Research Journal of
Microbiology

Academic
Journals Inc.

In vitro Susceptibility of *Naegleria fowleri* Trophozoites to Amphotericin B-combined Chlorpromazine

[1]S. Tiewcharoen, [2]J. Rabablert and [1]V. Junnu

[1]Department of Parasitology, Faculty of Medicine Siriraj Hospital,
Mahidol University, 2 Prannok Road, Bangkok-noi, Bangkok, 10700, Thailand
[2]Department of Biology, Faculty of Science, Silpakorn University,
Sanamchan Palace, Ampour Mung, Nakhon Pathom, 73000, Thailand

Abstract: The objective of this study was to study the susceptibility of *Naegleria fowleri* trophozoites to Amphotericin B-combined chlorpromazine investigated the activities of single drugs used in combination with amphotericin B compared to those of each drug alone *in vitro*. The 50% inhibitory concentrations (IC_{50}) and 100% minimal concentrations (MIC_{100}) were calculated for single drugs and the drugs combination with fixed combination ratios of IC_{50} of amphotericin B. Single drugs, amphotericin B had the best IC_{50} and MIC_{100} scores against *N. fowleri* trophozoites. chlorpromaxine, Artesunate and azitromycin had following IC_{50} and MIC_{100} scores against trophozoites. However, we found that chlorpromazine in combination with amphotericin B was the best synergistic drug against *N. fowleri* trophozoites. According to single drugs, chlorpromaxine, artesunate and azitromycin plus amphotericin B had also been synergistic drugs against *N. fowleri* trophozoites. It was suggested that the combined use of these agents may be beneficial in treating Primary amoebic meningoencephalitis.

Key words: Amphotericin B, chlorpromazine, *Naegleria fowleri*

INTRODUCTION

Free living amoebae of the genus *Naegleria* are found in a variety of natural environments including soil, ponds and fresh water and feed on bacteria present in these environments (Ma *et al.*, 1990). Several species have so far been identified, but only *Naegleria fowleri* has known to be an opportunistic pathogen, which causes primary amoebic meningoencephalitis, a severe disease of the central nervous system in human and mammals (John, 1982; Marcino-Cabral, 1988). Infection occurs usually by inhalation or aspirating of water contaminated with the amoeba trophozoites. The invasive amoebae enter the nervous system through the olfactory nerve and cause extensive inflammation, hemorrhage and necrosis in brain by disrupting neuronal tissues and other mammalian cells. Usually, the infection is fatal, resulting in death on 3 to 7 days after infection (Ma *et al.*, 1990; Shin and Ki, 2004).

The reports of successful treatment of PAM involved a 9 year old girl who was diagnosed early in the disease course and treated with intravenous and intrathecal amphotericin B, intravenous and intrathecal miconazole and oral rifampin. The patient

Corresponding Author: Jundee Rabablert, Department of Biology, Faculty of Science,
Silpakorn University, Sanamchan, Nakhon Pathom, 73000, Thailand
Tel: (66-34)253841 Fax: (66-34)253842

survived with minimal neurologic sequelae (Schuster and Rechthand, 1975). Additionally, the clinical use of amphotericin B in other patients is limited by a high frequency of renal toxicity and several other adverse effects (McCurdy *et al.*, 1968; Goodman and Koenig, 1970; Proffitt *et al.*, 1991). Moreover, not all patients treated with amphotericin B survived from PAM (Cursons *et al.*, 2003) due to several problems, one of the most important of which is finding therapeutic agents for clinician management. In addition to amphotericin B, the sensitivities of other drugs such as artemisinin, azithromycin, chlorpromazine, clotrimazole, fluconazole, itraconazole, ketoconazole, metragainst to *N. fowleri* had also been evaluated (Tiewcharoen *et al.*, 2002; Goswick and Brenner, 2003; Schuster and Visvesvara, 2004; Vargas-Zepeda *et al.*, 2005; Ondarza *et al.*, 2006, 2007; Soltow and Brenner, 2007).

It had been reported that interferon-α (IFN-α) and the nucleoside analog, acyclovir inhibited herpes simplex virus type 1 (HSV-1) replication. IFN-α and acyclovir can cross the blood brain barrier (Liu, 2005; Anand and Mitra, 2009). The IFN-α inhibited both DNA polymerase and alkaline DNase whereas acyclovir inhibited only DNA polymerase in post-transcription mechanism (Taylor *et al.*, 1998). No reports of IFN-α and acyclovir against *N. fowleri* were previously studied. Our group was interested to study the susceptibility of *N. fowleri* trophozoites to sensitivities of both drugs compared with antimicrobial and antipsychotic drugs either in drug alone or drug combination with Amphotericin B.

