THE EFFECTS OF THERAPEUTIC AGENTS

ON THE LEE (M67) STRAIN OF

NAEGLERIA FOWLERI

By

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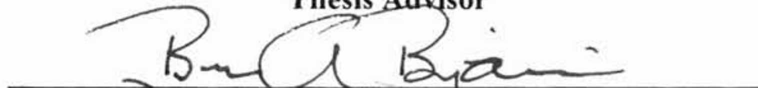

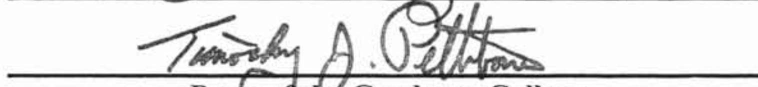

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LIST OF ABBREVIATIONS

CNS	**central nervous system**
CSF	**cerebrospinal fluid**
HCl	**hydrochloric acid**
MIC	**minimal inhibitory concentration**
PAM	**primary amebic meningoencephalitis**

CHAPTER I

INTRODUCTION

1.1 Background

Free-living amebae are present worldwide in soil, dust, and virtually all freshwater sources. These amebae usually feed on bacteria and do not cause disease. However, several genera of free-living amebae including *Naegleria, Acanthamoeba, Balamuthia,* and *Sappina* are capable of producing disease in the central nervous system (CNS) of humans and other animals. *Naegleria fowleri* produces primary amebic meningoencephalitis (PAM), a rapidly progressing meningoencephalitis which is almost always fatal. *Acanthamoeba* species are responsible for a chronic CNS infection known as granulamatous amebic encephalitis (GAE) and an eye infection called *Acanthamoeba* keratitis (John, 1993). *Balamuthia mandrillaris* causes a chronic CNS infection similar to that produced by *Acanthamoeba*, also termed GAE (John, 1998). *Sappina diploidea* was recently noted for causing an amebic encephalitis similar to PAM (Gelman *et al.*, 2001).

Free-living amebae were not considered pathogenic until 1959. In a series of experiments, Culbertson demonstrated that *Acanthamoeba* could produce an acute and fatal meningoencephalitis in laboratory animals when introduced by the intracerebral or nasal route (Culbertson *et al.*, 1958). In 1965, Fowler and Carter submitted the first published report of *N. fowleri* causing CNS disease in Australia. In this initial report of 4 patients, the authors suggested the etiologic amebae belonged to the genus *Acanthamoeba*. However, subsequent examinations determined these cases were most

likely caused by *N. fowleri*. In 1966, Butt reported the first human case of *N. fowleri* meningoencephalitis in the United States and coined the term primary amebic meningoencephalitis.

1.2 Taxonomy and Biology of *N. fowleri*

Naegleria is placed in the family Vahlkampfiidae, phylum Saccomastigophora, subphylm Sarcodina, and order Schizopyrenida. Currently, there are 17 species within the genus of *Naegleria* that have been confirmed based on partial SSUrDNA sequences. These include *N. fowleri, N. australiensis, N. lovaniensis, N. gruberi, N. jadini, N. andersoni, N. clarki, N. galeacystis, N. italica, N. jamiesoni, N. morganensis, N. niuginiensis, N. sturti, N. minor, N. carteri, N. robinsoni,* and *N. pussardi* (De Jonckheere and Brown, 1999). The only species identified as pathogenic in humans and other animals are *N. fowleri, N. italica,* and *N. australiensis* (De Jonckheere, 1994).

Trophozoite, flagellate, and cyst are the 3 stages seen in the lifecycle of *Naegleria*. Trophozoites measure 15-30 µm in diameter and are characterized by a large central nucleolus surrounded by a nuclear membrane without chromatin granules (John, 1998). Trophozoites are elongate and move by broadly rounded, granule-free processes that erupt from the surface known as lobopodia (Parija and Jayakeerthee, 1999). The cytoplasm is abundant with vacuoles and mitochondria. Trophozoites feed on bacteria and rapidly multiply by simple binary fission. Nuclear division is promitotic, meaning the nucleolus and nuclear membrane persist during nuclear division (John, 1993). Pathogenic strains of *Naegleria* have phagocytic structures called 'amoebostomes.' Amoebostomes are used to engulf particles including bacteria, erythrocytes, yeasts, other ameba, and cultured mammalian cells (John, 1993). The trophozoites are usually found

in tissue or cerebrospinal fluid (CSF) and is the only stage of the life cycle that is responsible for producing PAM.

The flagellate stage occurs as a response to changes in the environment. Factors inducing enflagellation include nutrient depletion, temperature, phase of growth, and culture agitation (John, 1998). In this form, *N. fowleri* are biflagellate and have an elongate cigar or pear-shaped appearance (John, 1993). The flagellates exist as non-dividing, non-feeding organisms, which with time will revert to trophozoites.

N. fowleri are also capable of encystment in response to adverse conditions such as starvation, cold temperatures, drying, and presence of drugs (John, 1998). Encystment is an adaptive mechanism enabling *Naegleria* to increase survival. The spherical cysts have 1 to 2 mucoid plugged pores, or ostioles, and range from 7-15 μm in diameter (John, 1998). Excystment is the process by which *Naegleria* exit their protective coat and occurs in response to a more favorable environment (John, 1993).

Naegleria spp. are distributed world-wide in soil and virtually all freshwater sources such as ponds, lakes, and rivers (Parija and Jayakeerthee, 1999). *N. fowleri* has also been isolated from poorly chlorinated swimming pools, sewage, hot springs, dust, thermal discharge of power plants, and heating and ventilation units (Marshall *et al.*, 1997; Martinez and Visvesvara, 1991). In addition, *N. fowleri* are thermophilic and can tolerate temperatures of 40 to 45°C (Marshall *et al.*, 1997).

1.3 Primary Amebic Meningoencephalitis (PAM)

PAM infection normally occurs in healthy children or young adults who have recently been swimming in warm water contaminated with *N. fowleri* (John, 1993). The amebae gain entry to the nasal cavity during inhalation or aspiration of contaminated

water. The amebae invade the olfactory mucosa, penetrate the submucosal nervous plexus, cross the cribriform plate, and gain access into the sub-arachnoid and perivascular spaces (Parija and Jayakeerthee, 1999). Presumably, the perivascular spaces facilitate the migration of amebae into the cerebral hemispheres, cerebellum, brain stem, and upper portions of the spinal cord (John, 1998). Within the brain, the amebae cause extensive inflammation, necrosis, and hemorrhage.

1.3.1 Clinical Features

The clinical features of PAM develop abruptly within 1 to 5 days after exposure to *N. fowleri*. The initial symptoms are characterized by severe frontal headache, fever (39-40° C), and anexoria, followed by nausea, vomiting, and signs of meningitis (John, 1998). Meningeal irritation may be accompanied by stiff neck, generalized seizures, and a positive Kernig's sign (Markell *et al.,* 1999). As symptoms persist, the patient may become confused, irritable, and restless before lapsing into a coma (John, 1993). Death ensues due to pulmonary edema or cardiorespiratory arrest within 3 to 7 days of the initial symptoms (Parija and Jayakeerthee, 1999). Other symptoms include convulsions, photophobia, abnormalities in taste or smell, increased intracranial pressure, and cerebellar ataxia (Marshall *et al.,* 1997).

1.3.2 Epidemiology

Although PAM is a rare disease, it has been reported worldwide. Most cases of PAM have been reported from developed rather than developing countries. This is probably due to misdiagnosis, inadequate information of PAM, and a very low autopsy rate in most developing countries. Of the reported cases of PAM, 75% have been from

Australia, Czechoslovakia, and the USA (John, 1993). In the USA, 67% of the reported cases have been from the coastal states of Florida, Virginia, and Texas (John, 1998).

PAM occurs more often during the summer months in warm climates due to warmer waters coupled with increased swimming activity. Thus, it is no surprise that PAM is more prevalent in normal, healthy children or young adults. Fatal cases have been reported following swimming in lakes, ponds, rivers, thermally polluted streams, coastal and freshwater, and poorly chlorinated swimming pools. In addition, cases of PAM have been reported resulting from exposure to cyst-contaminated dust (John, 1993). In a few instances, the source of exposure could not be determined (Sugita *et al.,* 1999).

1.3.4 Treatment of PAM

The mortality rate of patients infected with PAM is greater than 95% (Barnett *et al.,* 1996). This grave prognosis is due to the rapid progression of the disease and to the lack of effective chemotherapeutic agents. Seven patients have been reported to survive infection with *N. fowleri.* Two British children were treated with intravenous amphotericin B (Apley *et al.,* 1970). One Australian patient was given amphotericin B parenterally and intrathecally (Anderson and Jamieson, 1972). The fourth patient, a California girl was successfully treated employing amphotericin B and miconazole, both administered by intravenous and intrathecal routes, along with oral rifampicin (Seidel *et al.,* 1982). The fifth case reported was a 61-year-old male from Thailand who recovered completely following treatment of intravenous amphotericin B in combination with oral administration of rifampicin and ketoconazole (Poungvarin and Jariya, 1991). The sixth survivor was a male from Messina, Italy who was treated with amphotericin B via intravenous and intrathecal routes (Loschiavo *et al.,* 1993). The last reported survivor

was from Hong Kong and was treated with amphotericin B, rifampicin, and chloramphenicol (Wang *et al.,* 1993).

Presumably, amphotericin B is the most effective drug against *N. fowleri.* However, early diagnosis and prompt treatment remain a primary requirement for survival, as evidenced by 2 cases of PAM, which resulted in death in spite of treatment with amphotericin B (Stevens *et al.,* 1981).

1.4 Experimental Studies of Therapeutic Agents on *N. fowleri*

Since PAM was identified, a wide range of chemotherapeutic agents has been tested on *N. fowleri*. These agents include antifungals, antiprotozoals, antipsychotics, antimalarials, and several antibacterial agents. Published data on the effectiveness of anti-*naegleria* agents vary depending upon differences among the strains employed, the media and methods, times of exposure to the drug, and the growth phases of the *N. fowleri* cultures when the drug itself is added. The size of inoculum represents a potential variable that must be considered when determining pharmacological susceptibility of *N. fowleri.* In addition, definitions of the minimum inhibitory concentration (MIC) have varied throughout previous studies. For instance, Scaglia *et al.*, (1988) defined MIC as the lowest concentration inhibiting cellular growth without preventing subculturing, while Duma and Finley, (1976) defined MIC as the lowest concentration of drug at which growth was 50% less than that of the control as determined visually on the inverted microscope.

1.4.1 *In Vitro* Studies of Therapeutic Agents Against *N. fowleri*

In vitro experiments have provided evidence that antifungal agents are the most effective drugs against *N. fowleri*. Of the antifungals, amphotericin B has the most

efficacy against *N. fowleri* with MICs ranging from 0.018 - 1 μg/ml (Carter, 1969; Donald *et al.*, 1979; Duma and Finley, 1976; Lee *et al.*, 1979; Smego and Durack, 1984; Stevens *et al.*, 1981; and Scaglia *et al.*, 1988). Of all the agents evaluated against *N. fowleri*, amphotericin B has been the most studied and is the anti-*naeglerial* agent to which all other therapeutic agents are compared.

Scaglia *et al.*, (1988) concluded econazole, an antifungal, to be effective against *N. fowleri* and *N. australiensis* with an MIC of 4 μg/ml. Another antifungal, ketoconazole, was reported to have an MIC range of 0.31 - 5 μg/ml (Smego and Durack, 1984; Elmsly, 1980) on a New Zealand strain of *N. fowleri* isolated by Cursons *et al.*,(1979). Other studies determined the activity of the antifungals clotrimazole and miconazole with MICs ranging from 0.39-1.57 and 0.98-50 μg/ml, respectively, against several clinical isolates of *N. fowleri*, including cases from Virginia, Australia, and Florida, but their effects were less rapid and had less predictability than amphotericin B (Duma and Finley, 1976; Donald *et al.*, 1979; Elmsly, 1980, and Stevens *et al.*, 1981). In addition, chlorpromazine and trifluoperazine, which are antipsychotic agents, were shown to inhibit the growth of the Carter (1966) Australian isolate of *N. fowleri* at concentrations of 24 and 4.8 μg/ml, respectively (Schuster and Mandel, 1984). Lee *et al.*, (1979) concluded minocycline, a tetracycline antibiotic, to be effective against the HB-1 strain of *N. fowleri* with an MIC of 2.8 μg/ml. This study also determined minocycline to be synergistic with amphotericin B *in vitro*. Other agents found to exhibit synergism when combined with amphotericin B include rifampin, miconazole, and tetracycline (Donald *et al.*, 1979; Seidel *et al.*, 1982; Thong *et al.*, 1979a and 1979b).

Drugs tested against *N. fowleri in vitro* which were determined to have minimal or no activity include: chloroquine, chloramphenicol, clindamycin, dapsone, diethylcarbamazine, methotrexate, metronidazole, oxytetracycline, paromomycin, penicillin G, pentamidine, primaquine, quinine, streptomycin, sulfadiazine, sulfamethoxazole, and trimethoprim (Carter, 1969; Dhu, 1982; Duma and Finley, 1976; and Thong *et al.*, 1977).

1.4.2 *In Vivo* Studies in an Experimental Model of PAM

Previous *in vivo* studies have determined amphotericin B either alone or in combination with other drugs is the best treatment for experimental amebic meningoencephalitis. Ferrante (1982), concluded that 2.5 mg/kg amphotericin B produced 100% survival in mice infected with *N. fowleri.* Furthermore, amphotericin B at a concentration of 7.5 mg/kg produced 60% survival in mice inoculated with either an Australia isolate or Florida isolate of *N. fowleri* (Carter, 1969 and Stevens *et al.*, 1981).

Thong *et al.* (1979b) reported 2.5 mg/kg amphotericin B in combination with 150 mg/kg of rifampin produced 40% survival in a mouse model employing the Northcott strain. However, either 2.5 mg/kg amphotericin B or 150 mg/kg rifamycin alone was insufficient to treat an experimental PAM infection (Thong *et al.*, 1979b). In addition, Thong *et al.*, (1979a) demonstrated a delayed treatment of 72 hours combining 2.5 mg/kg amphotericin B with 150 mg/kg tetracycline produced 87.5% survival in mice inoculated with the Northcott strain of *N. fowleri*. In this same study, 2.5 mg/kg amphotericin B alone produced only 37.5% survival and tetracycline alone at 150 mg/kg produced no survival.

Agents tested against *N. fowleri in vivo* and found to be ineffective include: artemisinin drugs, ketoconazole, metronidazole, miconazole, miconazole in combination with amphotericin B, miconazole in combination with rifampin, nitrofurazone, paromomycin, pentamidine, pyrimethamine, pyrimethamine in combination with sulfadiazine, sulfadiazine, and tetracycline (Carter, 1969; Das *et al.*, 1970; Elmsly *et al.*, 1980; Ferrante, 1982; Gupta *et al.*, 1995; Stevens *et al.*, 1981; Thong *et al.*, 1978, 1979a, 1979b).

1.5 Significance of Study

The main emphasis of this research was to determine the *in vitro* and *in vivo* activity of novel chemotherapeutic agents against the Lee (M67) strain of *N. fowleri* in order to identify drugs that have a greater ability to inhibit the growth of *N. fowleri*. The agents chosen for these studies have a mechanism of action or a spectrum of activity that is similar to drugs already known to be active against *Naegleria*. Some agents were also included in this study because of their ability to penetrate the blood-brain barrier. The agents tested in this study include the antifungals: amphotericin B, liposomal amphotericin B, and ketoconazole. The protein synthesis inhibitors that were evaluated in this study were minocycline, azithromycin, quinupristin/dalfopristin, and linezolid. Other agents tested were rifampin, a RNA synthesis inhibitor, and trifluoperazine, which is an antipsychotic.

1.5.1 Properties of Antifungal Agents

The major therapeutic application for amphotericin B has been to treat systemic fungal infections, but it also has activity against some protozoans including *Naegleria*. Amphotericin B is reported to combine with membrane sterols, particularly cholesterol

and ergosterol, thereby increasing the fungal membrane permeability and allowing the cytoplasmic contents to escape from the cell (Schuster and Rechthand, 1975). Schuster and Rechthand (1975) have provided *in vitro* evidence that amphotericin B causes several ultrastructural changes in *N. fowleri.* The changes they observed were absence of pseudopods, abnormal mitochondria, distortions in nuclear shape, decrease in food vacuoles, and leakage of the plasma membrane (Schuster and Rechthand, 1975). These investigators also observed that these ultrastructural abnormalities were directly correlated with the time of exposure to amphotericin B. However, amphotericin B exhibits poor penetration of the CSF, which is the site of PAM infection. In addition, large amounts of amphotericin B cannot be administered to overcome this difficulty because it is highly toxic. Amphotericin B is considered the most toxic antibiotic drug in use today. It has been reported to cause a high rate of nephrotoxicity, acute liver failure, cardiac arrhythmias, and hematopoietic disorders such as anemia, leukopenia, and thrombocytopenia (Brenner, 2000).

Although amphotericin B is the most effective anti-*naeglerial* drug, its narrow therapeutic index continues to limit its clinical utility. To reduce toxicity, amphotericin B has been formulated in liposomes to allow the administration of higher doses of amphotericin B with less toxicity to mammalian cells by altering its pharmacokinetics and tissue distribution (Clemons and Stevens, 1998). Several preparations of liposomal amphotericin B have been developed, including Abelcet®, Amphotec®, and AmBisome®. Each of these preparations has been shown to be useful for the treatment of cryptococcal meningitis, *Leishmania* infections, and systemic fungal infections (Clemons and Stevens, 1998; Yardley and Croft, 1997). Studies have demonstrated that

higher dosages of liposomal amphotericin B must be administered in order to retain its therapeutic activity because its potency has been reduced by several fold compared to free amphotericin B (Clemons and Stevens, 1998). The effects of liposomal amphotericin B against *N. fowleri* have not been studied and should be evaluated as an alternative treatment for PAM infection.

Ketoconazole is the other antifungal agent evaluated in this study. Ketoconazole acts by inhibiting the conversion of lanosterol to ergosterol, which is the principal sterol in the fungal membrane (Naftalovich, 1991). Ketoconazole has been reported to inhibit the growth of *N. fowleri in vitro* (Smego and Durack, 1984). However, Elmsly *et al.*, (1980) reported ketoconazole was not effective in the treatment of experimental amebic meningoencephalitis. Ketoconazole is less utilized today to treat systemic fungal infections due to its greater potential for drug interactions by inhibiting the cytochrome P450 isozyme CYP3A4 (Brenner, 2000).

