The optical density was measured on days 2, 4, and 6, respectively. Values are means ± the standard errors of three experiments performed in triplicate.
and 19.3 days, respectively, compared to 11.2 days for control mice ($P < 0.01$) (Table 3).

**Histopathological findings of** *N. fowleri*-infected mice after drug treatment. To confirm the death of mice, mouse brain tissue was stained by hematoxylin and eosin and observed under a light microscope and also cultured with Nelson’s medium at 37°C in 25-cm² culture flasks. Both staining and culture revealed no *N. fowleri* trophozoites in the brain tissues of mice who lived through the experimental period (1 month) (Fig. 4C and D). On the other hand, however, *N. fowleri* trophozoites surrounded by many inflammatory cells were found in the brain tissues of mice that died during the experimental periods. In addition, many *N. fowleri* trophozoites were observed upon cultivation of the brain tissue (Fig. 4A and B).

**DISCUSSION**

Because of rapid progression of disease, difficulty in diagnosis, and lack of effective therapeutic agents, *N. fowleri* infections are difficult to control, and the mortality of patients is greater than 95% (22). Some investigators have reported cases successfully treated with amphotericin B, fluconazole, rifampin, and chloramphenicol (27, 29). Although amphotericin B is the only agent for the treatment of PAME, amphotericin B is a very toxic antibiotic, and it may cause renal toxicity, electrolyte disturbances, hematopoietic effects, and other organ damage, as well as chills, fever, nausea, vomiting, and headache (9, 16, 18). Most drugs used for the treatment of PAME are not curative or show only slight protection. Therefore, to find other potentially effective drugs for treatment, the in vitro and in vivo efficacies of various drugs against pathogenic *N. fowleri* should be evaluated.

In the present study, the growth curve of *N. fowleri* treated with three drugs, amphotericin B, miltefosine, and chlorpromazine, and the amoebicidal activity against each drug were tested. Amphotericin B and chlorpromazine showed 100% growth inhibition at concentrations of 0.78 and 12.5 μg/ml, respectively, on day 6. Miltefosine effectively sustained the growth inhibition throughout experimental period (6 days) and increased a little in amoebicidal activity, a finding that was similar to those of a previous study (20). Our results confirmed that all of the drugs used here effectively inhibited amoeba proliferation and had amoebicidal activity against *N. fowleri*.

In the in vivo study, chlorpromazine was the most effective drug among the three drugs against experimental *N. fowleri* infection. The in vivo activity and low toxicity of chlorpromazine, therefore, make it a potentially attractive drug for the treatment of PAME. In a mouse model, experiments were performed by treating of mice with drugs three times, on days 3, 7, and 11 postinfection, a treatment regimen that may more closely resemble the clinical setting, because patients do not present with symptoms until several days after infection (10). Although amphotericin B treatment resulted in 40% survival among *N. fowleri*-infected mice, chlorpromazine led to 75% survival in *N. fowleri*-infected mice and, furthermore, the effect in the in vivo setting was maintained for a long time, indicating
that the protective effect of chlorpromazine is better than that of amphotericin B. On the other hand, miltefosine was associated with a 55% survival rate. Although amphotericin B showed the best in vitro amoebicidal activity (cytotoxicity) against *N. fowleri*, the survival rate of mice treated with amphotericin B was 40%. However, the survival rates of mice treated with miltefosine or chlorpromazine were 55 and 75%.

It is very important to reduce the side effects of drugs, such as liver or renal toxicity, when a patient is treated with drugs. Our experimental setting (doses of 10 or 20 mg/kg, 4-day-interval, and three administrations) induced no liver and renal toxicity of mice and also resulted in a high survival rate among *N. fowleri*-infected mice. In our setting, the BUN levels of mice treated with each drug were within the physiological range in normal mouse (15 to 40 mg/dl) (3). Hepatocytes and renal cortical tubules of mouse also were of normal size and shape.

FIG. 3. Survival curve of *N. fowleri*-infected mice treated with amphotericin B (A), miltefosine (B), or chlorpromazine (C). Mice were inoculated intranasally with $10^4$ *N. fowleri* trophozoites. At 3 days postinoculation, the groups were treated with 10 mg of amphotericin B/kg or 20 mg of miltefosine and chlorpromazine/kg, respectively. Each group ($n = 20$ mice) was treated with drugs once (day 3), twice (days 3 and 7), or three times (days 3, 7, and 11). The control group received 100 µl of saline.
and showed no morphological changes. Thus, no toxicity of the liver and kidney in mice treated with drugs was observed. However, it is not known at present whether the dose, frequency, and intervals of administration are critical factors for successful treatment in PAME patients.