MATERIALS AND METHODS

Naegleria fowleri Cultivation

This research project was conducted from October 2008 to April 2009. Trophozoites of *N. fowleri* (strain Siriraj) were isolated from a clinical specimen of a patient from Siriraj Hospital in 1986. The *N. fowleri* trophozoites were grown axenically in Nelson medium supplemented with 5% FCS. Cultures were incubated at 37°C in T-75 cm^2 tissue culture flasks (Corning, USA). Trophozoites were harvested after 3-5 days during the logarithmic phase of growth by chilling the culture tubes in an ice-water bath for 15 min. After centrifugation at 2000 rpm for 5 min, the pellets were resuspended in culture medium and culture as previously described (Tiewcharoen *et al.*, 2008).

Pharmaceutical Agents

Drugs used in the study could be categorized to be five groups as followed. Group I, Antifungal drugs; Amphotericin B (Abbott, Saint-Remy-sur-Avre, France, S.A.; MW 924.1: $C_{47}H_{73}NO_{17}$), Fluconazole (Siam Bheasach, Bangkok, Thailand; MW 306.3: $C_{13}H_{12}F_2N_6O$) and Voricinazole (Vfend (R); Roerig/Pfizer Inc., NewYork, NY; MW 349.3105: $C_{16}H_{14}F_3N_5O$), Group II, Antibacterial drugs; azithromycin (Ben Venue Laboratories, Ohio, USA; MW 748.984, $C_{38}H_{72}N_2O_{12}$), Gentamicin sulfate (T.P. Drug Laboratories, Bangkok, Thailand; MW 477.59: $C_{21}H_{43}N_5O_7$) and Ciprofloxacin (Ranbaxy laboratories limited, Secunderabad, India; MW 331.4: $C_{17}H_{18}FN_3O_3$). Group III, Antiprotozoal drugs; Artesunate (Gulin Pharmaceutical Organization, Guangxi, China; MW 384.42: $C_{19}H_{28}O_8$), Quinine dihydrochlorinate (Guangzhou Person Pharmaceutical Organization, China; MW 396.90: $C_{20}H_{24}N_2O_2 \cdot HCl \cdot 2H_2O$) and Metronidazole (Biolab, Bangkok, Thailand; MW 171.16: $C_6H_9N_3O_3$). Group IV, Antiviral drug; acyclovir (Mayne Pharma Pty Ltd., Mulgrave, Australia; MW 225: $C_8H_{11}N_5O_3$). Group V, Antiphychotic drug; Chlorpromazine hydrochloride (The Government. Pharmaceutical Organization, Bangkok, Thailand; MW 355.3: $C_{17}H_{19}ClN_2S$). Group VI, Interferon-alpha

(IFN-α2b, MW 19.5 kDa, Reliance Biopharmaceuticals, Argentina). Pharmaceutical agents were dissolved in and diluted with sterile distilled water according to the manufacturer's specifications to provide the concentrations and doses used in this study.

Morphology and Growth of *N. fowleri* Trophozoites

One mililiters of the trophozoites resuspended in Nelson medium containing 5% FCS (1×10^4 cells) were cultured in each well of 12 well tissue culture plates (Corning). Then, the plates were sealed and incubated at 37°C. Tests were performed in duplicate and were repeated at least three times. Trophozoites were observed for morphological changes for 3 days post-inoculation. The number of dead and viable cells was counted for growth rate of the trophozoites with a haemocytometer using binocular microscope (Olympus, Japan) at 12, 24, 36, 48, 60 and 72 h of post-inoculation, respectively (Tiewcharoen *et al.*, 2008).

Susceptibility of *N. fowleri* to Single Drugs

Experiments were performed in sterile 12 well plates (Corning), each well contains 1.12 cm^2. Serial twofold dilutions of the single drugs, except Amphotericin B at conc. 0.976-$1000\,\mu g$ mL^{-1} were prepared in Nelson medium containing 5% FCS. Amphotericin B had ranging of concentrations at conc. 0.01-$0.1\,\mu g$ mL^{-1}. Control wells received 500 μL of medium alone. Five hundred microliters of calibrated trophozoite suspension (1×10^4 cells mL^{-1}) were added to each well and then the plates were sealed and incubated at 37°C. Tests were performed in duplicate and were repeated at least three times. Trophozoites were observed for cell viability at 24, 48 and 72 h of incubation under inverted microscope. At 72 h of incubation, the plates were kept at 4°C for 10 min. The cells were harvested and centrifuged at 2000 g for 2 min and then the culture medium was discarded. The cells were dissolved with 0.1 mL of PBS 7.2 containing 5% FBS. To assess the inhibitory effect of the drugs, 0.4% trypan blue in PBS was used. Dead trophozoites were stained after 5-15 min, while viable trophozoites remained unstained. The numbers of dead and viable cells were counted as described above.

Susceptibility of *N. fowleri* to Combined Drugs

Serial twofold dilutions of combined drugs were prepared from the various concentrations of the single drugs mixed with Amphotericin B at IC$_{50}$ scores in 500 μL of Nelson medium containing 5% FCS. Control well was medium alone. The 500 μL of trophozoite suspension ((1×10^4 cells mL^{-1}) was added to each well and the plates were incubated at 37°C. Total cell viability were counted as described above.