1.5.2 Properties of Protein Synthesis Inhibitor Agents

Minocycline is a tetracycline antibiotic, which competitively blocks the binding of tRNA to the 30s subunit of the bacterial ribosome, thereby preventing the addition of new amino acids to the growing peptide chain (Brenner, 2000). Minocycline is active against a wide variety of aerobic and anaerobic gram-positive and gram-negative bacteria. It is also used to treat diseases caused by mycoplasmas, chlamydiae, and rickettsiae (Brenner, 2000). Minocycline is more lipophilic than other tetracyclines and will pass into the CSF more readily than the other tetracyclines (Hardman and Limbird, 2001). Minocycline has been determined to inhibit the growth of *N. fowleri in vitro*, however the effects of minocycline *in vivo* have not been evaluated.

Azithromycin is a macrolide antibiotic that acts on the 50s ribosomal subunit. This drug blocks pepidyl transferase, the enzyme that catalyzes the formation of peptide bonds between the nascent peptide and the amino acid attached to the A site (Brenner, 2000). Previous *in vitro* studies found azithromycin to inhibit the growth of *Acanthamoeba,* another opportunistic ameba that causes an encephalitis (Schuster and Visvesvara, 1998). In addition, Araujo *et al.* (1988) reported that azithromycin protects against experimental toxoplasmic encephalitis in a murine model. These studies suggest azithromycin attains active concentrations in the inflamed CNS and may be promising against PAM infection.

Quinupristin/dalfopristin is a combination of these two semisynthetic pristamycin derivatives in a 30:70 ratio. Quinupristin and dalfopristin are protein synthesis inhibitors that act at the 50s subunit. Quinupristin binds to the 50s subunit resulting in the termination of protein synthesis. Dalfopristin binds to a site near quinupristin and causes a conformational change in the 50s ribosome, synergistically enhancing the binding of quinupristin at its target site (Hardman and Limbird, 2001). Quinupristin/dalfopristin is active against aerobic gram-positive organisms such as, vancomycin-resistant strains of *E. faecium* and methicillin-susceptiblie strains of *S. aureus* and *S. pyogenes* (Hardman and Limbird, 2001). In addition, Khan *et al.* (1999) determined quinupristin/dalfopristin to have *in vitro* and *in vivo* activity against the protozoan *Toxoplasma gondii.*

Linezolid belongs to the oxazolidinone class of antibacterial agents that act by inhibiting the process of bacterial protein synthesis by a novel mechanism. Linezolid terminates translation at the initiation step that involves the binding of N-formylmethionyl-tRNA to the 70s ribosome (Zurenko *et al.*, 2001). Since linezolid

inhibits the ribosome-assembly step of protein synthesis, there is no cross resistance with other antibacterials. Linezolid is active against gram-positive bacteria, including staphylococci, streptococci, enterococci, gram-positive anaerobic cocci, and gram-positive rods such as *Listeria monocytogenes* (Hardman and Limbird, 2001). It has little activity against most gram-negative aerobic or anaerobic bacteria. The therapeutic uses of linezolid are reserved for vancomycin-resistant *E. faecium*; nosocomial pneumonia caused by methicillin-susceptible and –resistant strains of *S. aureus*; community-acquired pneumonia caused by *S. pneumonia*; and complicated skin and skin-structure infections caused by streptococci and *S. aureus* (Hardman and Limbird, 2001). Regazzi *et al.* (2002) reported linezolid to have good CNS penetration and could be a promising candidate for the treatment of CNS infections. Linezolid should be considered in treating PAM infection due to its unique mechanism of action coupled with the ability to penetrate the CNS.

1.5.3 Properties of Other Therapeutic Agents

The phenothiazines, a group of drugs to which trifluoperazine belongs, are widely utilized in medical practice as antipsychotics, but trifluoperazine is also effective against many strains of staphylococci, *Shigella*, *Vibrio*, and *Plasmodium* species (Mazumder *et al.*, 2001). Previous *in vitro* studies have determined trifluoperazine to inhibit the growth of *N. fowleri, A. culbertsoni, A. polyphaga,* and *Balamuthia mandrillaris* (Schuster and Mandel, 1984; Schuster and Visvesvara, 1998). Interestingly, the *in vivo* effects of trifluoperazine have not been studied. The mechanism of action by which trifluoperazine inhibits ameba growth is unknown. Schuster and Mandel, (1984) reported that trifluoperazine might be interfering with cAMP production in *Naegleria* sp., but this

connection has not been demonstrated. Another hypothesis is that trifluoperazine may be acting at some other point under the influence of calmodulin, or since trifluoperazine is highly lipophilic it may be affecting the ameba plasma membrane (Schuster and Mandel, 1984). The ability of trifluoperazine to penetrate the blood brain barrier coupled with its ability to inhibit ameba growth warrants the evaluation of this drug *in vivo*.

Rifampin, a rifamycin derivative, is a broad spectrum antibiotic that has significant activity against many bacteria, including mycobacteria (Brenner, 2000). This drug acts by binding to the β subunit of DNA-dependent RNA polymerase thereby preventing the enzyme from binding to DNA and consequently inhibiting DNA transcription (Brenner, 2000). Rifampin has the ability to cross the blood-brain barrier and reach levels in the CSF that are 10-20 % of the levels in the serum. In addition, Thong *et al.,* (1979b) reported synergistic activity with a combination of rifampin and amphotericin B in experimental PAM studies.

CHAPTER II

RESEARCH DESIGN AND METHODS

2.1 Amebae and Cultivation

The LEE (M67) strain of *N. fowleri* was used in this study. Clifford Nelson isolated the LEE strain from the cerebrospinal fluid of a 15-year-old female who died from primary amebic meningoencephalitis in 1968 at the Medical College of Virginia in Richmond (John, *et al.*, 1993). The LEE (M67) strain is LEE that has been maintained by 67 passages in mice to retain maximum virulence. The *N. fowleri* LEE (M67) strain was cultured axenically, without agitation, in Mix medium, which consisted of 0.55% (w/v) Oxoid liver digest (Unipath LTD., Basingstoke, Hamphshire, England), 0.50% (w/v) proteose-peptone (Difco Laboratories, Detroit, MI), 0.25% (w/v) yeast extract (Difco Laboratories, Detroit, MI), and 0.30% dextrose (w/v) in Page's ameba saline (0.12 g NaCl, 0.004 g $MgSO_4.7H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.142 g Na_2HPO_4, 0.136 g KH_22PO_4 per liter of deionized water) (Page, 1988) supplemented with 4% (v/v) bovine calf serum (Sigma Chemical Co., St. Louis, MO) and 1 μg/ml hemin. All components were autoclaved except bovine serum and hemin, which were sterilized by filtration. The stock cultures of *N. fowleri* were maintained at 37° C employing Falcon (Becton Dickinson and Co., Franklin Lakes, NJ) 25 cm^2 polystyrene culture flasks, in 10 ml of medium.

2.2 Growth Studies

For growth studies, 130 ml of medium was prepared for each trial, inoculated with 10^4 amebae/ml from 72 hour stock cultures, and then 30 ml was distributed into 4 separate 50 ml tubes. All therapeutic agents to be studied were solubilized and diluted in stock solutions from which serial dilutions were made. Using an Eppendorf pipet 0.12 ml of each drug concentration was added directly to the 30 ml aliquot. Each agent was tested at 3 different concentrations with each concentration being performed in triplicate. The control flasks did not receive treatment, unless specified otherwise. The mixture was vortex-shaken and 10 ml was distributed into 3 separate culture flasks. The culture flasks were incubated at 37° C and examined daily for growth.

2.3 Cell Counting

To evaluate the effect of each drug on growth, cell counts were performed daily for a period of 96 hours and then at 168 hours. All cell counts were performed using a Coulter Counter (model Z_F, Coulter Electronics, Inc., Hialeah, Fl) by adding 0.2 ml of cell suspension to 9.8 ml of electrolyte solution consisting of 0.5% (v/v) formalin and 0.4% (w/v) NaCl in deionized water. Coulter settings for counting amebae were: 1/amplification 4; 1/aperture current 1; threshold 10; sample volume 0.5 ml. Cuvettes were vortex-shaken at a setting of 7 for 10 seconds to separate cell aggregates and then read within 5 minutes. Prior to counting, cuvettes were inverted a few times to resuspend settled cells and were counted after the bubbles dispersed. Four successive counts were obtained on each cuvette. The most deviant count for each cuvette was excluded; the mean of the remaining 9 counts was calculated (3 flasks of 3 counts each). The mean count for each trial was plotted using Sigma Plot. The minimum inhibitory

concentration (MIC) for each drug was determined from the graphs and was defined as the lowest concentration of drug that significantly inhibited amebae growth throughout a 7-day period. The data was analyzed by the student's T-test to determine whether the difference between the control and treated groups were significant.

2.4 Therapeutic Agents

The antimicrobial agents obtained from Sigma-Aldrich, Inc. (St. Louis, MO) include: amphotericin B powder, which consists of 45% amphotericin B, 35% deoxycholic acid sodium, and 20% sodium phosphate; amphotericin B solution, which consisted of 250 μg amphotericin B per ml of water, ketoconazole, minocycline hydrochloride, trifluoperazine dihydrochloride, and rifampicin crystalline.

AmBisome® (Amphotericin B liposome for injection) was purchased from Fujisawa Healthcare, Inc. (Deerfield, IL). AmBisome® for injection contains 50 mg of amphotericin B intercalated into a liposomal membrane, which consists of approximately 213 mg hydrogenated soy phosphatidylcholine; 52 mg cholesterol, NF; 84 mg distearoylphosphatidyl glycerol; 0.64 mg alpha tocopherol, USP; together with 900 mg sucrose, NF; and 27 mg disodium succinate hexahydrate as buffer.

Zithromax® (azithromycin for injection) was acquired from Pfizer Inc., (New York, NY). Zithromax® consists of azithromycin dihydrate and the inactive ingredients: citric acid and sodium hydroxide.

Synercid® I.V. (quinupristin and dalfopristin powder for injection) was obtained from Aventis. Synercid® is a sterile lyophilized formulation of quinupristin and dalfopristin in the ratio of 30:70 (w/w).

Zyvox® powder (linezolid) was a gift from Pharmacia pharmaceutical company. Zyvox® consists of linezolid powder.

2.5 *In Vitro* Drug Dilutions

All drug dilutions were prepared employing sterile techniques and were used immediately to minimize loss of activity.

Amphotericin B solution was serially diluted with sterile deionized water to provide final concentrations of 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. In order to determine a more accurate MIC, amphotericin B solution was further diluted to give final concentrations of 0.08 μg/ml, 0.04 μg/ml, and 0.02 μg/ml.

Liposomal amphotericin B was dissolved and serially diluted in sterile deionized water to give final concentrations of 1 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. Based upon the results for these concentrations, further experiments with liposomal amphotericin B were performed using final concentrations of 0.8 μg/ml, 0.4 μg/ml, and 0.2 μg/ml

Minocycline hydrochloride, rifampicin crystalline, and azithromycin were each dissolved and diluted in sterile deionized water to provide final concentrations of 100 μg/ml, 10 μg/ml, and 1 μg/ml.

Trifluoperazine dihydrochloride, quinupristin/dalfopristin, and linezolid were each dissolved and diluted in sterile deionized water to give final concentrations of 10 μg/ml, 5 μg/ml, and 1 μg/ml.

Ketoconazole was diluted in methanol to provide final concentrations of 0.5 μg/ml, 0.1 μg/ml, and 0.01 μg/ml. The controls received an equivalent amount of methanol in the ketoconazole experiments to determine whether methanol would affect amebae growth.

2.6. *In Vivo* Studies: Cell Harvesting

Amebae were harvested for mouse inoculations after being grown for 72 hours at 37° C in 10 ml of mix medium. Amebae were centrifuged at 2,000 X g for 10 minutes in a Beckman model TJ-6 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). The amebae were washed in Page saline using the centrifuge and resuspended in the appropriate amount of Page saline to provide a final concentration of $2x10^6$ amebae/ml.

2.7 Mouse Strain and Inoculations

Male 21-day-old CD-1 mice weighing approximately 23 g were used in all experiments and were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Prior to experimentation, mice were allowed 3 days to adapt to their new environment. Mice were housed in plastic cages with 10 mice per cage. All mice were given free access to water and feed (Purina Lab Chow, Ralston Purina Corp., St. Louis, MO).

Mice were inoculated by intranasal (i.n.) instillation of a 10 μl suspension of amebae in Page saline. While mice were under anesthesia by AErrane®(Isofluorane, USP, Baxter Caribe Inc., Deerfield, IL) 10 μl containing $2x10^4$ ameba was instilled into a single nare using an Eppendorf pipet.

2.8 Treatment of Experimental Amebic Meningoencephalitis

Treatment began 72 hours after intranasal challenge and persisted for 5 days. For each set of experiments, the 40 mice were randomly divided into 4 groups with each group receiving a different treatment. The control group received 0.1 ml intraperitoneal (i.p.) injections of 0.9% Sodium Chloride Injection (Abbott Labs, Chicago, IL) daily. The treated groups received 0.1 ml i.p. injections containing the active drug in sufficient

concentrations to provide the specified dosages (as stated). Injections were made with a 1 cc syringe and a 25 gauge 3/8 inch needle into the peritoneal cavity.

2.9 *In Vivo* Drug Dilutions

Working stock solutions of each drug were prepared using sterile deionized water for hydration and dilution, except for ketoconazole. The dilutions were stored at either 0° or 4° C in 1 ml aliquots and used within 1 week in order to minimize loss of activity.

Amphotericin B was dissolved in sterile deionized water to provide final doses of 2.5 mg/kg and 7.5 mg/kg. Liposomal Amphotericin B was dissolved in sterile deionized water to give final doses of 2.5 mg/kg, 7.5 mg/kg, and 25 mg/kg. Minocycline hydrochloride was dissolved in sterile deionized water to provide final doses of 25 mg/kg and 50 mg/kg. Azithromycin was dissolved in sterile deionized water to provide final doses of 25 mg/kg and 75 mg/kg. Trifluoperazine dihydrochloride was dissolved with sterile deionized water to give final doses of 2.5 mg/kg and 7.5 mg/kg. Quinupristin/dalfopristin was dissolved in sterile deionized water to give a final dose of 150 mg/kg. Ketoconazole was dissolved in 0.1 N HCl and further diluted with sterile deionized water to provide dosages of 10 mg/kg and 25 mg/kg. The ketoconazole control group received i.p. injections of 0.01 N HCl. Linezolid was dissolved in sterile deionized water to provide a final dose of 25 mg/kg and was prepared fresh each day. Linezolid at 75 mg/kg was dissolved in sterile deionized water to give the desired concentration in 0.2 ml injections.

Mice were held for 28 days after inoculation and the cumulative percent dead was recorded on a daily basis. The mean time death (MTD) was calculated for each group of mice by adding the day of death of each mouse and dividing by the number of mice that

died within the group. Brain tissue was cultured for amebae from the moribund and dead mice using 10 ml of mix medium with the addition of penicillin (500 U/ml) and streptomycin (500 ug/ml) in 25 cm^2 polystyrene culture flasks and incubated at 37° C.

CHAPTER III

RESULTS

3.1General Overview of *In Vitro* Studies

The *in vitro* studies were performed to determine the activity of a number of anti-microbial agents against *N. fowleri*. The Lee (M67) strain of *N. fowleri* was employed in all studies. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that significantly inhibited the growth of *N. fowleri* throughout a 7-day period.

3.1.1 Temperature Studies

Pilot studies were performed to determine the growth of the *N. fowleri* Lee M(67) strain incubated at different temperatures. The temperatures tested in this study were 23, 30, and 37° C. As shown in Figure 1, the maximal growth of *N. fowleri* was obtained at 37° C. The growth of *N. fowleri* was slightly reduced at 30° C and considerably reduced at 23° C. Based on these results, the Lee (M67) strain of *N. fowleri* was incubated at 37° C in all studies thereafter.

