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Therapeutic effect of rokitamycin in vitro and on experimental meningoencephalitis due to *Naegleria fowleri*

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ABSTRACT

Inhalation of freshwater containing the free-living amoeba *Naegleria fowleri* leads to a potentially fatal infection known as primary amoebic meningoencephalitis (PAME). Amphotericin B is the only agent with clinical efficacy in the treatment of PAME in humans, however this drug is often associated with adverse effects on the kidney and other organs. In an attempt to select other useful therapeutic agents for treating PAME, the amoebicidal activities of antibacterial agents including clarithromycin, erythromycin, hygromycin B, neomycin, rokitamycin, roxithromycin and zeocin were examined. Results showed that the growth of amoeba was effectively inhibited by treatment with hygromycin B, rokitamycin and roxithromycin. Notably, when *N. fowleri* trophozoites were treated with rokitamycin, the minimal inhibitory concentration was 6.25 μg/mL on Day 2. In the treatment of experimental meningoencephalitis due to *N. fowleri*, survival rates of mice treated with roxithromycin and rokitamycin were 25% and 80%, respectively, over 1 month. The mean time to death for roxithromycin and rokitamycin treatment was 16.2 days and 16.8 days, respectively, compared with 11.2 days for control mice. Finally, rokitamycin showed both in vitro and in vivo therapeutic efficacy against *N. fowleri* and may be a candidate drug for the treatment of PAME.

1. Introduction

Naegleria fowleri belongs to the free-living amoebae and feeds on bacteria and nutrients in moist soil and freshwater. This organism is generally not very well known and the method for laboratory diagnosis is unfamiliar. Human infections were first reported in 1965 by Fowler and Carter who studied four patients with primary amoebic meningoencephalitis (PAME) in South Australia [1]. PAME caused by *N. fowleri* is an acute fatal disease of the brain that generally occurs in previously healthy children and young adults with a history of swimming in lakes or ponds. The period between contact with the organism and onset of clinical symptoms such as fever, headache and rhinitis may vary from 2–3 days to as long as 7–15 days [2–4].

Many antimicrobial, antifungal and antiparasitic drugs have been screened for therapeutic activity against *N. fowleri* in vitro and in vivo. Amphotericin B is the only agent with established in vitro activity and clinical efficacy for PAME [5,6]. Other antimicrobials that have been tested, mostly in vitro, are clotrimazole, itraconazole, fluconazole, ketoconazole and chlorpromazine, with varying degrees of efficacy [7,8]. Azithromycin has been described for the effective treatment of experimental PAME in mice [9]. In recent years, miltefosine, which was used as an anticancer drug, and voriconazole, which was used in systemic fungal infections, were found to be effective in in vitro studies [10].

Although *N. fowleri* is very sensitive to amphotericin B in vitro, only a few patients have recovered following intrathecal or intravenous injections of this drug alone or in combination with miconazole [6]. Often a serious acute reaction is noted after the infusion, which consists of fever, shaking chills, hypotension, anorexia, nausea, vomiting, headache, dyspnoea and tachypnoea [11–13]. Nephrotoxicity is a major issue and can be severe or irreversible. Moreover, not all patients treated with amphotericin B have survived PAME [14]. Unfortunately, at the present time there is no satisfactory treatment for PAME.

In this study, the in vitro and in vivo effects of various antibacterial agents on pathogenic *N. fowleri* were investigated to identify other effective drugs for *N. fowleri* infection. Seven antibacterial agents were used (clarithromycin, erythromycin, hygromycin B, neomycin, rokitamycin, roxithromycin and zeocin), which have only been tested in vitro or have not been previously studied in the treatment of *N. fowleri* infection. Clarithromycin and

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erythromycin are macrolide antibiotics, which prevent bacteria from growing by interfering with their protein synthesis [15,16]. Hygromycin B is an aminoglycoside antibiotic, blocking polypeptide synthesis and inhibiting elongation [17]. Neomycin binds to the 30S or 50S subunit of the ribosome and consequently causes miscoding that inhibits initiation and elongation during protein synthesis. Neomycin is usually combined with other antibiotics [18]. Roxithromycin and rokitamycin are semisynthetic macrolide antibiotics [19,20]. Zeocin is a copper-chelated glycopeptide antibiotic, acting by binding to DNA and cleaving it [21].

2. Materials and methods

2.1. Amoeba culture

Naegleria fowleri trophozoites (Carter NF69 strain, ATCC accession no. 30215) were cultured under axenic conditions in Nelson's medium at 37 °C [22]. Before using, *N. fowleri* was tested for its ability to induce experimental meningoencephalitis in mice.