Statistics

Data were analyzed or plotted and statistics were calculated with Microsoft Excel. The results were expressed as the means±standard errors of the means (SEM). Student's t test was used to determine the significance, which was taken as a value of $p \leq 0.05$.

RESULTS

Morphology and Growth Kinetic of *N. fowleri* Trophozoites

Morphology, *N. fowleri* trophozoites growing in axenix medium exhibited eruptive movement by producing hemspherical bulges of hyaline cytoplasm (Fig. 1a). A single nucleus with a large, centrally placed nucleolus was present in the trophozoites. The trophozoite measured from 8 to 20 μm with a mean of 10 μm based on the measurements of
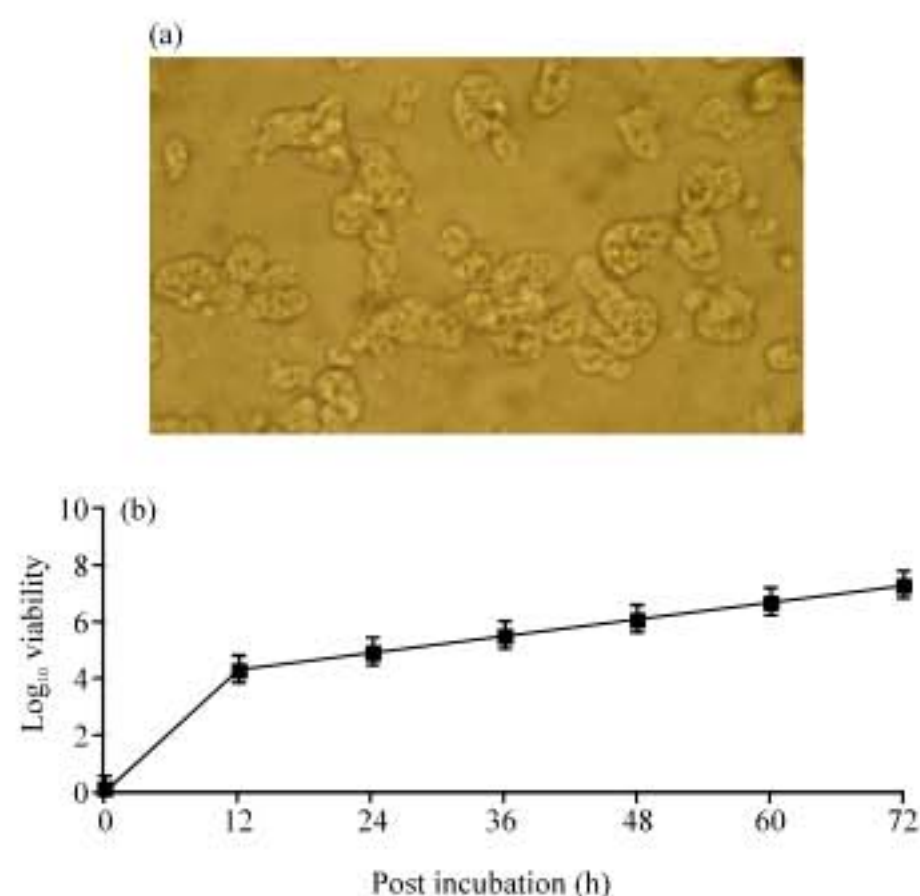
Fig. 1: (a) Micorscopic characteristic of *N. fowleri* and (b) growth kinetic of *N. fowleri*. *N. fowleri* trophozoites was cultured in Nelson's medium with 5% FCS at 37°C for 72 h. Morphological shapes was observed under inverted microscope, 400X and the viability of the cells were counted at indicated times using Trypan blue exclusion method. Results were the average of three experiments and errors were standard deviation

at least 20 trophozoites. Growth kinetic, *N. fowleri* trophozoites (5×10^3 cells at started point) were detected at about 6 to 72 h interval with doubling times. The yields of trophozoites were detectable approximately 1×10^7 cells after 72 h post-inoculation (Fig. 1b).

Susceptibility of *N. fowleri* Trophozoite to Drugs Alone and Drugs Combination

Naegleria fowleri trophozoites were cultured in 12 well-plates in the presence of the various concentrations of either drugs alone or drug combination at 37°C for 72 h. At 72 h-post incubation, the IC_{50} and MIC_{100} scores for tested drugs against *N. fowleri* trophozoites were expressed in µg mL^{-1} appear in Table 1.

Antifungal Drugs

Single drugs, amphotericin B had the best IC_{50} and MIC_{100} scores at 0.0246 ± 0.004 µg mL^{-1} and 0.098 ± 0.0037 µg mL^{-1} (Fig. 2a). At MIC_{100} scores of drug combination, treatment with fluconazole plus Amphotericin B (MIC_{100} scores at 126.114 ± 0.63 µg mL^{-1}) to *N. fowleri* trophozoites showed 3.99 fold-lower than treated the trophozoites with fluconazol alone (MIC_{100} scores at 503.75 ± 0.35 µg mL^{-1}). The sensitivity of voriconazole plus Amphotericin B was effective to the trophozoites with MIC 100 scores at 126.072 ± 0.62 µg mL^{-1}, while voriconazole alone (1004.19 ± 0.45 µg mL^{-1}) had no detectable effect on the trophozoites (Fig. 2b).