3.1.2 Amphotericin B Studies

The concentrations of amphotericin B used in this study were based upon those obtained from previous *in vitro* studies with *N. fowleri* (Carter, 1969; Donald *et al.*, 1979; Duma and Finley, 1976; Lee *et al.*, 1979; Smego and Durack, 1984; Stevens *et al.*, 1981; and Scaglia *et al.*, 1988). Figure 2 depicts the effects of amphotericin B on *N. fowleri* growth throughout a 7-day course. As shown, 0.1 and 1 μg/ml of amphotericin B were

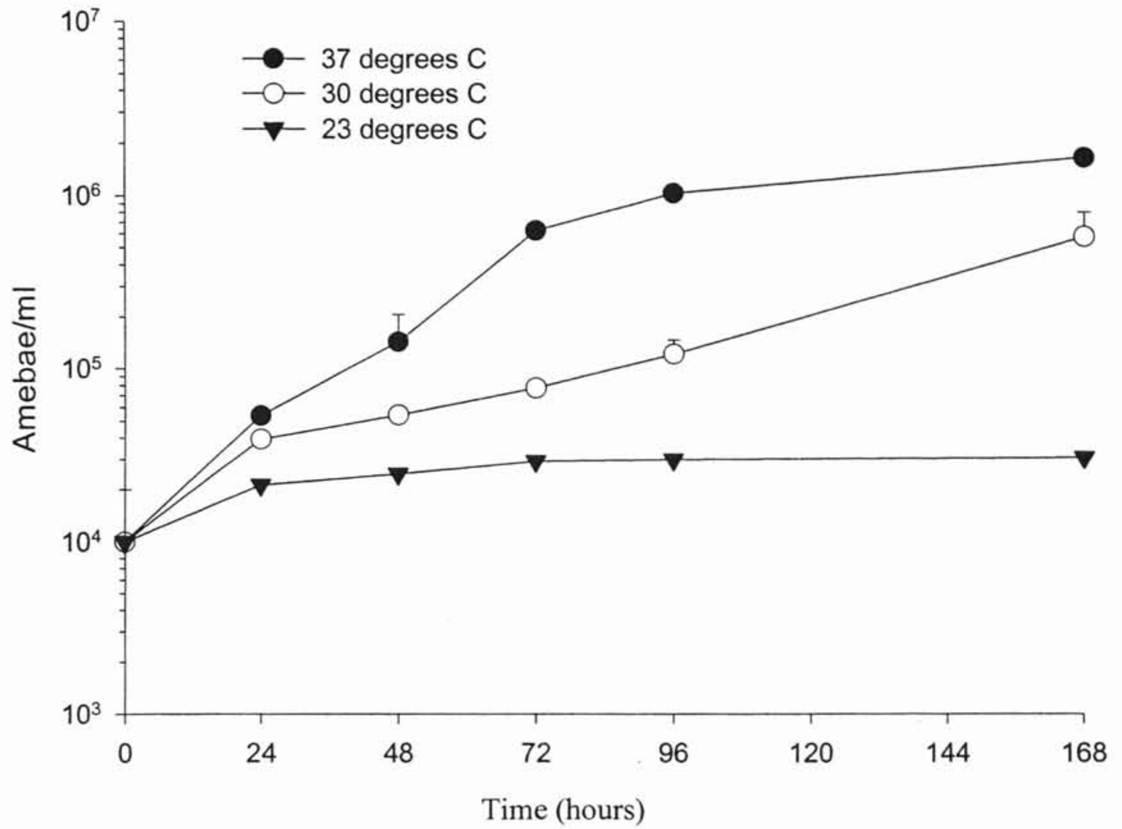

Figure 1. Growth of *N. fowleri* incubated at different temperatures over a 7-day period. The data points represent the mean of 2 experiments performed in triplicate. The error bars represent the standard error of the mean for individual data points.

significantly different from the control and effectively inhibited the growth of *N. fowleri*. A concentration of 0.01 μg/ml amphotericin B had limited activity on the growth of *N. fowleri*. Figure 3 demonstrates the percent inhibition of *N. fowleri* growth over time. As shown, 0.1 and 1 μg/ml inhibited the growth of *N. fowleri* greater than 92% from 48 hours and thereafter. The concentration of 0.01 μg/ml amphotericin B had a maximal percent inhibition of 22% at 48 hours and began to deteriorate thereafter. Based upon the results of this study, different concentrations of amphotericin B were used in order to determine a more precise MIC. The results of this study are shown in Figures 4 and 5. Figure 4 demonstrates *N. fowleri* growth in the presence of 0.08, 0.04, and 0.02 μg/ml of amphotericin B over time. As shown in Figure 4, the concentration of 0.08 μg/ml amphotericin B significantly inhibited the growth of *N. fowleri* and was determined the MIC. The concentration of 0.04 μg/ml amphotericin B had limited activity on growth, whereas 0.02 μg/ml of amphotericin B did not inhibit the growth of *N. fowleri*. Figure 5 represents the percent inhibition of amphotericin B on amebae growth over a 7- day period. Amphotericin B at 0.02 μg/ml had a range of inhibition from 50% to 15%, whereas 0.04 μg/ml of amphotericin B inhibited growth from 84% to 41% throughout the 7-day period. Amphotericin B at 0.08 μg/ml had the most effect on growth with a range of inhibition from 65% to 97% throughout the 7-day course.

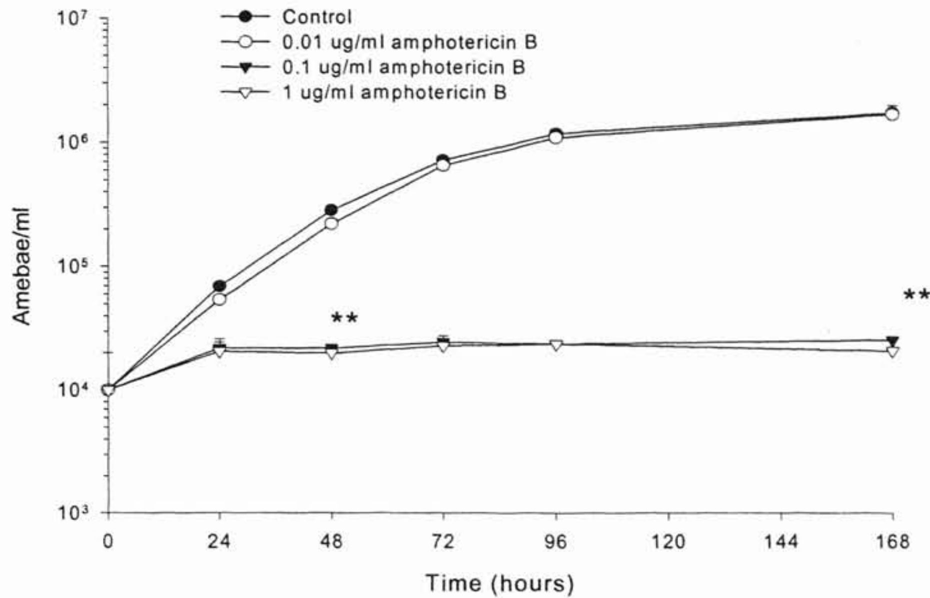

Figure 2. Effect of *N. fowleri* growth to a range of amphotericin B concentrations over a 7-day interval. The data points illustrate the mean of 2 experiments performed in triplicate. The error bars represent the standard error mean of the individual data points. ** significant at $p < 0.01$

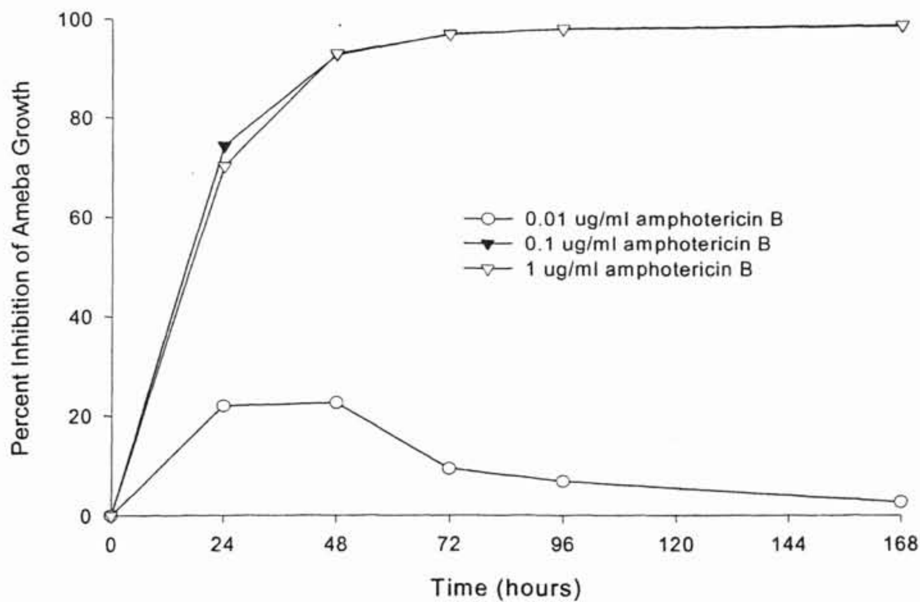

Figure 3. Percent inhibition of *N. fowleri* growth by amphotericin B over 7 days.

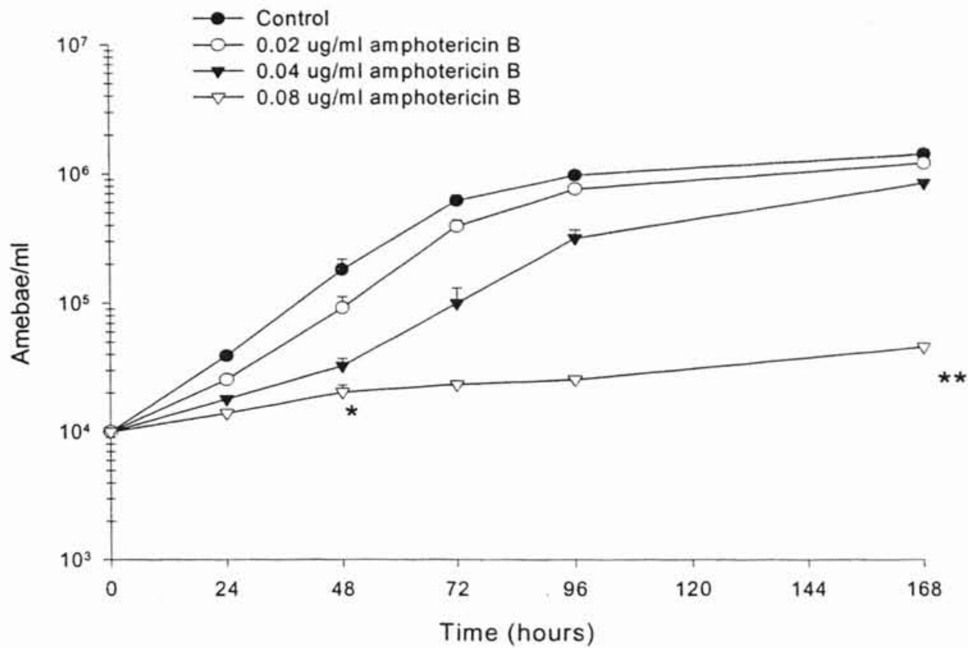

Figure 4. Effect of *N. fowleri* growth to a range of amphotericin B concentrations over a 7-day interval. The data points represent the mean of 2 experiments performed in triplicate. The error bars represent the standard error mean of the individual points.
* significant at $p < 0.05$ ** significant at $p < 0.01$

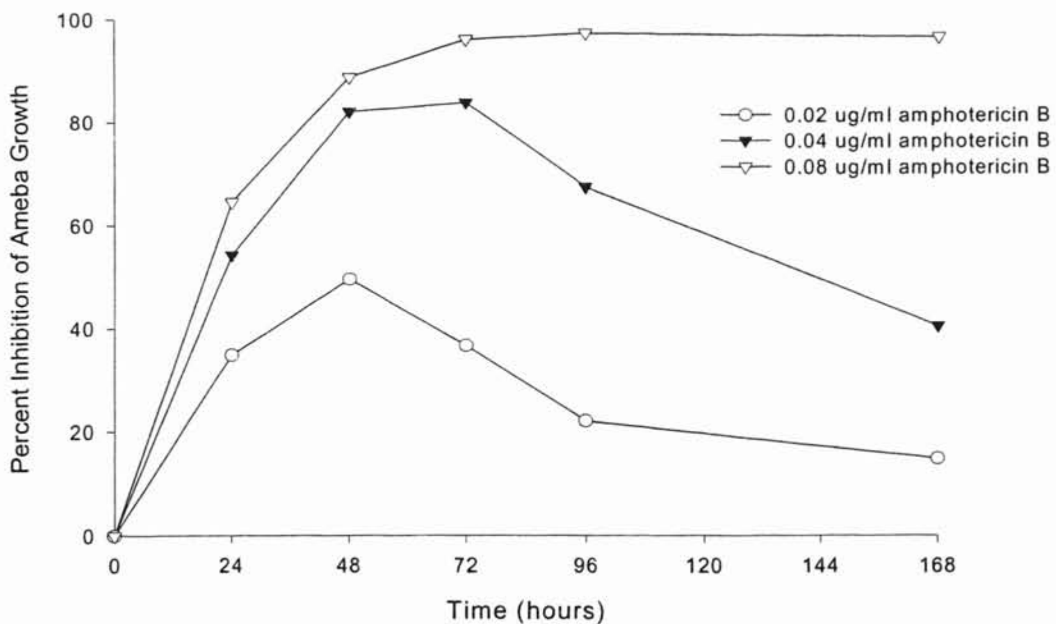

Figure 5. Percentage inhibition of *N. fowleri* growth in the presence of a range of amphotericin B concentrations over a 7-day period.

3.1.3 Liposomal Amphotericin B Studies

The concentrations of liposomal amphotericin B used in this study were the same as free amphotericin B in order to compare the 2 agents. Figure 6 illustrates the effects of liposomal amphotericin B on *N. fowleri* throughout a 7-day interval. The concentrations of 0.01 and 0.1 μg/ml liposomal amphotericin B did not effectively inhibit the growth of *N. fowleri*, whereas 1 μg/ml significantly inhibited amebae growth and was determined to be the MIC of this study. Figure 7 represents the percentage inhibition of amebae growth in the presence of liposomal amphotericin B. As shown, a concentration of 1 μg/ml inhibits 98% of amebae growth throughout 168 hours. A concentration of 0.1 μg/ml had a maximal percentage inhibition of 52% at 24 hours, whereas a concentration of 0.01 μg/ml of liposomal amphotericin B did not inhibit growth at any time. Based upon the results of this study, further studies were performed in order to determine a more accurate MIC. Figure 8 demonstrates the effect of 0.8, 0.4, and 0.2 μg/ml of liposomal amphotericin B on *N. fowleri* growth. As shown in Figure 8, 0.2 and 0.4 μg/ml of liposomal amphotericin B had limited activity on the growth of *N. fowleri*. Liposomal amphotericin B at 0.8 μg/ml significantly inhibited growth of *N. fowleri* and was determined to be the MIC. Percent inhibition of liposomal amphotericin B on *N. fowleri* growth is represented in Figure 9. The concentration of 0.8 μg/ml had a percent inhibition greater than 95% at 48 hours and thereafter. The concentration of 0.4 μg/ml inhibited 92% of amebae growth at 48 hours and then decreased to 44% by 96 hours. Liposomal amphotericin B at 0.2 μg/ml produced maximal inhibition of 43% at 24 hours and was not significantly inhibitory thereafter.

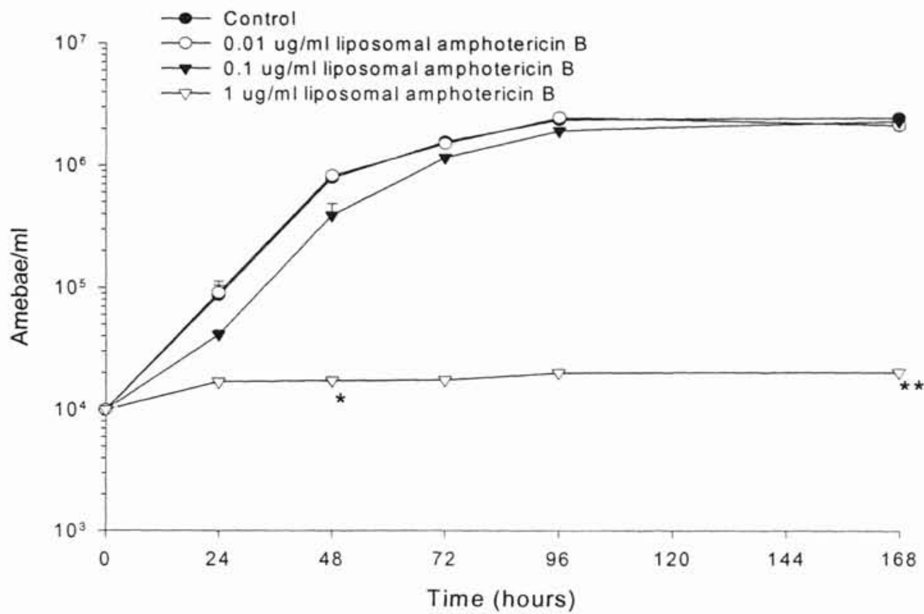

Figure 6. Effects of a range on concentrations of liposomal amphotericin B on *N. fowleri* growth over a 7-day period. The data points represent the mean of 2 experiments performed in triplicate. The error bars demonstrate the standard error mean of the individual points. * significant at $p < 0.05$ ** significant at $p < 0.01$

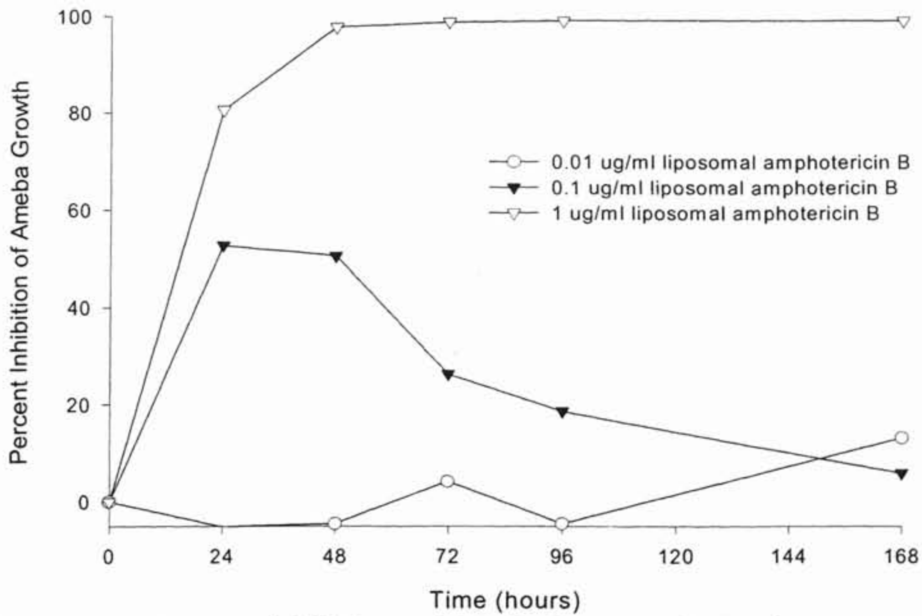

Figure 7. Percent inhibition of *N. fowleri* growth in the presence of liposomal amphotericin B

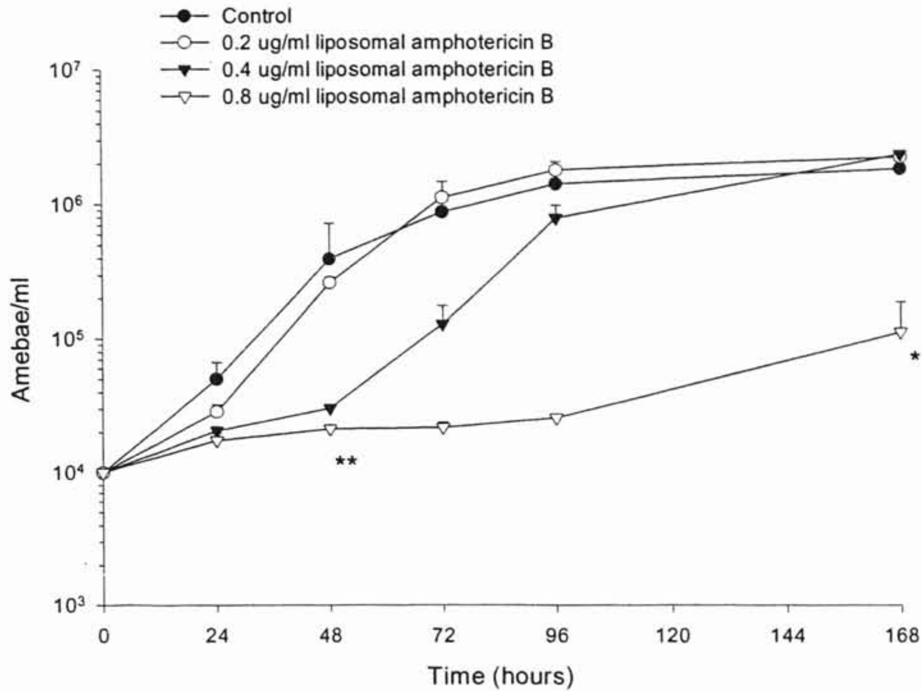

Figure 8. The effects of a range of concentrations of liposomal amphotericin B on the growth of *N. fowleri* over a 7-day period. The data points represent the mean of 2 experiments performed in triplicate. The error bars demonstrate the standard error mean of the individual points. * significant at p<0.05 ** significant at p < 0.01