2.2. Pharmaceutical agents

Erythromycin, hygromycin B, neomycin, roxithromycin and zeocin were purchased from Sigma–Aldrich Inc. (St. Louis, MO). Rokitamycin was purchased from Asahi Kasei Pharma Corporation (Tokyo, Japan). Clarithromycin was purchased from Abbott Laboratories (Chicago, IL). Erythromycin and rokitamycin were made up as stock solutions containing 20 mg/mL and 8 mg/mL in 95% (v/v) ethanol, respectively. Clarithromycin (1 mg/mL), roxithromycin (0.5 mg/mL), hygromycin B (2.5 mg/mL), neomycin (10 mg/mL) and zeocin (1 mg/mL) were dissolved in Nelson's medium. A preliminary test with ethanol was performed to ensure that no trophozoite inhibition occurred at the concentrations used.

2.3. Determination of the minimal inhibitory concentration (MIC) of drugs against N. fowleri

Serial two-fold dilutions of the drugs were prepared in Nelson's medium. Control wells received 100 μL of Nelson's medium. All drug solutions 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). The calibrated trophozoite suspension in Nelson's medium at a density of 1×10^4 mL^{-1} was added to each well and the plates then were sealed and incubated at 37 °C. The amoebae were treated with drugs for 6 days. Cell growth and the MIC (the lowest concentration of drug that completely inhibited *N. fowleri* growth) were determined for periods of 2, 4 and 6 days using a light microscope and a haemocytometer. Tests were performed in triplicate and repeated at least three times.

2.4. Lactate dehydrogenase (LDH) release assay after drug treatment

To evaluate amoebicidal activity (cytotoxicity against *N. fowleri*) of the drugs, the LDH release assay was performed according to a previous study [23]. *Naegleria fowleri* trophozoites were cultured as a monolayer in Nelson's medium using 96-well culture plates at 37 °C. The details of the experiments were as follows: 1×10^4 *N. fowleri* trophozoites only; or 1×10^4 *N. fowleri* trophozoites treated with each drug at concentrations of 1.56–100 μg/mL. For the LDH release assay, 50 μL of reacted supernatant in each well was transferred onto 96-well assay plates (Nunc A/S). Then, 50 μL of the reconstituted assay buffer from the CytoTox96® Non-radioactive Cytotoxicity Assay Kit (Promega Corp., 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 an enzyme-linked immunosorbent assay (ELISA) reader. The optical density of supernatant containing LDH released from *N. fowleri* was measured at 2, 4 and 6 days. The formula for in vitro cytotoxicity was as follows:

$$\text{cytotoxicity}\,(\%) = \frac{\text{sample release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

2.5. Experimental PAME in mice

Trophozoites cultured for 3 days in Nelson's medium at 37 °C were harvested and inoculated into mice. *Naegleria fowleri* trophozoites (1×10^4) were inoculated intranasally into 7-week-old BALB/c female mice (purchased from KIST, Daejeon, South Korea) under secobarbital anaesthesia. Intranasal inoculation of *N. fowleri* trophozoites showed 100% mortality of mice. All the following experiments were carried out with 20 mice per group. When infected mice died or death was imminent, an autopsy or a biopsy was performed. PAME was observed grossly in the brain of mice, and trophozoites were recovered from tissue cultivation.

2.6. Dosage of drugs for treatment of N. fowleri-infected mice

Intraperitoneal drug treatments began 3 days following *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 at a concentration of 20 mg/kg.

2.7. Analysis of blood urea nitrogen (BUN) levels in serum of mice

BUN levels from mice sera after treatment with drugs were measured to determine renal toxicity. The analyses were performed at the Department of Clinical Pathology, Ajou University Hospital, South Korea, using a Hitachi 747 Analyzer. Blood was collected once a day for 3 days from mice treated with each drug at 10, 20 and 50 mg/kg. Histopathological examination of the kidney and liver were performed for the toxicity evaluation after treatment with scheduled dosage of drugs.

2.8. Survival rate and mean time to death (MTD) of mice for evaluation of drug effect

Mice were held for 1 month after inoculation with amoeba and the cumulative percentage of death was recorded on a daily basis. The MTD was also determined for each treatment group. To verify the cause of death, brain tissue from dead mice was cultured at 37 °C in Nelson's medium and trophozoites were observed microscopically.

2.9. Histopathological examination of mice tissue

The organs of dead or live mice were evaluated by histopathological examination. Mice organs were stored in 10% buffered neutral formalin, washed in chloroform, decalcified, dehydrated and embedded in paraffin. Paraffin-embedded tissues on slides were stained with haematoxylin and eosin and examined with a light microscope. If possible, a complete cross-section of each organ was evaluated (liver, kidney and brain).