Antibacterial Drugs

Single drugs, azithromycin, gentamicin sulphate and ciprofloxacin were effective to the *N. fowleri* trophozoites with the IC_{50} scores at 6.767 ± 0.04, 15.662 ± 0.03 and

Table 1: Mean (±SD) µg mL^{-1} of IC$_{50}$ and MIC$_{100}$ scores of either single drugs alone or single drugs plus with Amphotericin B against Naegleria fowleri Siriraj strain

Drugs	Single drugs alone		Amphotericin-combined drugs	
	IC$_{50}$	MIC$_{100}$	IC$_{50}$	MIC$_{100}$
Antifungal				
Amphotericin B	0.0246±0.0040	0.0980±0.0037	-	-
Fluconazole	31.9290±0.9300	503.7500±0.3500	7.8369±0.740	126.1140±0.630
Voriconazole	125.6890±0.9000	1004.1900±0.4500	3.9380±0.050	126.0720±0.620
Antibacterial				
Azithromycin	6.7670±0.0400	510.3080±0.6200	1.2430±0.021	31.2060±0.640
Gentamicin sulphate	15.6620±0.0300	1002.6700±0.7100	15.6376±1.600	1001.3600±0.650
Ciprofloxacin	63.5210±0.7100	1001.3700±0.9200	31.3570±0.120	1000.4000±1.120
Antiprotozoa				
Artesunate	3.9277±0.0400	8.1705±0.0600	1.9683±0.030	4.02795±0.14
Quinin sulphate	62.4790±0.0300	1000.7800±0.6100	15.8798±0.350	1000.67300±0.92
Metronidazole	251.2220±0.6100	1012.1050±0.7400	125.4360±0.610	1009.01000±0.81
Antiviral				
Aciclovir	31.3122±0.060	1000.1530±0.2400	8.8244±0.020	504.79500±0.83
Antiphycotic				
Chlorpromaxine	0.9800±0.0100	7.9600±0.0100	0.4937±0.010	1.97390±0.01

63.521±0.71 µg mL^{-1}, respectively. Azithromycin was effective to the trophozoites MIC$_{100}$ at 510.308±0.62 µg mL^{-1}, while gentamicin sulphate or ciprofloxacin was ineffective to the trophozoites at concentrations of MIC$_{100}$ scores (Table 1 and Fig. 2c). Combined treatment with azithromycin plus amphotericin B (131.206±0.64 µg mL^{-1}) to the trophozoites was 3.89-fold less than treatment with azithromycin alone (510.308±0.62 µg mL^{-1}), suggesting that both amphotericin B and azithromycin had the synergistic effect to *N. fowleri* trophzoites. In contrast, we observed the sensitivity of combined gentamicin sulphate was not different from gentamicin sulphate alone at MIC$_{100}$ scores to the trophozoites. Neither amphotericin B-combined ciprofloxacin nor ciprofloxacin had detectable effect to the trophozoites. It was suggested that either ciprofloxacin or gentamicin sulphate had not synergistic effect to *N. fowleri* trophozoites.

Antiprotozoal Drugs

As shown in Table 1 and Fig. 2d, artesunate was effective to the *N. fowleri* trophozoites than quinine sulphate and metronidazol at IC$_{50}$ and MIC$_{100}$ scores, respectively. The sensitivities of combined artesunate with amphotericin B to the trophozoites were 2-fold less than artesunate alone at IC$_{50}$ and MIC$_{100}$ scores. In contrast, no susceptible activity of trophozoites to quinine sulphate alone or combined quinine sulphate was observed. Metronidazone alone or drug combination with amphotericin B had no also detectable effect on to the trophozoites. It was suggested that artesunate and amphotericin B had the synergistic effects on *N. fowleri* trophozoites.