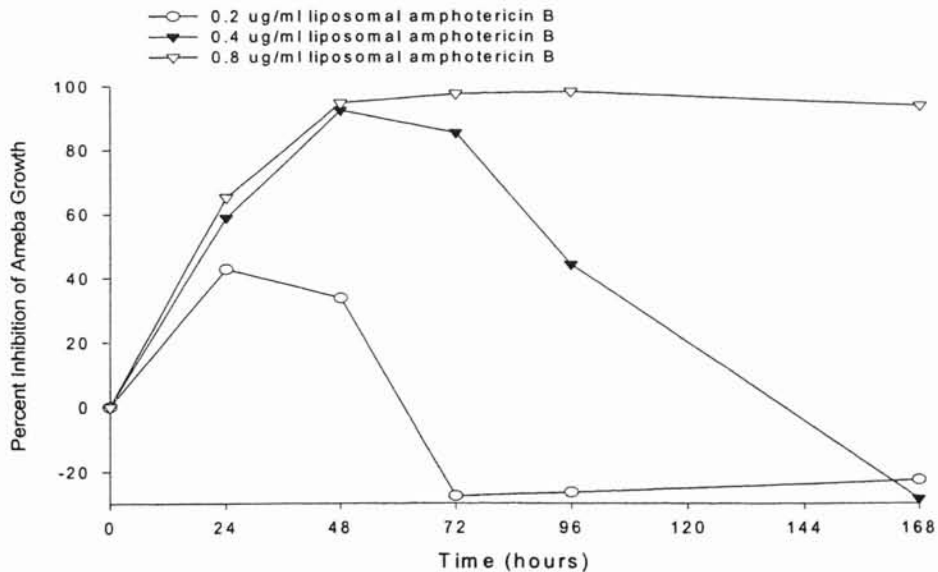

Figure 9. Percent inhibition of *N. fowleri* growth in the presence of a range of concentrations of liposomal amphotericin B.

Studies were conducted to determine whether there was a difference in the effectiveness of agitated vs. unagitated liposomal amphotericin B. Figure 10 depicts the effects of agitated liposomal amphotericin B on the growth of *N. fowleri*. As shown in Figure 10, 1 μg/ml liposomal amphotericin B significantly inhibited growth of *N. fowleri*, whereas 0.1 and 0.01 μg/ml were insignificant at inhibiting growth. Therefore, agitation of the medium did not significantly affect the *in vitro* activity of liposomal amphotericin B against *N. fowleri*, except that agitation did increase the doubling rate of the amebae at the concentrations where growth was not inhibited.

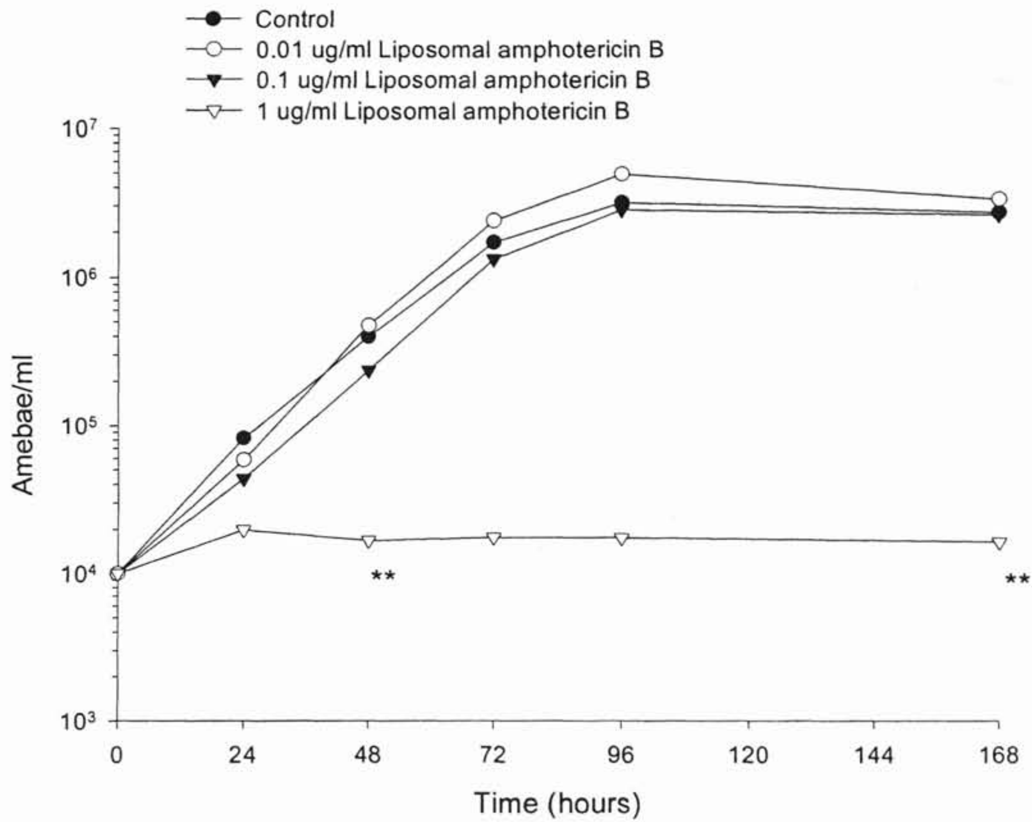

Figure 10. Effects of agitated liposomal amphotericin B on the growth of *N. fowleri*. The data points represent the mean of an experiment performed in triplicate.
** significant at $p< 0.01$

3.1.4 Ketoconazole Studies

The concentrations used in this study were based upon those obtained from previous *in vitro* studies with ketoconazole (Smego and Durack, 1984). The results of ketoconazole on the growth of *N. fowleri* are illustrated in Figure 11 and ketoconazole percent inhibition of ameba growth is represented in Figure 12. As shown in Figure 11, a concentration of 0.01 μg/ml was insufficient in repressing ameba growth with a percent inhibition ranging from 34% to 22% throughout 168 hours (Figure 12). As shown in Figure 11, the concentrations of 0.1 and 0.5 μg/ml of ketoconazole significantly inhibited ameba growth. The range of percentage inhibition from 48 to 168 hours was 90-96% and 92-97%, respectively (Figure 12). The MIC was determined to be 0.1 μg/ml of ketoconazole.

3.1.5 Minocycline Studies

The concentrations of minocycline used in this study were derived from prior *in vitro* experiments with *N. fowleri* (Lee *et al.*, 1979). Figure 13 represents the growth of *N. fowleri* in the presence of minocycline. The concentrations of 10 and 100 μg/ml significantly repressed the growth of *N. fowleri.* Minocycline at 1 μg/ml did not inhibit amebae growth. Figure 14 demonstrates the percentage inhibition of ameba growth in the presence of minocycline during a 7-day period. Minocycline at a concentration of 1 μg/ml produced maximal inhibition of 73% at 48 hours, but this effect began to deteriorate thereafter. The concentrations of 10 and 100 μg/ml inhibited ameba growth at greater than 92% throughout the 7-day course. Maximal percent inhibition of 98% was obtained at 96 hours at a concentration of 10 μg/ml of minocycline and was determined the MIC of this study.

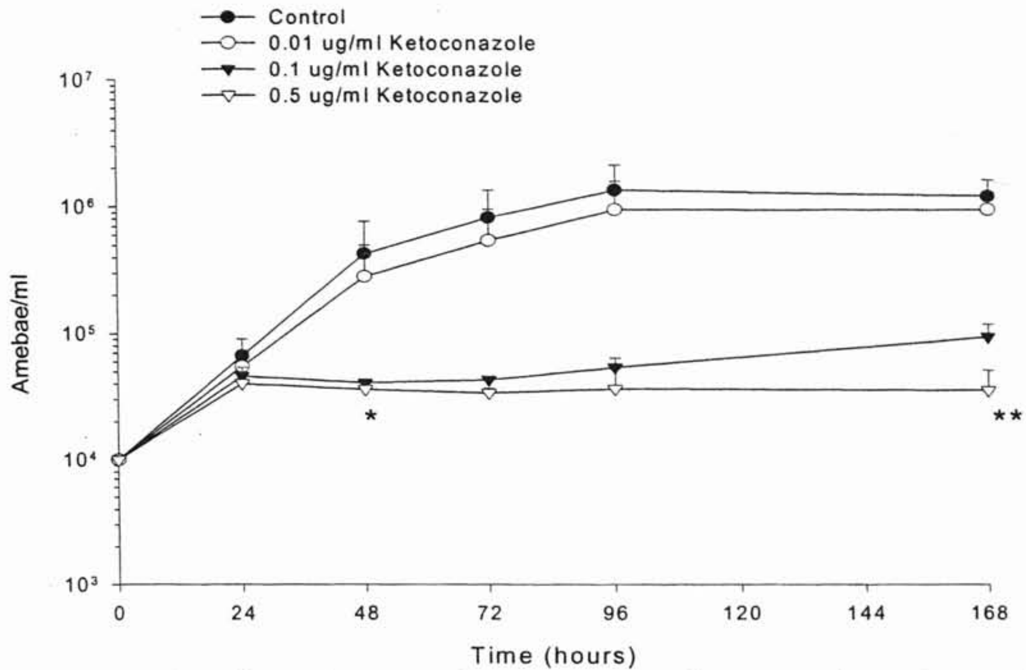

Figure 11. The effects of a range of concentrations of ketoconazole on the growth of *N. fowleri.* The data points illustrate the mean of 2 experiments performed in triplicate. The error bars represent the standard error mean of the individual data points.
* significant at $p < 0.05$ ** significant at $p < 0.01$

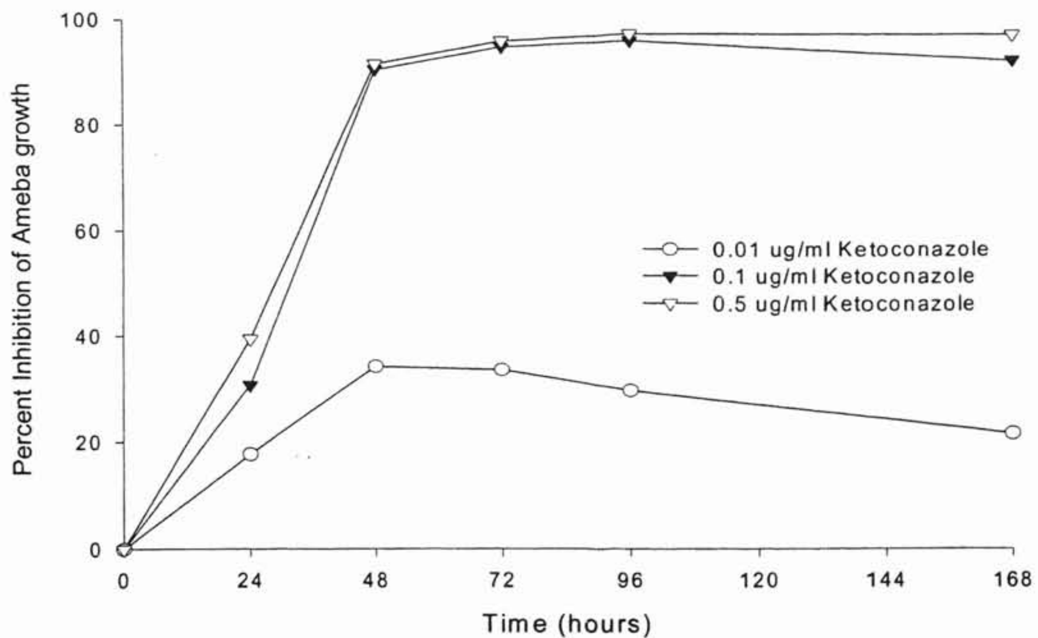

Figure 12. The percent inhibition of *N. fowleri* growth in the presence of ketoconazole.

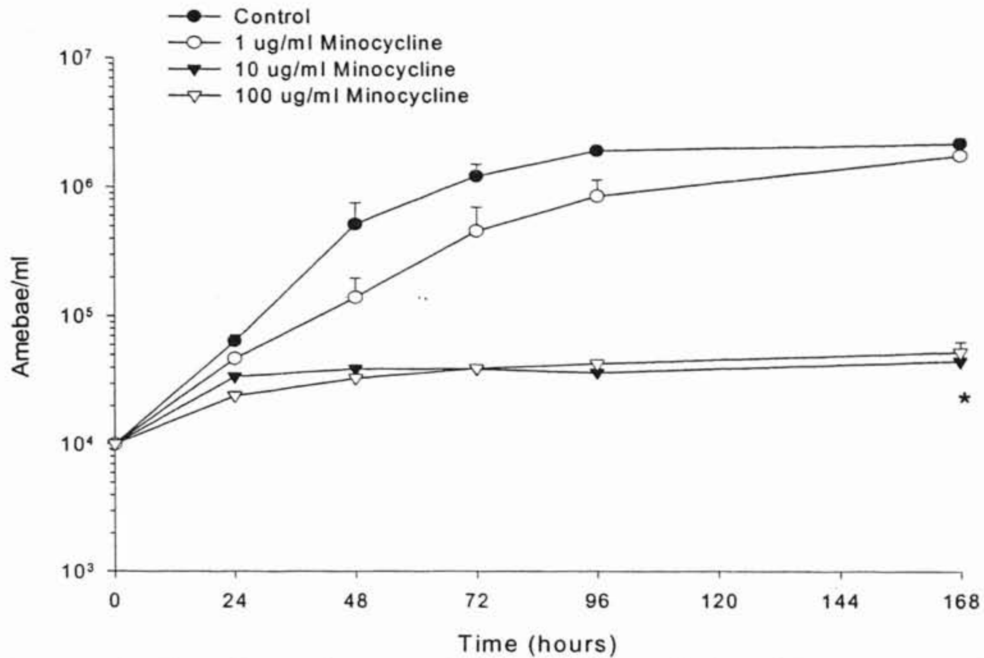

Figure 13. The effects of a range of concentrations of minocycline on *N. fowleri* growth over a 7-day interval. The data points demonstrate the mean of 2 experiments performed in triplicate. The error bars illustrate the standard error mean of the individual data points. * significant at $p < 0.05$

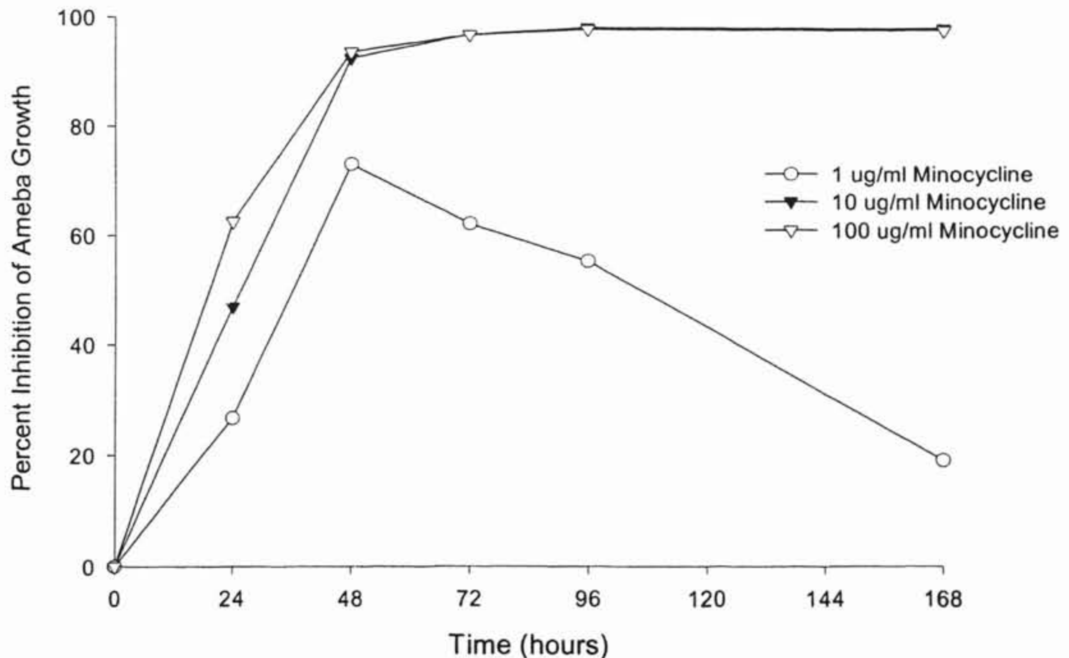

Figure 14. Percent inhibition of *N. fowleri* growth in the presence of minocycline over a 7-day period.

3.1.6 Azithromycin Studies

Azithromycin concentrations used in this study were obtained from previous *in vitro* studies with the other opportunistic amebae, *Acanthamoeba* and *Balamuthia* (Schuster and Visvesvara, 1998). Figure 15 depicts the effects of azithromycin on *N. fowleri* growth throughout a 7-day interval. As shown, azithromycin at 10 and 100 μg/ml significantly inhibited the growth of *N. fowleri*. A concentration of 1μg/ml azithromycin was not significantly different from the untreated control. The percentage inhibition of ameba growth in the presence of azithromycin is represented in Figure 16. At the lower concentration 1 μg/ml, inhibition was less than 20% the 7 day study. Percent inhibition of 10 and 100 μg/ml of azithromycin was greater than 90% on 48 hours and thereafter. The MIC was determined to be 10 μg/ml of azithromycin.