Miltefosine, originally developed as an antineoplastic, is used for the treatment of visceral and cutaneous leishmaniasis (25), Chagas’ disease (19), cryptococcosis (30), and Trichomonas vaginalis infection (4). Schuster et al. (20) reported that miltefosine showed effective in vitro activity against free-living amoebae, such as N. fowleri, Acanthamoeba spp., and Balamuthia mandrillaris. Recently, miltefosine has been used in the case of an Acanthamoeba granulomatous amoebic encephalitis infection in a patient (28). Walochnik et al. (28) reported that the patient that was finally diagnosed with miliary tuberculosis, tuberculous meningitis, granulomatous amoebic encephalitis, and Acanthamoeba skin lesions that did not improve after tuberculostatic treatment or treatment with streptomycin, fluconazole, trimethoprim-sulfamethoxazole, amphotericin B, fluconazole, and sulfadiazine, which are drugs that are potentially effective against Acanthamoeba spp. However, the patient was finally treated successfully with oral and topical miltefosine and intrathecal and intravenous amikacin. In our study, miltefosine was shown to have amoebicidal activity in N. fowleri and increased the survival rate against PAME in experimental mice model.

Chlorpromazine developed as an antiemetic agent (5) is used for the clinical management of patients in shock (32) and for reducing the replication of adenovirus (13). Since 1984, it has been shown that chlorpromazine has in vitro activity against pathogenic free-living amoebae such as N. fowleri, Acanthamoeba culbertsoni, and Acanthamoeba polyphaga (21). Particularly, Schuster and Mandel (21) reported that chlorpromazine showed effective in vitro activity against Acanthamoeba spp. Mattana et al. (15) also demonstrated the in vitro effectiveness of chlorpromazine against Acanthamoeba castellanii. In the present study, chlorpromazine showed in vivo efficiency as well as amoebicidal activity against N. fowleri. Also, when the N. fowleri-infected mice were treated with 20 mg of chlorpromazine/kg three times at 4-day intervals, the survival rate for mice with PAME was 75%. At that time, no

### TABLE 3. Survival and MTD of N. fowleri-infected mice treated with various drugs

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>No. of surviving mice (%)</th>
<th>MTD (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>20</td>
<td>8 (40)</td>
<td>17.3</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>20</td>
<td>11 (55)</td>
<td>19.3</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>20</td>
<td>15 (75)</td>
<td>17.2</td>
</tr>
<tr>
<td>Control†</td>
<td>20</td>
<td>0 (0)</td>
<td>11.2</td>
</tr>
</tbody>
</table>

† All groups were treated with drugs three times at days 3, 7, and 11, respectively. The control group received 100 μl of saline under the same conditions.

§ Mice were held for 28 days after inoculation, and the cumulative percentage was recorded on a daily basis.

The MTD was calculated based only on dead mice.

FIG. 4. Brain tissue of mice infected with N. fowleri trophozoites. (A and B) The brain tissue of dead mouse after drug treatment shows N. fowleri trophozoites and inflammatory cells. In the boxed area in panel B, arrows indicate N. fowleri trophozoites surrounded by inflammatory cells (A, ×50; B, ×400). (C and D) The brain tissue of mice that survived during the experimental periods (1 month) after drug treatment had no N. fowleri trophozoites and showed no change (C, ×50; D, ×400). The tissues were stained by hematoxylin and eosin.
liver and kidney toxicities in mice were observed. Unfortunately, the mechanism of chlorpromazine action is still unclear. However, we suggest that the activity of chlorpromazine may change amoeba calcium regulatory protein or may be due to the lipophilic action of the drugs on the amoeba plasma membrane. The accumulation of chlorpromazine in the central nervous system makes it a potentially useful chemotherapeutic agent for the treatment of PAME in humans that is caused by *Naegleri fowleri* (15, 21).

Further studies are needed to investigate the mechanisms of action of chlorpromazine or miltefosine and whether chlorpromazine exhibits additive, synergistic, or antagonistic effects in mouse experiments when combined with miltefosine or other drugs. Finally, on the basis of both in vitro and in vivo activities against *Naegleri fowleri*, chlorpromazine and miltefosine may be candidate drugs for the treatment of *N. fowleri* infection.

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