Antiviral Drugs

As shown in Table 1 and Fig. 2e, the IC$_{50}$ of combined acyclovir (8.8244±0.02 µg mL^{-1}) to the *N. fowleri* trophozoites was 3.548 fold than acyclovir alone (31.3122±0.06 µg mL^{-1}). The MIC$_{100}$ of combined acyclovir to the *N. fowleri* trophozoites was concentrations of 504.795±0.83 µg mL^{-1}, while acyclovir alone was ineffective to the trophozoites. It was suggested that combination treatment with acyclovir and amphotericin B had the synergistic effect on *N. fowleri* trophozoites. This is the first data showed that acyclovir in combination with amphotericin B could be used for *N. fowleri* infection.
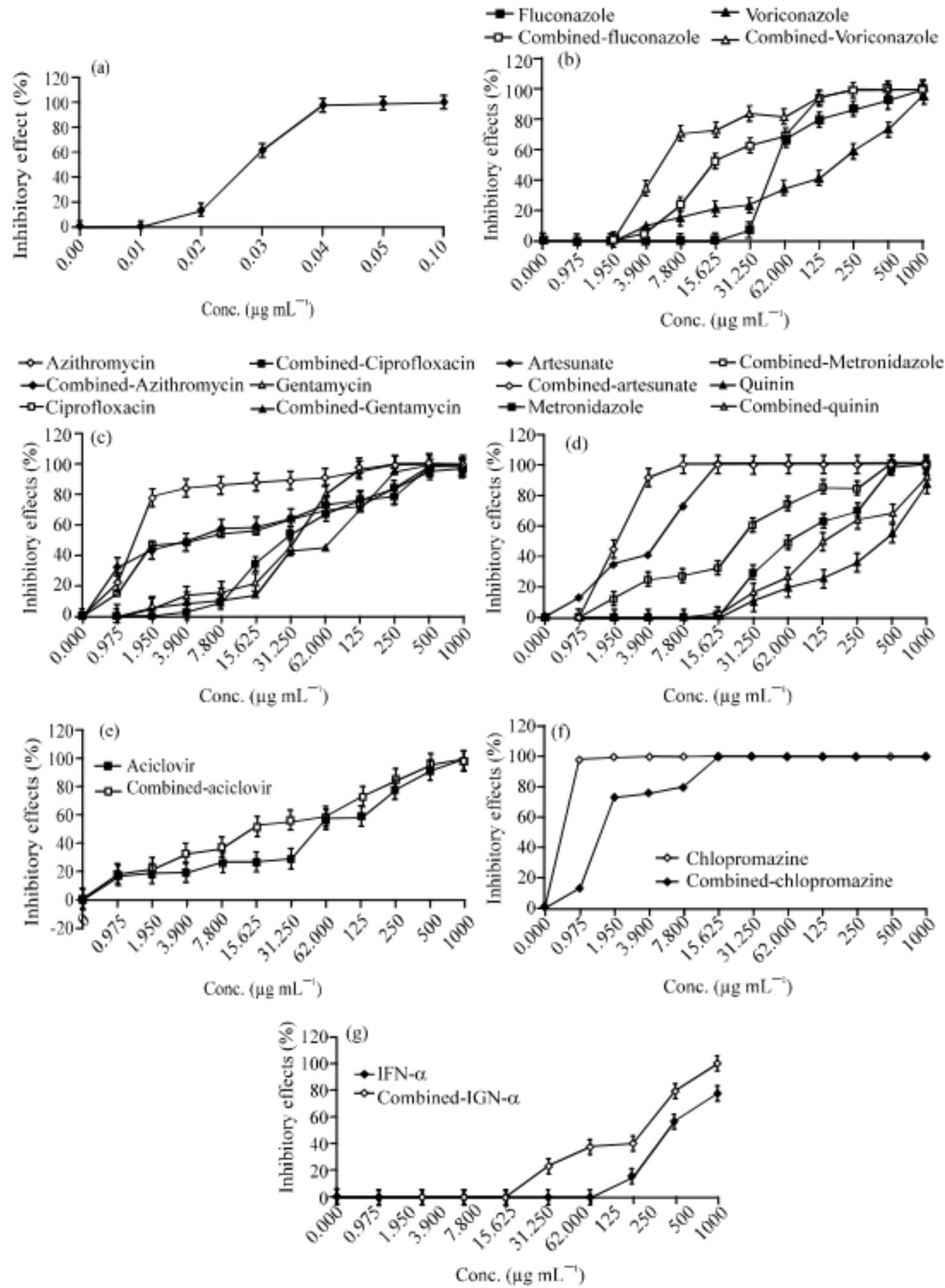
Fig. 2: (a) Susceptibility of *N. fowleri* to Amphotericin B alone, (b) antifungal drugs alone or plus with Amphotericin B, (c) antibacterial drugs alone or plus with Amphotericin B, (d) antiprotozoal drugs alone or plus with Amphotericin B, (e) antiviral drugs alone or plus with Amphotericin B, (f) antiphychotic drugs alone or plus with Amphotericin B and (g) IFN-α alone or plus with Amphotericin B. *N. fowleri* trophozoites were studied in the presence or various concentrations of either single drug alone or single drug plus Amphotericin B at 37°C for 72 h. The number of viable trophozoites was counted by Trypan blue exclusion method. Results were the average of three experiments and errors were standard deviation

Antiphycotic Drugs

As shown in Table 1 and Fig 2f, chlorpromazine had an IC_{50} score at 0.980 ± 0.01 µg mL^{-1} with a MIC_{100} of 7.96 ± 0.01 µg mL^{-1}, thereby combined Chlorpromazine had an IC_{50} score at 0.4937 ± 0.01 µg mL^{-1} with a MIC_{100} of 1.9739 ± 0.01 µg mL^{-1}. The MIC_{100} scores of combined chlorpromaxine to the trophozoites were 4 fold lower than chlorpromazine alone.