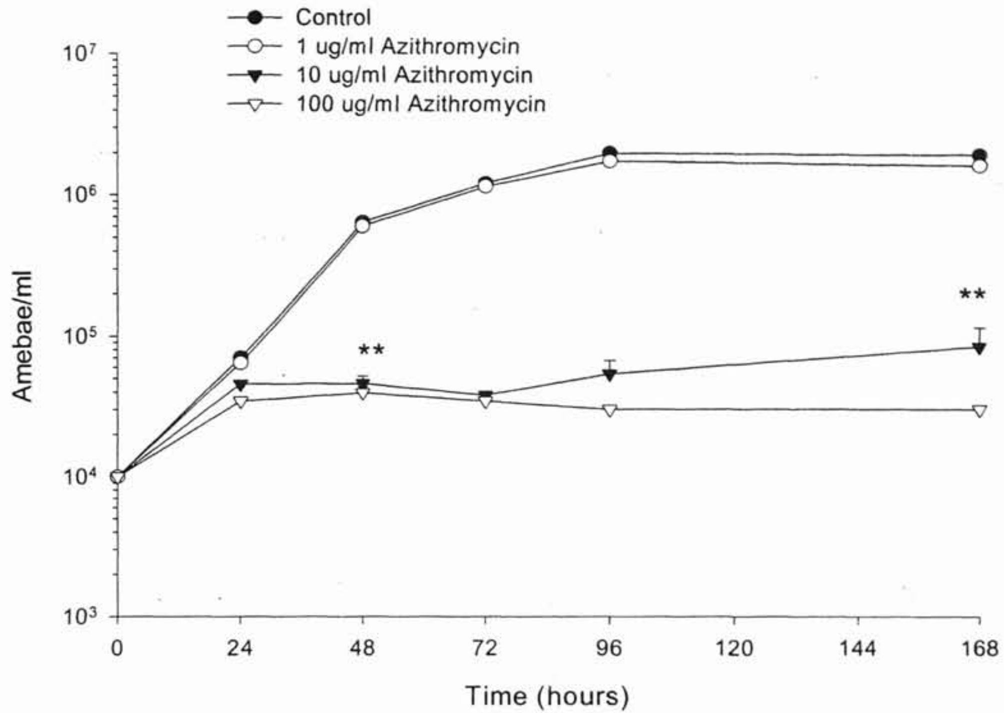

Figure 15. The effects of a range on concentrations of azithromycin on the growth of *N. fowleri* over a 7-day period. The data points demonstrate the mean of 2 experiments performed in triplicate. The error bars represent the standard error mean of the individual data points. ** significant at $p < 0.01$

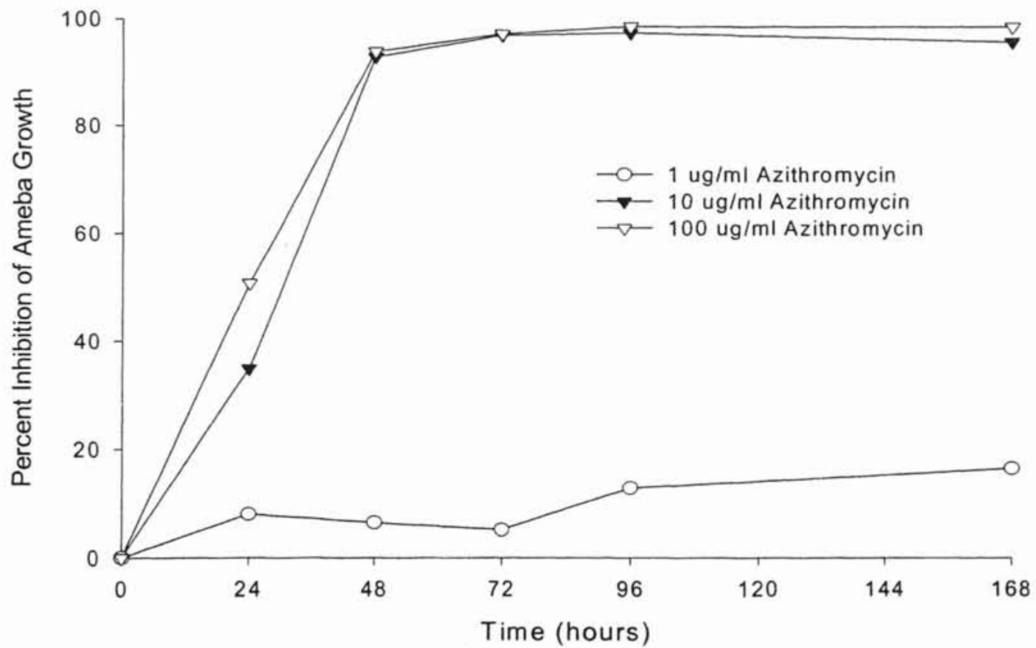

Figure 16. Percent inhibition of *N. fowleri* growth in the presence of azithromycin over a 7-day interval.

3.1.7 Quinupristin/dalfopristin *In Vitro* Studies

The concentrations of quinupristin/dalfopristin employed in this study were obtained from prior *in vitro* studies with the protozoan *Toxoplasma gondii* (Khan *et al.*, 1999). The results of quinupristin/dalfopristin are illustrated in Figures 17 and 18. Figure 17 depicts the effects of quinupristin/dalfopristin on *N. fowleri* growth throughout 7 days. As shown, the concentration of 10 μg/ml significantly inhibited the growth of *N. fowleri* and was determined the MIC of this study. The lower concentrations of quinupristin/dalfopristin, 5 and 1 μg/ml were marginal in their effect on *N. fowleri* growth. Figure 18 represents the percentage inhibition of quinupristin/dalfopristin on amebae growth. As shown, at 48 hours 10 μg/ml of quinupristin/dalfopristin inhibited 58% of growth and continued to inhibit growth throughout day 7. Quinupristin/dalfopristin at a concentration of 5 μg/ml inhibited 22% of growth at 48 hours, while the percent inhibition of 1 μg/ml was a mere 6% at 48 hours.

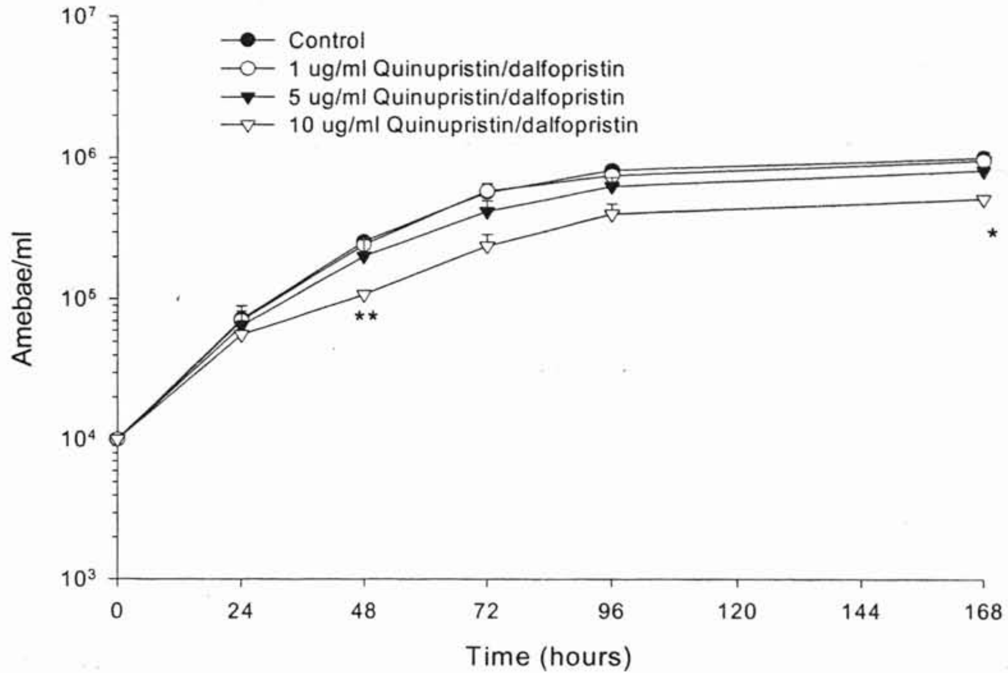

Figure 17. The effects of a range on concentrations of quinupristin/dalfopristin on the growth of *N. fowleri* over a 7-day period. The data points illustrate the mean of 2 experiments performed in triplicate. The error bars represent the standard error mean of the individual data points. * significant at $p < 0.05$ ** significant at $p < 0.01$

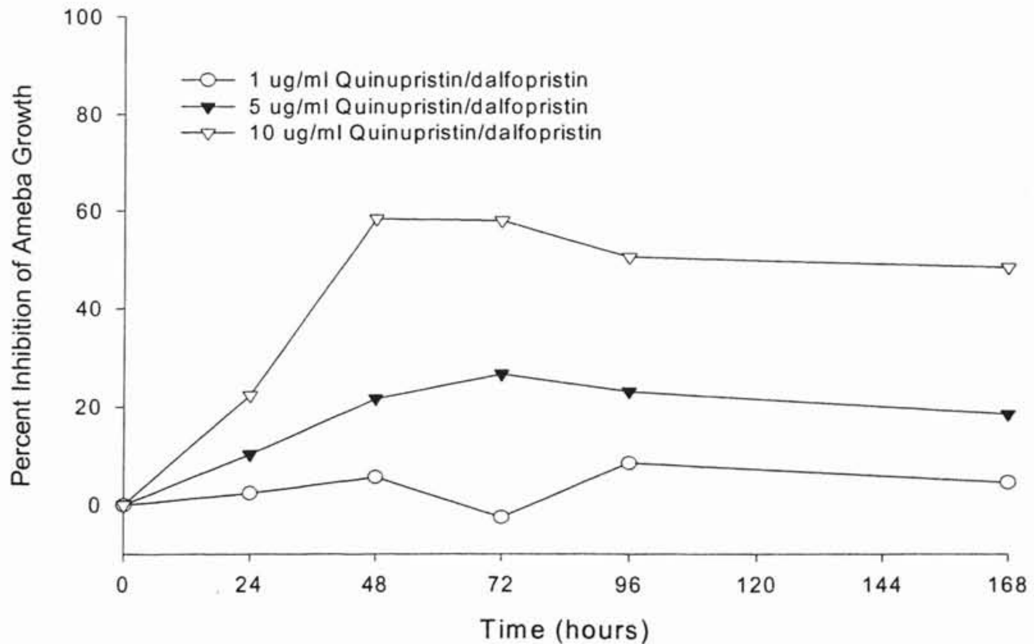

Figure 18. Percent inhibition of *N. fowleri* growth in the presence of quinupristin/dalfopristin over a 7-day course.

3.1.8 Linezolid Studies

Linezolid concentrations used in this study were obtained from prior *in vitro* experiments employing *Mycobacterium marinum* (Braback *et al.*, 2002). The effects of linezolid on *N. fowleri* growth are represented in Figure 19. As shown, linezolid at concentrations of 1, 5, and 10 μg/ml had no effect on *N. fowleri* growth. Linezolid was determined to have an MIC greater than 10 μg/ml.

3.1.9 Trifluoperazine Studies

Trifluoperazine concentrations used in this study were determined by previous *in vitro* studies on *N. fowleri, Acanthamoeba,* and *Balamuthia* (Schuster and Visvesvara, 1998; Schuster and Mandel, 1984). Figure 20 demonstrates the results of trifluoperazine on amebae growth. Trifluoperazine at a concentration of 1 μg/ml did not effectively inhibit *N. fowleri* growth. A concentration of 5 μg/ml of trifluoperazine had marginal effects on ameba growth. Trifluoperazine at a concentration of 10 μg/ml significantly suppressed the growth of *N. fowleri* and was determined the MIC. Figure 21 represents the percentage inhibition of *N. fowleri* growth by trifluoperazine. Trifluoperazine at 1 μg/ml had a range of inhibition from 9-39% with maximal inhibition at 168 hours. At a concentration of 5 μg/ml the range of percent inhibition was 29-48% with the highest inhibition at 168 hours. The range of percent inhibition of 10 μg/ml trifluoperazine was 48-91% with optimal inhibition at 72 hours.

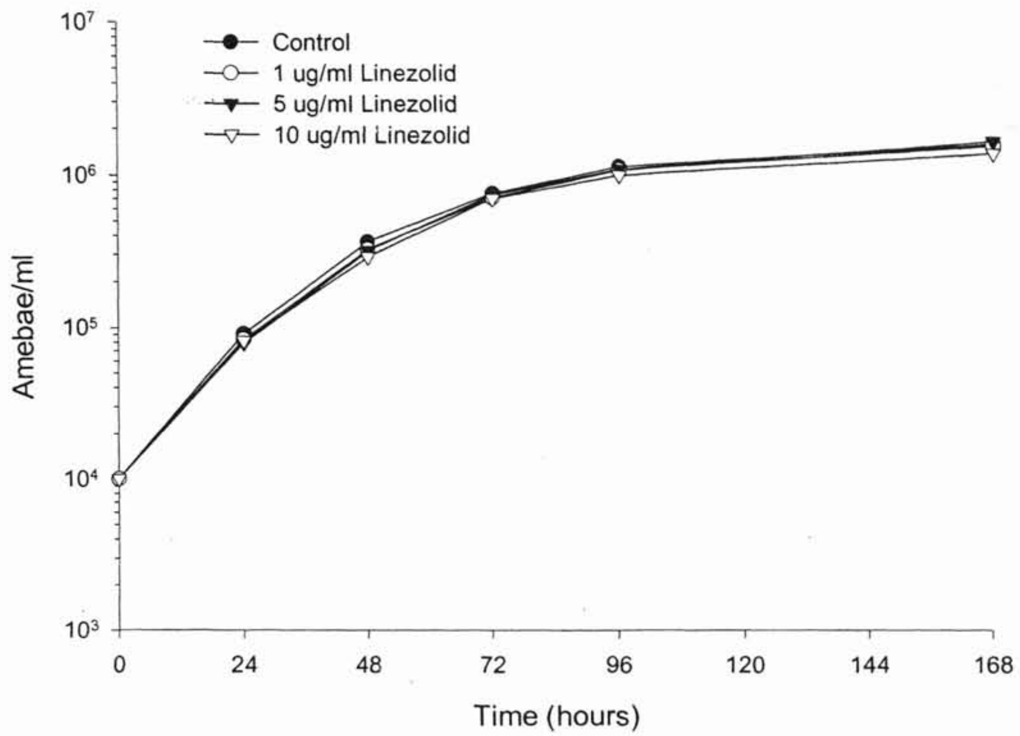

Figure 19. The effects of a range of concentrations of linezolid on the growth of *N. . fowleri* over a 7-day interval. The data points represent the mean of an experiment performed in triplicate.

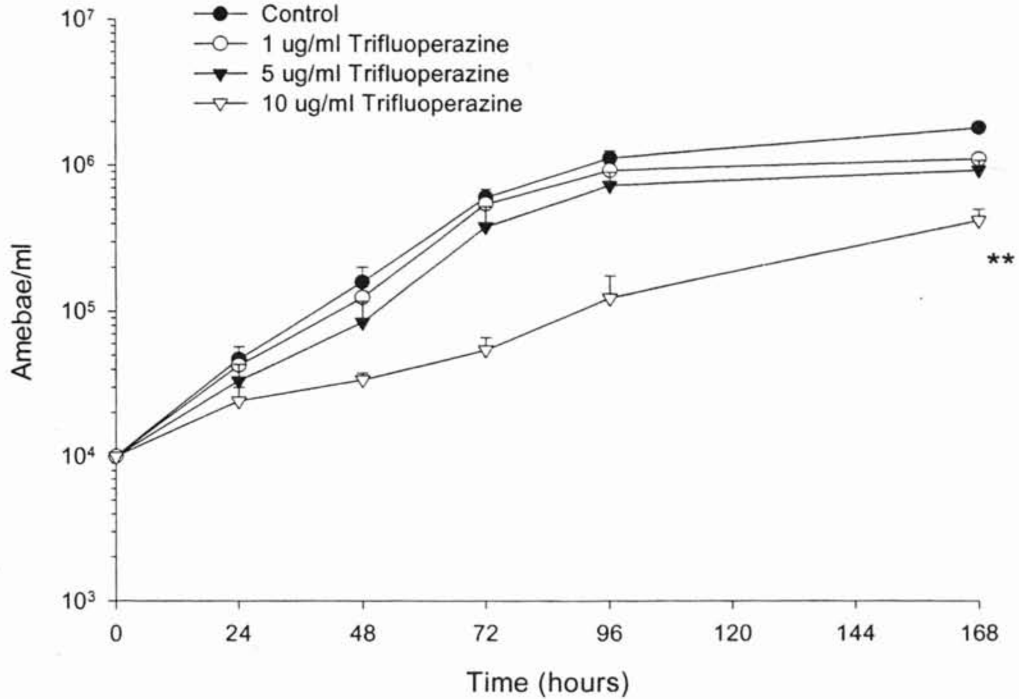

Figure 20. The effects of a range of concentrations of trifluoperazine on the growth of *N. fowleri* over a 7-day period. The data points represent the mean of 2 experiments performed in triplicate. The error bars illustrate the standard error mean of the individual data points. ** significant at $p < 0.01$

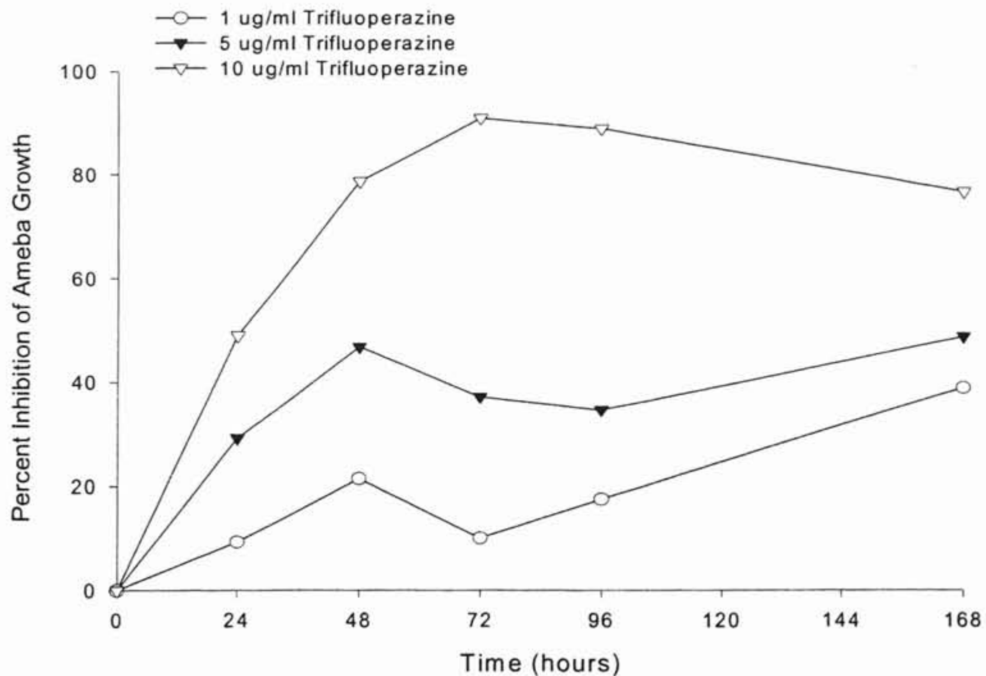

Figure 21. Percent inhibition of *N. fowleri* growth in the presence of trifluoperazine over a 7-day interval.

3.1.10 Rifampin Studies

The concentrations of rifampin used in this study were 1, 10, and 100 μg/ml and were based on previous *in vitro* studies with *N. fowleri* (Thong *et al.*,1977 and Donald *et al.,* 1979). Figure 22 illustrates the effects of rifampin on *N. fowleri* growth. As shown, rifampin did not significantly inhibit the growth of *N. fowleri* at any of the concentrations tested. Rifampin was determined to have an MIC greater than 100 μg/ml.

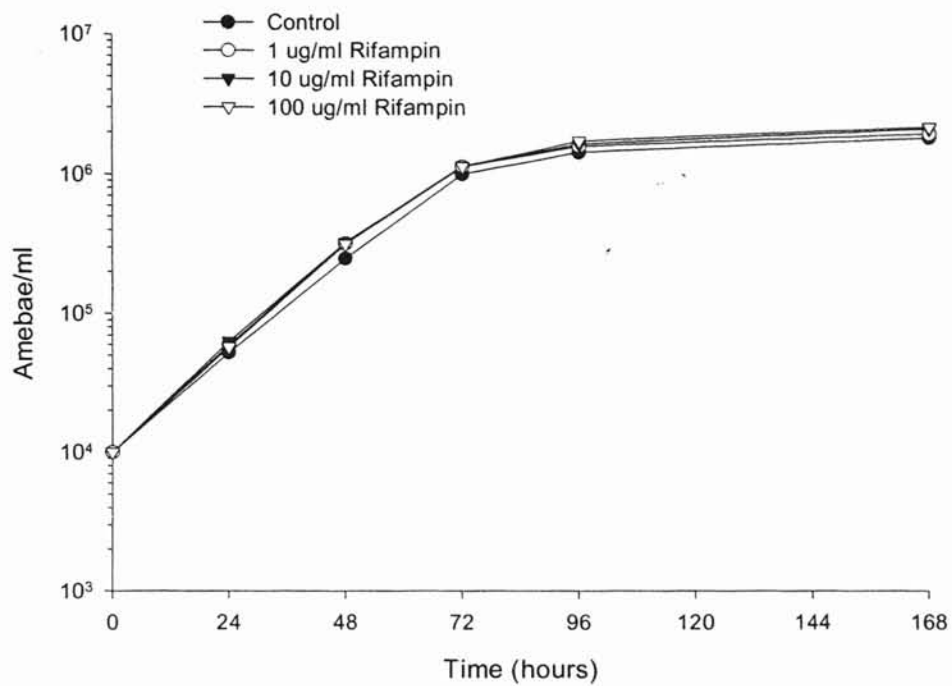

Figure 22. The effects of a range of concentrations of rifampin on *N. fowleri* growth. The data points represent the mean of an experiment performed in triplicate.

Table I. Summary of *In Vitro* Results

Drug	MIC (μg/ml)	Percent Inhibition at 48 Hours
Amphotericin B	0.08	97
Liposomal amphotericin B	0.8	95
Ketoconazole	0.1	90
Minocycline	10	92
Azithromycin	10	90
Quinupristin/dalfopristin	10	58
Trifluoperazine	10	48
Rifampin	>100	
Linezolid	>10	

3.2 Experimental PAM Studies in a Murine Model

Anti-microbial agents that were effective *in vitro* were evaluated in an experimental model of PAM infection. Mice were infected with *N. fowleri* by intranasal inoculation. Treatment was begun 72 hours after intranasal challenge and persisted for 5 days. The control groups received 0.1 ml i.p. injections of saline and the treated groups received 0.1 ml i.p. injections of the active drug, unless stated otherwise. Mice were observed for 28 days and the cumulative percent dead were recorded on a daily basis. To confirm the cause of death, brain tissue was cultured for *N. fowleri* from dead or moribund mice.

3.2.1 Preliminary *In Vivo* Results

Preliminary experiments were performed to determine the inoculum size that produced 100% mortality in the control group. The first inoculum size used was 10^4 amebae/mouse. Amphotericin B was employed in this experiment using concentrations of 1.75 and 4 mg/kg/day. The results of amphotericin B treatment are presented in Figure 23. As shown, the control group had 20% survival and a mean time death of 9.4 days. Amphotericin B at 1.75 mg/kg/day demonstrated 30% survival and had a prolonged mean time to death of 13.8 days as compared to the control group. Mice treated with 4 mg/kg/day of amphotericin B had a 87.5% protection rate and a mean time death of 9 days. Based on the results of this experiment, the inoculum size was increased to 2×10^4 amebae per mouse. This inoculum size produced 100% mortality of untreated mice and was used in all *in vivo* studies thereafter.

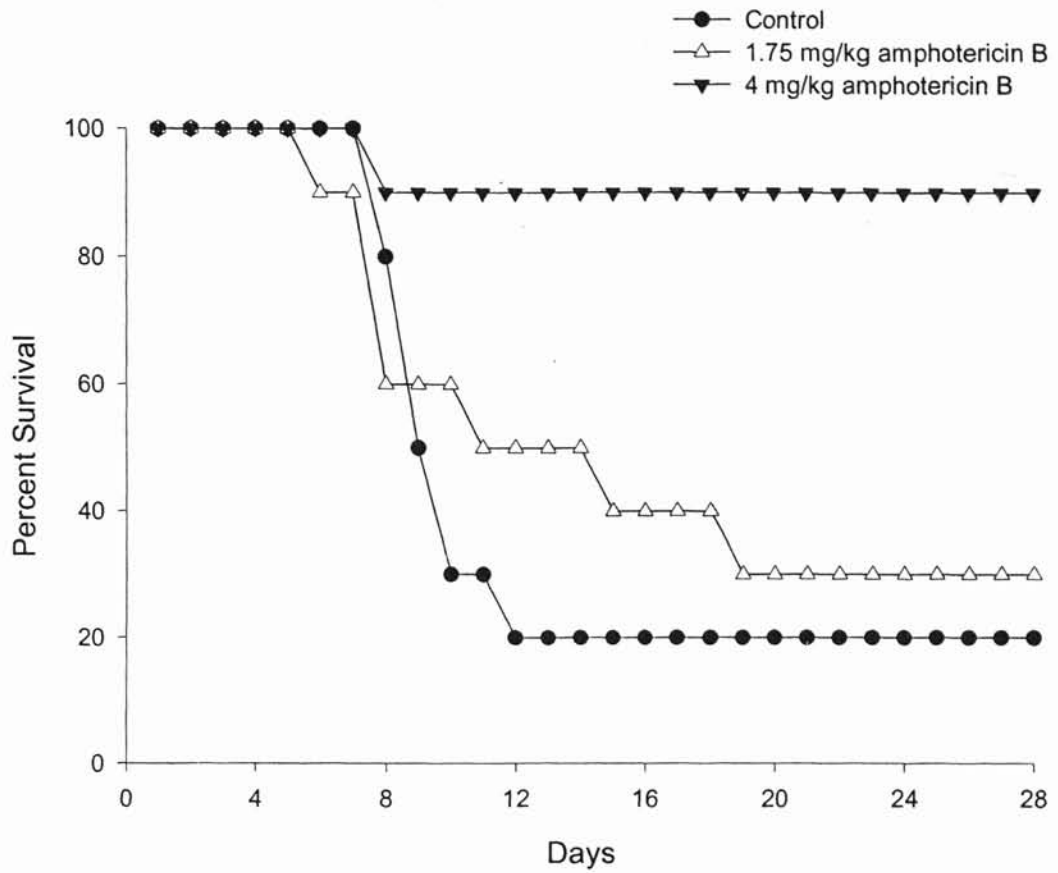

Figure 23. Preliminary results: Treatment of mice inoculated with $1x10^4$ *N. fowleri*/ mouse with amphotericin B.

3.2.2 Amphotericin B *In Vivo* Studies

The concentrations of amphotericin B used in this study were chosen by previous *in vivo* experiments with *N. fowleri* (Carter, 1969; Thong *et al.*, 1979a; Ferrante, 1982). The effects of amphotericin B on experimental amebic meningoencephalitis are illustrated in Figure 24. Mice in the untreated group had no survivors and a mean time death of 9.3 days. Amphotericin B at 2.5 mg/kg/day protected 30% of mice from death with a mean time death of 7 days. The higher dose of amphotericin B (7.5 mg/kg/day) had a survival rate of 50% and a mean time death of 7.8 days.

3.2.3 Liposomal Amphotericin B *In Vivo* Studies

Liposomal amphotericin B concentrations were equivalent to free amphotericin B in order to compare the 2 agents. Figure 25 demonstrates the results of 2.5, 7.5, and 25 mg/kg/day of liposomal amphotericin B on experimental PAM. The untreated group had a mean time death of 8.2 days with no mice surviving infection with *N. fowleri.* Liposomal amphotericin B at concentrations of either 2.5, 7.5, or 25 mg/kg of body weight per day protected 30% of infected mice from death. The mean time death was 8.3 days for 2.5 and 25 mg/kg liposomal amphotericin B and 8.1 days for 7.5 mg/kg liposomal amphotericin B.

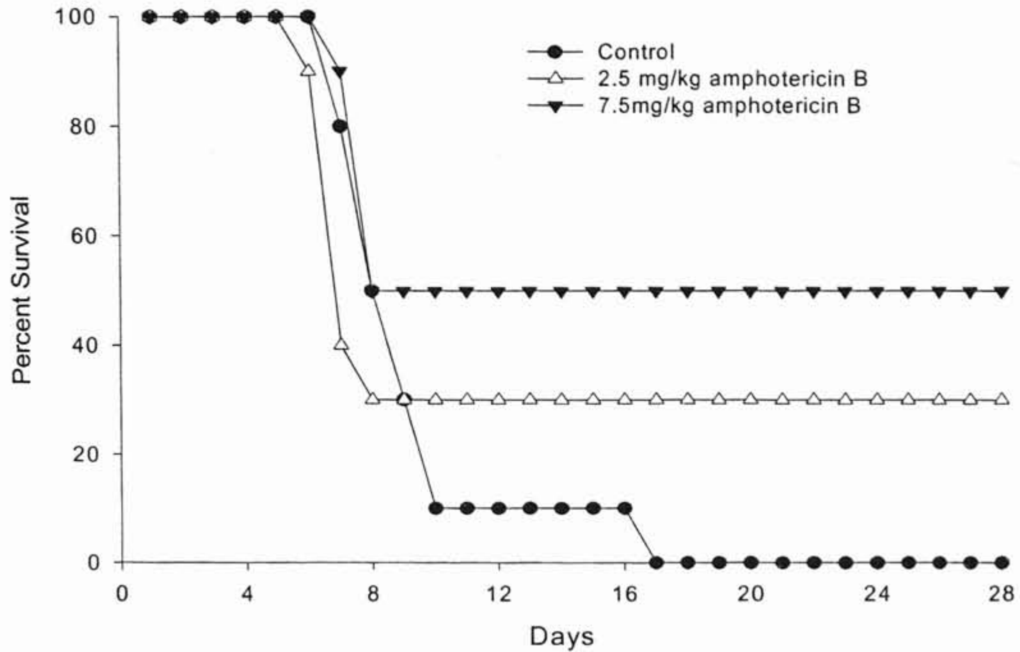

Figure 24. The effects of amphotericin B on experimental amebic meningoencephalitis. The graph represents the percent survival of mice infected with *N. fowleri* over a 28-day course.

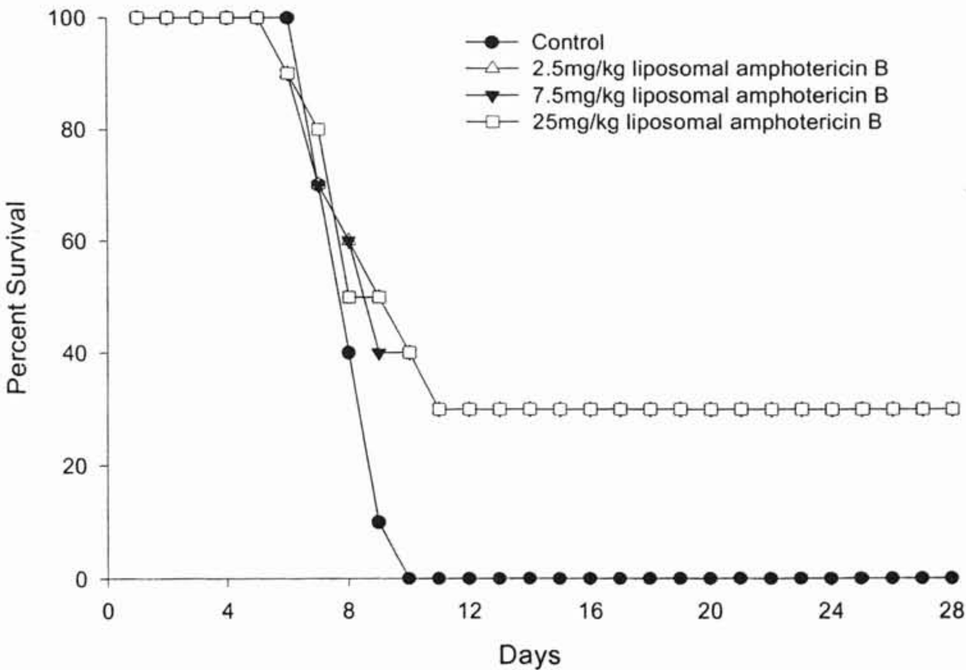

Figure 25. The effects of liposomal amphotericin B on experimental amebic meningoencephalitis. The graph illustrates the percent survival of mice infected with *N. fowleri* over a 28-day course.

3.2.4 Ketoconazole *In Vivo* Studies

The doses of ketoconazole used in this study were obtained from previous *in vivo* experiments with *N. fowleri* (Elmsly *et al.*,1980). In this experiment, the control group received i.p. injections of 0.01 N HCl because ketoconazole was solubilized in 0.1 N HCl. The effects of ketoconazole on experimental amebic meningoencephalitis are depicted in Figure 26. The control group had no survival with a mean time death of 8 days. Mice treated with 10 mg/kg/day of ketoconazole produced 20% survival with a mean time death of 7.6 days. The dose of 25 mg/kg/day of ketoconazole produced 30% survival in infected mice and a mean time death of 7.7 days.

3.2.5 *In Vivo* Minocycline Studies

Minocycline dosages used in this study were obtained from previous *in vivo* experiments on the protozoan *Toxoplasma gondii* (Romand *et al.,* 1993). The results of experimental amebic meningoencephalitis treatment with minocycline are illustrated in Figure 27. Mice treated with 25 mg/kg/day of minocycline took longer to develop symptoms. Although mortality was still 100%, the mean time death was 8.5 days compared to 7.8 days in the untreated group. Mice treated with 50 mg/kg/day minocycline had a mere 10% survival and a prolonged mean time death of 9.3 days as compared to the untreated mice.

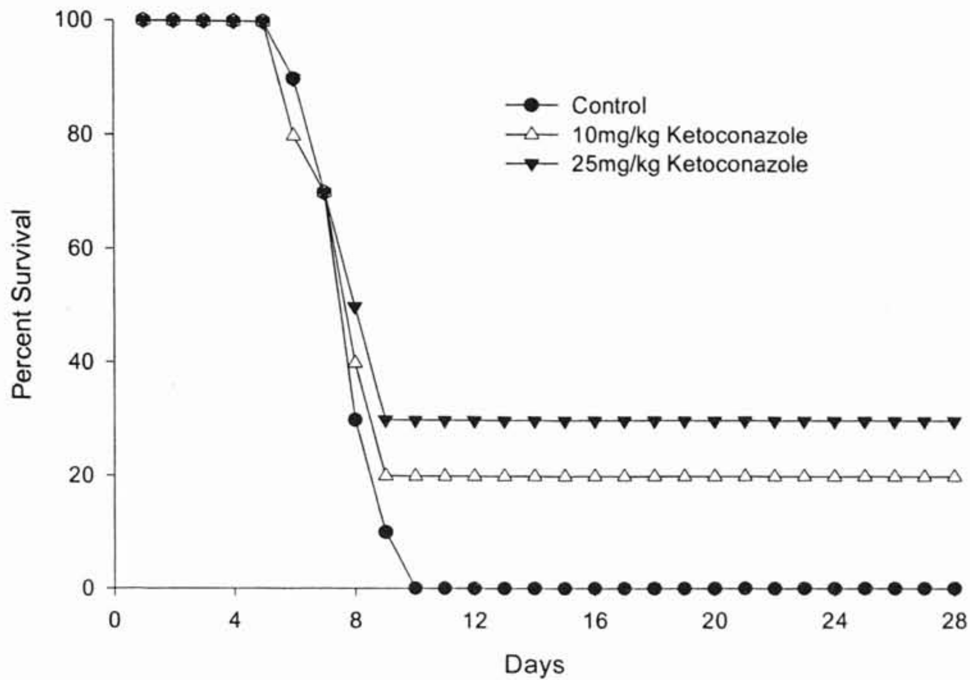

Figure 26. The effects of ketoconazole on experimental PAM infection. The graph represents the percent survival of mice infected with *N. fowleri* over a 28-day interval.