Interferon-Alpha

IFN-α had an IC_{50} score at 5.8 log 10 IU with a MIC_{100} of >7 log 10 IU to *N. fowleri* trophozoites (data not shown). The trophozoites were susceptible to IFN-α in combination with amphotericin B (MIC_{100} of 6.18 log 10 IU mL^{-1}), while they was not susceptible to IFN-α alone (Fig. 2g). It was suggested that IFN-α in combination with amphotericin B had the synergistic effect on the trophozoites.

DISCUSSION

In the present study, we examined the morphology and growth kinetic of trophozoites of *N. fowleri* Siriraj-strain grew in Nelson's medium at 37°C. Morphologically, the trophozoites were similar to those of *N. fowleri* and other *Naegleria* sp., with respect to the nucleus and cytoplasmic organelles (Visvesvara *et al.*, 2005). Growth kinetic, total double numbers of *N. fowleri* Siriraj strain trophozoites were also similar to those of other *N. fowleri* trophozoites at approximate 6 h-post inoculation, as previous described (Ondarza and Tamayo, 2004). Inhibition of growth kinetic of *N. fowleri* amoeba using single drugs or drug combination was the one of the choice for PAM therapy in human.

Many investigators screened a lot of drugs for *in vitro* and *in vivo* therapeutic activity against *N. fowleri*. Amphotericin B was used as the first therapeutic drug for *N. fowleri* infection (Goodman and Koenig, 1970). Other drugs such as chlorpromazine, clotrimazole, fluconazole, itraconazole, ketoconazole, metronidazole, micronazole, rifampicin and trifluoperazine had also been tested *in vitro* (Tiewcharoen *et al.*, 2002; Schuster and Mandel, 1984; Vargas-Zepeda *et al.*, 2005; Ondarza *et al.*, 2006, 2007). Artemisinin and azithromycin has been described as effective treatment of experimental PAM in mice (Goswick and Brenner, 2003). Recently, miltefosine which was developed and used as an anticancer drug and voriconazole, which was used in systemic fungal infections were found to be effective in *in vitro* studies (Schuster and Visvesvara, 2004).

In this study, we evaluated the sensitivities of antifungal (amphotericin B, fluconazole and voriconazole), antibacterial (azitromycin, gentamicin sulphate and ciprofloxacin) antiprotozoa (artesunate) and antiviral (anticyclovir) and antipsychotic (chloromazine) drugs as well as interferon-alpha against *N. fowleri* trophozoites. Additionally, we also evaluated the effects of synergistic drugs occur when the effect of two agents used in combination compared with the sum of the individual effects of each agent given alone. The greater synergy between two agents, the less of each agent is required to produce a specific effect. Synergy may enable the use of lower doses to produce a defined level of efficacy, thereby increasing safety and tolerability. Synergy may also enable two agents, both of which are <50% efficacious, to be combined to produce 100% efficacy. Furthermore, we were also interested the sensitivity of single drugs or drugs combination to *N. fowleri* during 1-3 day interval in order to follow drug-resistant *N. fowleri* trophozoites.

Antifungal agents, the MIC scores of amphotericin B (0.098 ± 0.0037) had still been the highest potential against *N. fowleri*. Schuster and Rechthand (1975) reported that the action of amphotericin B binded to ergosterol in the fungal cell membrane immediately, resulting in

depolarization of the membrane and formation of pores that leads to K$^+$ leakage and fungal cell death. The mechanism of amphotericin B against *N. fowleri* was established. This drug was bind to membrane sterols, leading to membrane permeability, leakage and strongly inhibited three NADPH-dependent flavoprotein disulfide reductases CysR (NADH-dependent reductase), GR and TR, either by blocking the electron transfer from NADPH to FAD, or by reacting directly with the reducing catalytic site of *N. fowleri* (Ondarza *et al.*, 2007). The MIC$_{100}$ scores of fluconazole alone and combined fluconazole were 503.75±0.35 and 126.114±0.63 µg mL^{-1}, respectively. It was suggested that the sensitivity of combined fluconazole was 3.99 times less than fluconazole against *N. fowleri* trophozoites. No mechanism of fluconazole against *N. fowleri.* trophozoites has been reported before. Earlier studies showed that fluconazole has been used for treatment of cryptococcal meningitis, dissemininated candidiasis and invasive candidial infection (Clark *et al.*, 1996). It has been proven to penetrate the blood-brain barrier remarkably well (Yang *et al.*, 1996). However, this drug has also shown an unexpected effectiveness for reducing organ dysfunction and mortality in septic shock, probably by increasing bactericidal activity and the recruitment of neutrophils (Jacobs *et al.*, 2003). In this study, the amphotericin B combined fluconazole were more effective than fluconazole alone against *N. fowleri.* These findings are identical to previous *in vitro* studies reported that the amphotericin B and fluconazole had amoebocidal effect against *N. fowleri.* trophozoites (Tiewcharoen *et al.*, 2002). This results were supported by the successful treatment of *N. fowleri* infection by using intravenous amphotericin B, fluconazole and oral rifampin in a 10 year-old boy patient (Vargas-Zepeda *et al.*, 2005). In the present, the mechanism of fluconazole against *N. fowleri* has not been revealed. Clinical studies in the treatment of fungal infection sustained by *Candida* sp., the effect of fluconazole acts on ergosterol biosynthesis by inhibition of cytochrome P450 14a-demethylase (P450 14DM) (Bachmann *et al.*, 2002). The MIC$_{100}$ scores of voriconazole in combination with amphotericin B to the trophozoites was 126.072±0.62 µg mL^{-1}. Our groups did not observe detectable effect of voricnazole on the trophozoites. In contrast, Schuster *et al.* (2006) revealed that voriconazole had the greater potency to *N. fowleri* trophozoites than fluconazole. As with all azole antifungal agents, voriconazole worked principally by inhibition of cytochrome P450 14a-demethylase (P45014DM). This enzyme is in the sterol biosynthesis pathway that leads from lanosterol to ergosterol compared to fluconazole, voriconazole inhibited P45014DM to a greater extend (Sanati *et al.*, *1*997). Antibacterial agents, the MIC$_{100}$ scores of azithromycin (510.308±0.62 µg mL^{-1}) were 3.89 times greater than those of combined azithromycin (131.206±0.64 µg mL^{-1}) against *N. fowleri* trophozoites. It has been reported that azithromycin, a derivative of erythromycin with improved activity against Gram-negative bacteria,inhibited bacterial protein synthesis by binding to the 50S ribosomal subunit and blocking peptide bond formation and translocation including exhibits a marginal inhibition effect in a model system derived from *Escherichia coli*, in which a peptide bond is formed between puromycin and AcPhe-tRNA bound at the P-site of poly(U) (Dinos *et al.*, 2001).