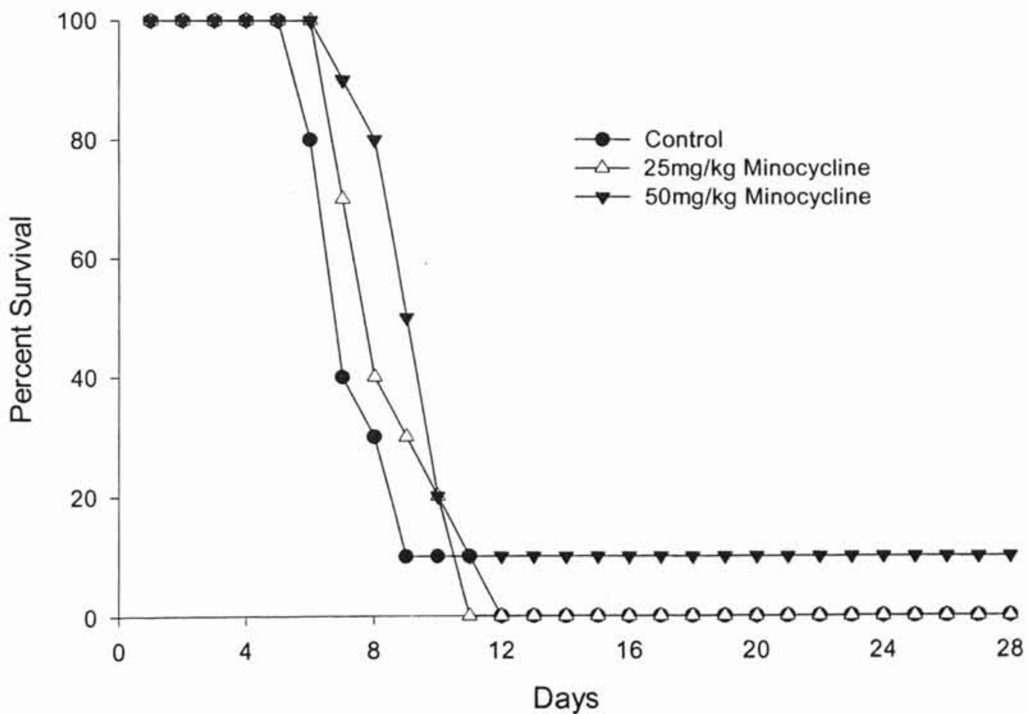

Figure 27. The effects of minocycline on experimental amebic meningoencephalitis. The graph illustrates the percent survival of mice infected with *N. fowleri* over a 28-day period.

3.2.6 Azithromycin *In Vivo* Studies

Azithromycin doses were chosen based on prior studies on toxoplasmosis in mice (Araujo *et al.*, 1992). Figure 28 represents the effects of treatment with azithromycin on experimental amebic meningoencephalitis. The untreated group had no survivors with a mean time death of 7.5 days. Azithromycin administered at dose of 25 mg/kg/day protected 40% of infected mice and increased the mean time death to 14.8 days. Azithromycin at 75 mg/kg/day protected 100% of the infected mice against death up to 28 days at which time the experiment was terminated.

3.2.7 Quinupristin/Dalfopristin *In Vivo* Studies

The 150 mg/kg/day dose of quinupristin/dalfopristin was based on experimental mice studies with *Toxoplasma gondii* (Khan *et al.*, 1999). The effects of quinupristin/dalfopristin on experimental amebic meningoencephalitis are demonstrated in Figure 29. Infected mice treated with 150 mg/kg/day of quinupristin/dalfopristin produced 50% survival, whereas mice in the untreated group had no survivors. The mean time death was 8.2 days for the untreated group and 7.8 days for quinupristin/dalfopristin treated group.

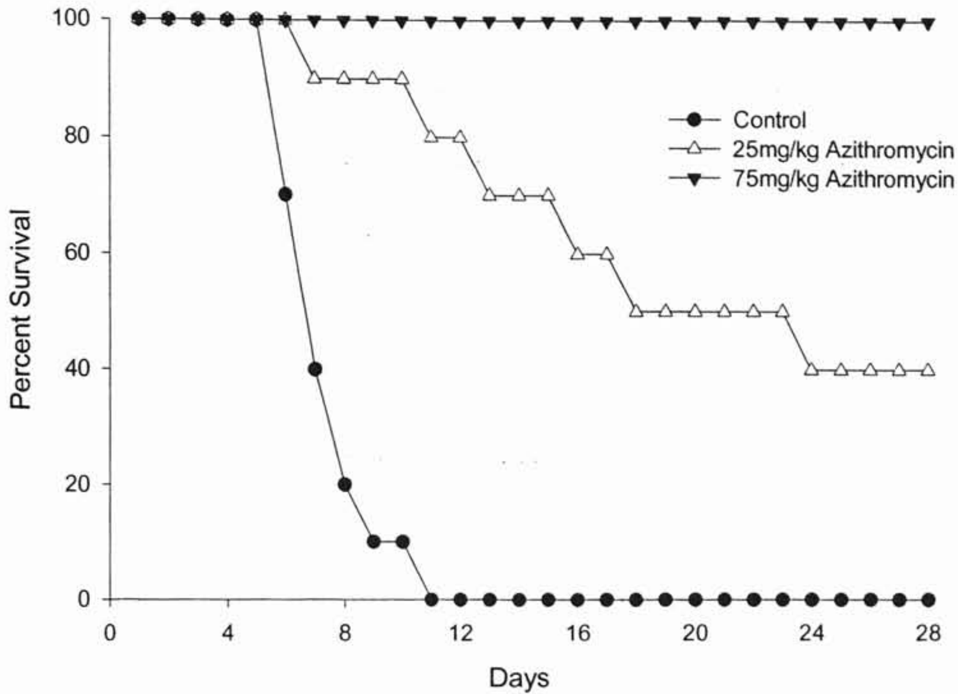

Figure 28. The effects of azithromycin on experimental PAM infection. The graph represents the percent survival of mice infected with *N. fowleri* throughout a 28-day period.

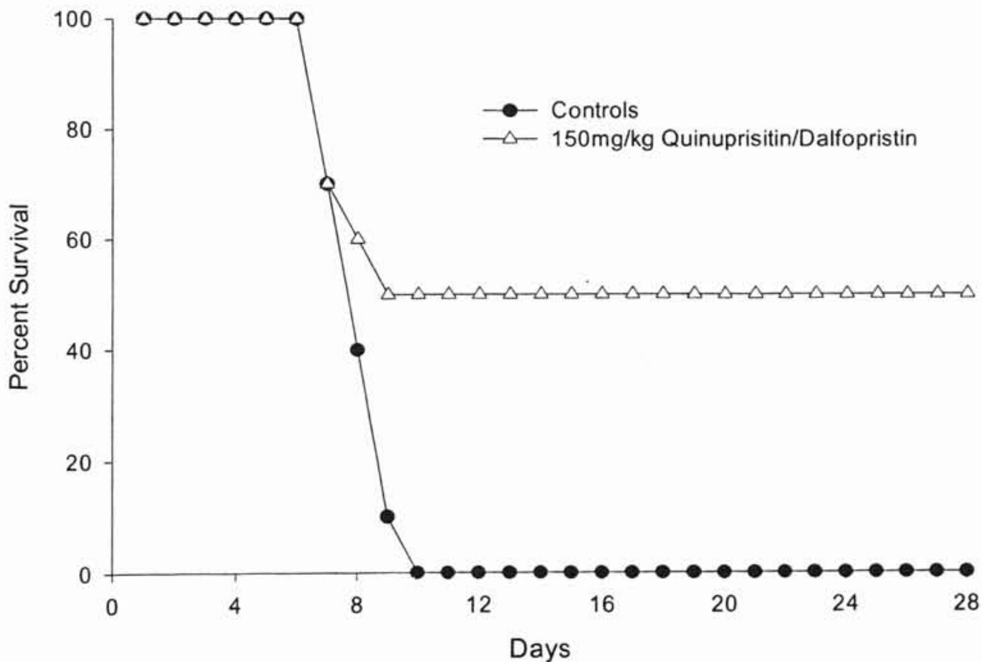

Figure 29. The effects of quinupristin/dalfopristin on experimental amebic meningoencephalitis. The graph illustrates the percent survival of mice infected with *N. fowleri* throughout a 28-day course.

3.2.8 Linezolid *In Vivo* Studies

Linezolid doses were obtained from studies employing experimental bacterial infections (Ford *et al.,* 1996). The effects of linezolid on experimental PAM are demonstrated in Figure 30. Linezolid at 75 mg/kg/day was administered in 0.2 ml i.p. because this concentration was insoluble in the smaller volume. Infected mice receiving 75 mg/kg/day linezolid had only 10% survival with a mean time death of 7.7 days. Mice treated with 25mg/kg/day linezolid also produced 10% survival, but the mean time death was prolonged to 8.3 days. The untreated group had no survivors and a mean time death of 8 days.

3.2.9 *In Vivo* studies with Trifluoperazine

The doses of trifluoperazine employed in this study were based on those used in previous *in vivo* experiments with bacteria (Mazumder *et al.,* 2001). The effects of trifluoperazine on experimental amebic meningoencephalitis are represented in Figure 31. The untreated group had a mean time death of 7.7 days with no survivors. Mice treated with 2.5 mg/kg/day of trifluoperazine had a mean time death of 8 days and 20% protection from death. Mice treated with 7.5 mg/kg/day of trifluoperazine were similar to the lower dose of trifluoperazine the mean time death was 7.9 days with a 20% protective effect from infection. Mice treated with 7.5 mg/kg/day trifluoperazine were observably more lethargic and drowsy when compared to all other therapeutic agents used *in vivo.*

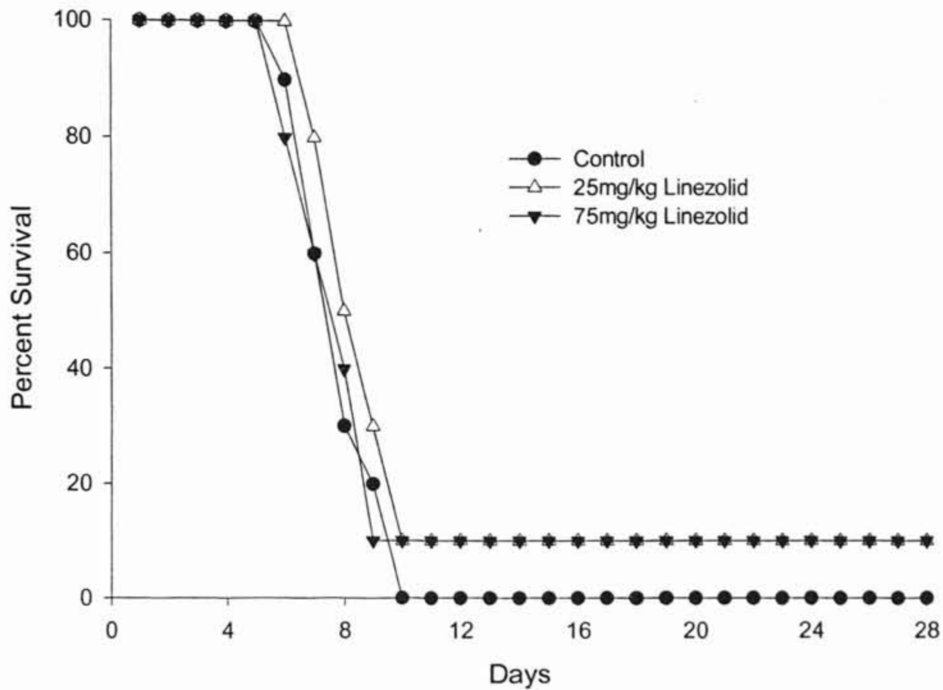

Figure 30. The effects of linezolid on experimental PAM infection. The graph demonstrates the percent survival of mice infected with *N. fowleri* throughout a 28-day interval.

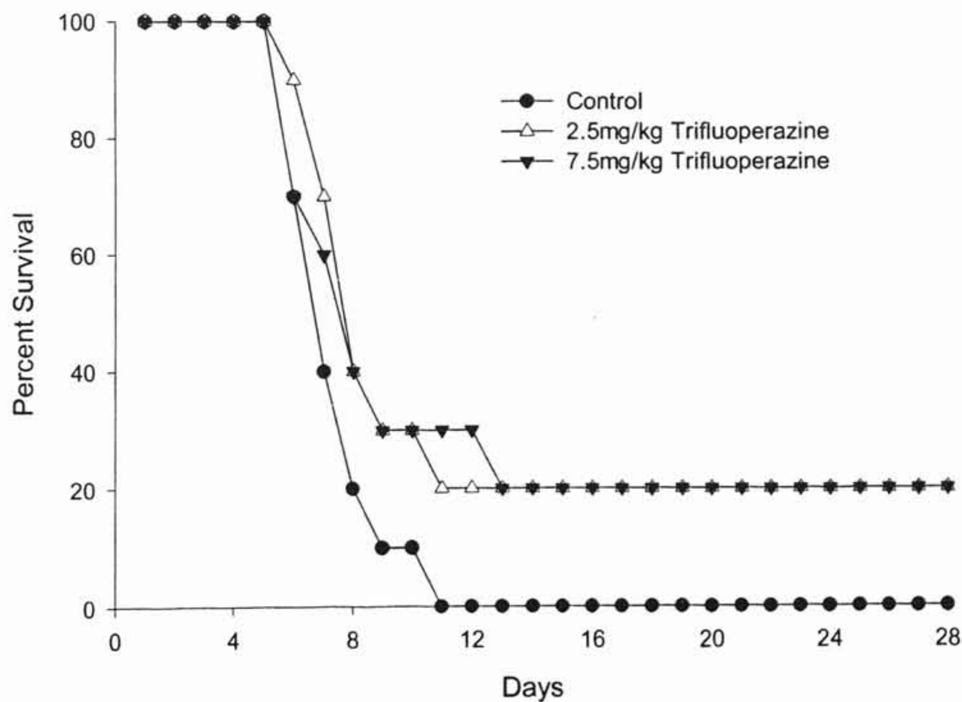

Figure 31. The effects of trifluoperazine on experimental amebic meningoencephalitis. The graph represents the percent survival of mice infected with *N. fowleri* throughout a 28-day period.

Table II. Summary of *In Vivo* Results

Drug	(mg/kg)	% Survival on (day) 7	10	14	21	28	Mean Time Death (day)
Untreated controls		60	5	2	0	0	8.1
Amphotericin B	2.5	40	30	30	30	30	7
	7.5	90	50	50	50	50	7.8
Liposomal Amphotericin B	2.5	70	40	30	30	30	8.3
	7.5	70	40	30	30	30	8.1
	25	80	40	30	30	30	8.3
Ketoconazole	10	70	20	20	20	20	7.6
	25	70	30	30	30	30	7.7
Minocycline	25	70	10	0	0	0	8.5
	50	90	20	10	10	10	9.3
Azithromycin	25	90	90	70	50	40	14.8
	75	100	100	100	100	100	>28
Quinupristin/dalfopristin	150	70	50	50	50	50	7.8
Linezolid	25	80	10	10	10	10	8.3
	75	60	10	10	10	10	7.7
Trifluoperazine	2.5	70	30	20	20	20	8
	7.5	60	30	20	20	20	7.9

CHAPTER IV

DISCUSSION

4.1 Treatment of PAM Infection

At present, there exists no satisfactory treatment of the rapidly fatal disease primary amebic meningoencephalitis (PAM). The antibiotics used to treat bacterial meningitis are ineffective in *N. fowleri* infections, as are the antiamebic drugs (John, 1993). A variety of drugs have been used to combat infections caused by *Naegleria,* yet treatment has been mostly unsuccessful. Of the drugs that have been evaluated against *N. fowleri,* amphotericin B, an antifungal agent, is the only agent with established clinical efficacy against PAM infection in humans (Apley, *et al.,* 1970; Anderson and Jamieson, 1972; Seidel *et al.,* 1982; Poungvarin and Jariya, 1991; Loschiavo *et al.,* 1993; Wang *et al.,* 1993). Several human patients received other drugs in combination with amphotericin B successfully treated for PAM, even though the therapeutic value of these drugs has not been demonstrated in *N. fowleri* infection.

4.2 Experimental Studies of Antifungal Agents on *N. fowleri*

Of all the agents evaluated against *N. fowleri,* amphotericin B has been the most studied and is the anti-*naeglerial* agent to which all other agents are compared. Based upon this study and prior data of other investigators, amphotericin B still appears to be the most potent and effective agent against *N. fowleri in vitro.* The present study observed that the MIC of amphotericin B was 0.08 μg/ml, which caused a percent inhibition of greater than 95%. This observation is in general agreement with results

from previous *in vitro* studies, which reported MICs of amphotericin B ranging from 0.018 to 1 μg/ml (Carter, 1969; Donald *et al.*, 1979; Duma and Finley, 1976; Lee *et al.*, 1979; Smego and Durack, 1984; Stevens *et al.*, 1981; and Scaglia *et al.*, 1988). The reported MICs for amphotericin B vary depending upon differences among the strains of *N. fowleri* employed, size of inoculum, and the growth phase of *N. fowleri* cultures when the drug itself is added.

Most prior *in vivo* studies on experimental PAM have evaluated the effectiveness of amphotericin B against *N. fowleri*. The results of these studies vary widely depending upon the size of inoculum used, the strain of *N. fowleri* employed, and the time at which treatment began. Of these previous *in vivo* experiments, most treatment regimens were started either before inoculation or 24 hours post-inoculation with *N. fowleri*.

The present studies utilized a delayed treatment in which compounds were administered 72 hours after intranasal inoculation of *N. fowleri* and continued for 5 days. The 72 hour delayed treatment of experimental amebic meningoencephalitis corresponds to the approximate time an infected patient would present with symptoms and begin treatment. The results of this *in vivo* study produced 30% survival of infected mice receiving 2.5 mg/kg of amphotericin B and 50% survival of mice receiving 7.5 mg/kg of amphotericin B daily for 5 days. However, amphotericin B did not significantly increase the mean time to death in this study.

Another study reported that amphotericin B at 7.5 mg/kg administered 24 hours pre-inoculation and continuing for 14 days protected 60% of mice from death due to *N. fowleri* infection (Carter, 1969). Because amphotericin B treatment was begun before mice were infected, the clinical relevance of the study is less significant. Another study

reported amphotericin B at a dose of 2.5 mg/kg daily for 10 days protected only 10% of mice infected with the Northcott strain of *N. fowleri*, when therapy was begun 24 hours post-inoculation (Thong, *et al.,* 1979b). In contrast, Ferrante (1982) presented evidence that mice infected with the Northcott strain receiving 2.5 mg/kg of amphotericin B beginning 24 hours post-inoculation and continuing for 10 days produced 100% survival. Thong *et al.,* (1979a) is the only other published report of delayed treatment in an experimental amebic meningoencephalitis model. The authors reported that infected mice receiving 2.5 mg/kg of amphotericin B beginning 72 hours post-inoculation and continuing for 7 days had a survival rate of 37.5%, which is similar to the results of this study. Overall, the present study produced results with amphotericin B that were similar to other investigators.