The susceptibility of *N. fowleri* trophozoites of gentamicin sulphate alone and combined gentamicin sulphate at MIC$_{100}$ were not observed. In general, gentamicin inhibited bacterial protein synthesis mainly through binding with the 30S ribosomal subunit and acted through two different mechanisms. In one mechanism at high concentrations, it could interfere with the correct amino acid polymerization and elongation. Another mechanism predominated at low concentrations in which amino acid codons were misread by tRNA and proofreading is impaired. This leaded to incorrect amino acid sequencing and nonsense proteins

(Auslander *et al.*, 1973). Like genamicin sulphate, ciprofloxacin was ineffective to *N. fowleri* trophozoites at the concentrations of MIC_{100}. Ciprofloxacin was a fluoroquinolone derivative of nalidixic acid which targets DNA gyrase of the bacterial cell (Lebel, 1988). During DNA synthesis, DNA gyrase facilitated the movement of replication by releasing the tension of the coiled DNA. However, when ciprofloxacin was used, binding occurs to form a fluoroquinolone-gyrase-DNA complex. This complex stopped the replication. DNA gyrase was also involved in the supercoiling of the bacterial DNA into cell, thus, ciprofloxacin will inhibit DNA gyrase from packing the DNA into bacterial cell. As supercoiling were essential for cell growth, the inhibition eventually cause the bacterial cell to lyse (Griggs *et al.*, 2005).

Antiprotozoal drugs; artesunate alone showed high potency at IC_{50} (3.9277 ± 0.04 µg mL^{-1}) and MIC_{100} (8.1705 ± 0.06 µg mL^{-1}). Artesunate is one of semi-synthetic derivatives of artesinin isolated from decoctions of traditional Chinese medicine *Artemisia annua* L. (quingho, sweet wormwood) (Meshnick and Kamchonwongpaisan, 1996). During recent years artemisinin and its derivatives have emerged as the most effective and safe drugs for the treatment of severe and chloroquine-resistant malaria (Haynes, 2001) Artemisinin and its derivatives have been demonstrated to affect a number of other cellular biochemical processes, such as anti-proliferation, anti-angiogenic effects, induction of cell apoptosis and oxidative stress (Wartenberg *et al.*, 2003; Huan-huan *et al.*, 2004). More recently, there is increasing evidence to support the notion that these compounds may have potential role on anti-inflammation and immune regulation. For instance, artemisinin suppresses NF-κB-mediated NO synthesis in human astrocytoma T67 cells (Aldieri *et al.*, 2003) and the inhibitory effects of dihydroarteannuin, one of artemisinin derivatives, on elevated TNF-α production may result from the blockade of NF-κB signaling pathway in peritoneal macrophages and renal tissue from lupus BXSB mice (Li *et al.*, 2006). 3-(12-beta-artemisininoxy) phenoxyl succinic acid (SM735), a novel artesinin derivative, was also demonstrated to play a potential immunosuppressive role (Zhou *et al.*, 2005). Furthermore, artemisinin potently suppressed release of TNF-α and IL-6 induced by CpG-containing oligodeoxy-nucleotides, lipopolysaccharide or heat-killed *Escherichia coli* (Wang *et al.*, 2006). Artemisinin inhibits TNF-α induced nuclear NF-κB translocation, DNA-binding activity and gene transcriptional activity, as well as, degradation and phosphorylation of IκBα in rheumatoid synoviocytes (Xu *et al.*, 2007). However, the inhibitory effects of artesunate against *N. fowleri* are unknown. Quinine alone was effective to *N. fowleri* at the concentrations of IC_{50} = 62.479 ± 0.03 µg mL^{-1} and MIC_{100} = 1000.78 ± 0.61 µg mL^{-1}, respectively. Combined-quinine showed high potency at IC_{50} (15.8798 ± 0.35 µg mL^{-1}) and MIC_{100} (1000.673 ± 0.92 µg mL^{-1}) to *N. fowleri* trophozoites. This data revealed the sensitivity of amphotericin B-combined quinin at IC_{50} scores were 3.934 times to *N. fowleri* than Quinin alone, suggesting that both drugs were synergistic drugs to the amoebae. Research on the mechanism of quinine and how the *Naegleria* has acquired quinine resistance is still ongoing. Sanchez *et al.* (2008) investigated passive distribution, in accordance with the intracellular pH gradients and intracellular binding could account for only a small fraction of the high amount of quinine accumulated by human malaria parasite *Plasmodium falciparum*. The results of trans-stimulation kinetics suggested that high accumulation of quinine had been brought about by a carrier-mediated import system. This import system might be weakened in parasites with reduced quinine susceptibility. Other data show that polymorphisms within PfCRT are causatively linked with an increased verapamil-sensitive quinine efflux that, depending on the genetic background, resulted in reduced quinine accumulation. As an antiviral agent, quinin inhibits the glycosylation of the HIV envelope glycoprotein gp120 which occurs within the golgi apparatus (Lelievre *et al.*, 2007).