Amphotericin B acts by combining with the sterols ergosterol and cholesterol in the plasma membrane, and forms pores which causes leakage of the intracellular contents. Schuster and Rechthand (1975) studied the cytologic effects of amphotericin B on *N. fowleri.* The investigators discovered that *N. fowleri* exposed to amphotericin B would round up and fail to form pseudopodia. The ultrastructural abnormalities included alteration of nuclear shape, increased rough and smooth endoplasmic reticulum, swelling and degeneration of mitochondria, appearance of autophagic vacuoles, and a decrease in the number of food vacuoles over that normally observed in *N. fowleri.*

Although amphotericin B remains the first choice for treatment of PAM, amphotericin B therapy is restricted by its acute toxicity and low therapeutic index. Amphotericin B is probably the most toxic antibiotic in use today. It has been reported to cause nephrotoxicity in 80% of patients who are treated with it (Brenner, 2000). In

addition, amphotericin B has been associated with acute liver failure, cardiac arrhythmias, and hematopoietic disorders such as anemia, leukopenia, and thrombocytopenia (Brenner, 2000). In an effort to reduce toxicity, amphotericin B has recently been formulated into liposomes, which greatly reduces the drug's toxicity and extends its half-life (Adler-Moore and Proffitt, 1993). However, it is necessary to administer larger doses of liposomal amphotericin B in order to obtain the same efficacy as free amphotericin B in experimental fungal infections.

Because of the high sensitivity of *N. fowleri* to amphotericin B and since this agent has considerable toxic effects, the present study evaluated the potential substitution of liposomal amphotericin B for free amphotericin B. The results of this study confirmed liposomal amphotericin B is highly active against *N. fowleri in vitro,* as it inhibited amebae growth greater than 95% at a concentration of 0.8 µg/ml. Since free amphotericin B had 10 times more *in vitro* activity when compared to liposomal amphotericin B, it was speculated that amphotericin B was not being released from the liposomes *in vitro*. Therefore, experiments were conducted to determine whether agitation of the medium would increase the release of amphotericin B from the liposomes *in vitro*. The results of the experiments with agitated liposomal amphotericin B were identical to those obtained with unagitated liposomal amphotericin B experiments. These results indicate that agitation of the medium does not increase the *in vitro* activity of liposomal amphotericin B, and free amphotericin B is more potent than liposomal amphotericin B on *N. fowleri in vitro*.

Experiments with mice demonstrated that liposomal amphotericin B is effective in the treatment of experimental amebic meningoencephalitis. In this model, the

administration of either 2.5, 7.5, or 25-mg/kg liposomal amphotericin B protected 30% of infected mice. Hence, a dose-response relationship was not observed in this study. The present study indicates that there are some differences in potency and therapeutic efficacy between free amphotericin B and liposomal amphotericin B. Liposomal amphotericin B was not as effective as free amphotericin B considering that 7.5 mg/kg of free amphotericin B produced 50% survival and the same dose of liposomal amphotericin B produced only 30% survival of infected mice. The unusual kinetics of liposomal amphotericin B may partly explain the lack of dose-response found in this study. However, Alder-Moore and Proffitt (1993) reported that liposomal amphotericin B could effectively deliver amphotericin B to the brain in sufficient concentrations to either eliminate or markedly reduce the burden in fungal infections. The pharmacokinetics and activity of liposomal amphotericin B in the treatment of *N. fowleri* infection remain to be elucidated.

The other antifungal tested in this study was ketoconazole, which has a mechanism of action similar to amphotericin B. Ketoconazole acts by impairing the biosynthesis of ergosterol, the main sterol of fungal cell membranes, allowing increased permeability and leakage of cellular components (Hardman and Limbird, 2001). The MIC of ketoconazole was 0.1 μg/ml with a percent inhibition of *N. fowleri* growth greater than 90%. The only other reported *in vitro* study of ketoconazole found a similar effect on *N. fowleri* with a MIC of 0.31 μg/ml (Smego and Durack, 1984).

In the present study, ketoconazole treatment was associated with increased survival of infected mice and demonstrated a dose-response relationship. The survival rate was 20% for mice receiving 10 mg/kg ketoconazole and 30% survival for those

treated with 25 mg/kg ketoconazole. The mean time to death for ketoconazole treated mice was not different from untreated mice. Elmsly *et al.*, (1980) evaluated the efficacy of ketoconazole treatment in an experimental PAM model. However, the dosage varied during the experimental treatments up to 20 mg/kg i.p. once or twice daily. Therapy was initiated immediately after inoculation with *N. fowleri.* The authors reported ketoconazole neither reduced mortality nor prolonged survival. The reason these authors were unable to show efficacy of ketoconazole could be due to the extremely high inoculum used in their study, which was 10^5 amebae/mouse and was almost 10 times more than that used in the present study. The clinical use of ketoconazole in PAM infections may be limited due to the poor penetration of ketoconazole into the CSF and the numerous drug-interactions it causes. Ketoconazole was used in combination with a low dose of amphotericin B and rifampin in 1 human survivor of PAM (Poungvarin and Jariya, 1991). However, the mechanism of action of ketoconazole in *N. fowleri* has not been elucidated.

4.3 Experimental Studies with Protein Synthesis Inhibitors on *N. fowleri*

Minocycline was included in the present study because of a previously described *in vitro* sensitivity of *N.* fowleri to this drug (Lee *et al.,* 1979). Minocycline is a tetracycline derivative that inhibits protein synthesis by blocking the binding of tRNA to the 30S subunit of the bacterial ribosome (Brenner, 2000). The results of this study show that minocycline effectively inhibits *N. fowleri* growth at a concentration of 10 μg/ml with a percent inhibition greater than 92%. Lee *et al.,*(1979) is the only other study that has evaluated the *in vitro* effects of minocycline on *N. fowleri*. The authors reported minocycline to inhibit the growth of *N. fowleri* with a MIC of 2.8 μg/ml. The

differences in MICs are probably due to the dissimilarity between the methods employed and the definitions of MIC. Lee *et al.*, (1979) defined MIC as the concentration of drug required to prevent the cytopathic effect of *N. fowleri* on monkey kidney cell (MKC) cultures to which an inoculum of approximately 1,000 *N. fowleri* had been added. In addition, the inoculum size used in their study was 10 times lower (10^3 vs. 10^4 amebae/mouse) than that used in the present study. Nevertheless, both studies determined that minocycline effectively inhibited the growth of *N. fowleri*.

The present *in vivo* study of minocycline administered at a dose of 25 mg/kg had little efficacy, as 100% of mice died with a mean time death of 8.5 days. Minocycline at a dose of 50 mg/kg produced only 10% survival in infected mice and the mean time death was prolonged to 9.3 days. Although minocycline provided limited protection against *N. fowleri*, mice receiving either dose of minocycline lived longer than the untreated controls which had a mean time death of 7.8 days.

The present *in vivo* results are comparable to another experimental PAM study conducted with tetracycline, which is in the same class as minocycline. Thong *et al.*, (1978) reported tetracycline administered up to 150 mg/kg had minimal activity in the treatment of experimental PAM. The authors reported 100% mortality of infected mice and a prolonged mean survival time, which is comparable to the present study. Minocycline was expected to be more potent than tetracycline in treating PAM infection because minocycline is more lipophilic and it reaches higher concentrations in the CNS (Hardman and Limbird, 2001). The fact that minocycline has *in vitro* activity, but was not effective *in vivo* is possibly related to the amount of minocycline that is actually

reaching the site of infection. However, Derouin *et al.*, (1992) found that minocycline was 100% effective in the treatment of encephalitis due to *Toxoplasma gondii.*

The macrolide antibiotic, azithromycin, was selected for this study on the basis of a previously described sensitivity of *Acanthamoeba* to this drug (Schuster and Visvesvara, 1998). The present study determined azithromycin to be active against *N. fowleri in vitro*, as it inhibited growth greater than 90% at a concentration of 10 μg/ml. Schuster and Visvesvara (1998) concluded azithromycin inhibited *Acanthamoeba* growth with a MIC of 0.1 μg/ml. Thus, *Acanthamoeba* species appear to be more sensitive to azithromycin than *N. fowleri*.

The present *in vivo* studies have indicated superior activity of azithromycin compared to all other agents evaluated against *N. fowleri*. These results revealed that azithromycin administered at a dose as low as 25 mg/kg protected 40% of infected mice from infection. In addition, the mean time death was doubled to 14.8 days compared to untreated control mice, which had no survivors and a mean time death of 7.5 days. Treatment with 75 mg/kg of azithromycin resulted in a remarkable survival rate: 100% of infected mice were still alive with no apparent signs of infection at 28 days when the experiment was terminated. Another study found azithromycin to be highly active in a toxoplasmic encephalitis murine model (Araujo *et al.*, 1988).

The results of this study and the toxoplasmosis study have suggested that azithromycin attains active concentrations in the inflamed CNS. The present results also imply that azithromycin possess a superior efficacy compared to amphotericin B in experimental PAM studies. Such results are probably due to the fact of a very long half-life of azithromycin and the high accumulation of azithromycin in the tissues that give it

a greater advantage (Hardman and Limbird, 2001). The mechanism whereby azithromycin inhibits or kills *N. fowleri* is not known. It is interesting to postulate that either azithromycin alone or in combination with amphotericin B could possibly be an improved treatment of PAM infection in humans. However, further studies should be conducted with azithromycin and amphotericin B to determine the activity of these drugs in combination against *N. fowleri*.

Quinupristin/dalfopristin is a novel antimicrobial agent that has a mechanism of action similar to azithromycin. Due to the discovery of azithromycin on *N. fowleri*, it was considered of interest to investigate the effects of quinupristin/dalfopristin against *N. fowleri*. This *in vitro* study suggested that quinupristin/dalfopristin has moderate activity against *N. fowleri*, as a concentration of 10 μg/ml produced a maximal inhibition of ameba growth of 60%. Due to the *in vitro* results, the *in vivo* studies employed a high dose of quinupristin/dalfopristin, which was 150 mg/kg daily. The *in vivo* results revealed that quinupristin/dalfopristin administered at this high dose produced 50% survival of infected mice. The mean time to death was not different from the untreated control group.

Quinupristin/dalfopristin has been primarily used for the treatment of bacteremia, pneumonia, and skin and soft tissue infections due to drug-resistant gram-positive cocci (Brenner, 2000). Recently, quinupristin/dalfopristin was determined to exhibit *in vitro* and *in vivo* activity against the protozoan *Toxoplasma gondii* (Khan *et al.*, 1999). The investigators reported quinupristin/dalfopristin at 100 and 200 mg/kg administered i.p. for 10 days significantly protected 50 and 100% the infected mice against death, respectively.

Linezolid was evaluated in the present study due to its unique mechanism of action. Linezolid is a novel antibacterial agent that acts by inhibiting the formation of the 70S complex by binding to the 50S ribosome near to the interface with the 30S subunit (Diekema and Jones, 2002). This study was the first to evaluate the effects of linezolid on *N. fowleri*. In addition, linezolid has been reported to exhibit good penetration of the CSF and could be a potential treatment of PAM (Regazzi *et al.*, 2002).

The present *in vitro* study evaluated linezolid at concentrations of 1, 5, and 10 μg/ml and found it to have no inhibitory effects on *N. fowleri* growth. Regardless of the ineffectiveness of linezolid *in vitro*, linezolid was evaluated *in vivo* at doses of 25 and 75 mg/kg. As expected, linezolid offered only 10% protection to infected mice treated with either dose. In addition, there was no difference in the mean time to death between the treated and untreated mice. The minimal activity of linezolid *in vitro* and *in vivo* suggests a permeability problem in linezolid penetration across the *Naegleria* cell membrane. Therefore, linezolid does not seem to be useful in the treatment of PAM.

4.4 Experimental Studies with Other Therapeutic Agents on *N. fowleri*

Trifluoperazine is a phenothiazine derivative that is commonly used in medical practice as an antipsychotic. In addition, trifluoperazine has recently been discovered to have significant antimicrobial activity (Mazumder *et al.*, 2001). Trifluoperazine was evaluated in this study based upon previous *in vitro* studies with *N. fowleri*. The *in vitro* study indicated 10 μg/ml of trifluoperazine had an inhibitory effect greater than 90% on *N. fowleri* growth. The lower concentrations of trifluoperazine (5 and 1 μg/ml) were also moderately effective against *N. fowleri* growth. Schuster and Mandel (1984) first studied the *in vitro* activity of trifluoperazine against several strains of *N. fowleri*. The

investigators reported 4.8 μg/ml trifluoperazine inhibited the growth of different *N. fowleri* strains 90% or greater. Another *in vitro* study found trifluoperazine inhibited *Acanthamoeba* growth by 70 to 90% at 5 and 10 μg/ml. These studies show that trifluoperazine has similar activity against *N. fowleri* and *Acanthamoeba in vitro.*

The present experiments with mice portrayed trifluoperazine to have limited efficacy in the treatment of experimental amebic meningoencephalitis. In this model, administration of either 2.5 or 7.5 mg/kg of trifluoperazine protected only 20% of infected mice. Thus, a dose-response relationship was not observed in this study. In addition, the mean time of death was not significantly different among the treated and untreated mice. This study was the first attempt to treat experimental amebic meningoencephalitis infection with trifluoperazine.

Due to the high *in vitro* sensitivity of *N. fowleri* to trifluoperazine, the *in vivo* results were somewhat disappointing. Since trifluoperazine is highly lipophilic, the low *in vivo* activity probably correlates with the antimicrobial action of trifluoperazine within the brain. The mode of action of trifluoperazine has not been fully established in *Naegleria.* Schuster and Twomey, (1983) studied the *in vitro* effects of trifluoperazine on nonpathogenic *Naegleria gruberi*. The investigators found trifluoperazine to have an inhibitory effect on flagellation and on the trophic growth of *N. gruberi.* Schuster and Mandel, (1984) postulated several different *in vitro* modes of action for trifluoperazine on *N. fowleri* growth. First, trifluoperazine may be interfering with cAMP production in *Naegleria* sp., although this association has not been established. Second, trifluoperazine could be inhibiting the calcium regulatory protein, calmodulin at some point in

Naegleria. The other theory of trifluoperazine action on *Naegleria* is since trifluoperazine is highly lipophilic, it may affecting the ameba plasma membrane.

Rifampin did not have *in vitro* activity at any of the concentrations evaluated against *N. fowleri* in the present study. The MIC of rifampin was determined to the greater than 100 μg/ml. These results are similar to a previous *in vitro* study by Donald *et al.*, (1979). The authors tested rifampin at a range of concentrations between 10 and 500 μg/ml against the MsT strain of *N. fowleri* and found no inhibition of growth at any of the concentrations tested. Their study determined rifampin to have a MIC of greater than 500 μg/ml. In contrast, Thong *et al.*, (1977) found rifampin at a concentration of 100 μg/ml to significantly inhibit *N. fowleri* growth.

Due to the *in vitro* results of this study and the results of Donald *et al.*, (1979), rifampin was not included in the *in vivo* studies. Rifampin was previously studied in an experimental murine model of PAM by Thong *et al.*, (1979b). The investigators administered rifampin at 50, 100, and 150-mg/kg 24 hours post-inoculation daily for 10 days to infected mice. Their study determined rifampin had minimal activity with a 100% mortality rate of treated mice. However, the authors demonstrated synergistic activity between rifampin and amphotericin B in an experimental model of PAM. In addition, rifampin has been successfully utilized in 3 human cases of PAM in triple combination with amphotericin B and 1 of the following compounds: ketoconazole, miconazole, or chloramphenicol.

4.5 Summary and Future Directions

In conclusion, the antifungal agents appear to have the most *in vitro* activity against *N. fowleri,* as the MICs of the antifungals were all less than 1 μg/ml. In addition,

minocycline, azithromycin, quinupristin/dalfopristin, and trifluoperazine also demonstrated *in vitro* activity against *N. fowleri* with MICs of 10 μg/ml. Rifampin and linezolid did not exhibit *in vitro* activity against *N. fowleri* at the concentrations tested and the MICs were determined to be greater than 100 and 10 μg/ml, respectively.

However, the sensitivity of *N. fowleri* to a therapeutic agent *in vitro* does not directly correlate with the drug's *in vivo* activity. These results have indicated superior activity for azithromycin treatment of experimental PAM infection. Amphotericin B treatment had the next best *in vivo* activity. Lipid preparations of amphotericin B are inferior to conventional amphotericin B, and the lipid preparations did not exhibit a dose-response relationship in this study. Ketoconazole was effective in the treatment of experimental PAM and clearly demonstrated a dose-response relationship. A high dose of quinupristin/dalfopristin offered protection to mice infected with *N. fowleri*. Trifluoperazine demonstrated limited *in vivo* efficacy, and also did not exhibit a dose-response relationship. Finally, minocycline and linezolid offered minimal protection in this experimental PAM model.

It is proposed that the experimental PAM model using a delayed treatment will provide a more reliable basis for therapeutic agents that will be effective in patients suffering from PAM infection. Azithromycin has great promise in the clinical treatment of patients suffering from PAM infection. Further studies should be conducted in an experimental model of PAM to evaluate whether a combination of azithromycin and amphotericin B are synergistic. In addition, the therapeutic agents: ketoconazole, quinupristin/dalfopristin, and trifluoperazine, which displayed marginal activity, should be evaluated for synergy with amphotericin B. It is possible that amphotericin B, by

increasing the permeability of the ameba membrane, could allow the other therapeutic agents to more effectively penetrate the ameba.

Even though minocycline and linezolid did not offer protection to infected mice, these agents should also be considered in synergy studies with amphotericin B. According to Thong *et al.,* (1979a and 1979b), rifampin and tetracycline did not protect infected mice when administered alone, but the effects of these drugs were potientated when combined with amphotericin B.

Finally, the study with liposomal amphotericin B had marginal efficacy in mice infected with *N. fowleri,* with no dose-response relationship. It would also be of interest to evaluate liposomal amphotericin B combined with other therapeutic agents for potientation or synergistic effects.

Future studies to determine the mechanism of action of azithromycin and other active drugs on *N. fowleri* would also be helpful in identifying other potential therapeutic agents for treating PAM infection.

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VITA 2

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