Metronidazole had IC_{50} and MIC_{100} effects against *Naegleria* trophozoites at 251.222 ± 0.61 and 1012.105 ± 0.74 µg mL^{-1}. Combined metronodazole against the trophozoites expressed as IC_{50} and MIC_{100} of 125.436 ± 0.61 and 1009.01 ± 0.81, respectively. The inhibitory effect of combined metronidazole was less 2 folds than metronidazole alone. Metronidazole is a prodrug. It is converted in anaerobic organism by the redox enzyme pyruvate ferredoxin oxidoreductase. The nitro group of metronidazole is chemically reduced by ferredoxin (or a ferredoxin-linked metabolic process) and the products are responsible for disrupting the DNA helical structure, thus inhibiting nucleic acid synthesis. Metronidazole is selectively taken up by anaerobic bacteria (Church *et al.*, 2006) and sensitive protozoal organisms because of the ability of these organisms to reduce metronidazole to its active form intracellularly (Wassman *et al.*, 1999).

Antiviral agents, the sensitivity of acyclovir alone was infective to *N. fowleri* trophozoites at day 3 post-inoculation. Drug combination between acyclovir and amphotericin B to *N. fowleri* trophozoites had the MIC_{100} scores of 504.795 ± 0.83 µg mL^{-1}. The mechanism of acyclovir had been studied in HSV-infected human cell. The acyclovir molecules entered the cell and were converted to acyclovir monophosphate by the HSV enzyme Thymidine Kinase (TK). Enzymes in the human cell added two more phosphates to eventually form the active drug acyclovir triphosphate. The acyclovir triphosphate competed with 2-deoxyguanosine triphosphate (dGTP) as a substrate for viral DNA polymerase, as well as acting as a chain terminator. In actual infection, the HSV released its naked capsid that delivers DNA to the human nucleus; the active drug acyclovir triphosphate exerted its action on the viral DNA located in the nucleus (Balfour, 1999). It was proposed that both drugs had synergistic drugs against *N. fowleri* trophozoites, thereby acyclovir and amphotericin B inhibited chain elongation and protein synthesis of *N. fowleri* trophozoites, respectively.

Antipsychotic agents, the phenothiazine compounds chlorpromazine hydrochloride either alone or in combination with amphotericin B had *in vitro* activity against *N. fowleri* trophozoites. The MIC_{100} scores of chlorpromazine and combined chlorpromazine to *N. fowleri* trophozoites were 7.96 ± 0.01 and 1.9739 ± 0.01 µg mL^{-1}, respectively. The MIC_{100} scores of chlorpromazine were 4.03 folds rather than combined chlorpromazine. Chlorpromazine inhibited 80% cysteine reductase and glutathione reductase of the *N. fowleri* trophozoites and decreased 80% trophozoites during the 24 h incubation (Kim *et al.*, 2008) where as the amphotericin B and miconazole inhibited were the most potent inhibitors of trypanothione reductase and inhibited 100% trophozoites (Ondarza *et al.*, 2007). Moreover, chlorpromazine reflected sensitivity of amoeba calcium regulatory protein to the phenothiazine compounds or was due to the lipophilic action of the drugs on the amoeba plasma membrane (Schuster and Mandel, 1984).

Interferon, the sensitivity of IFN-α to *N. fowleri* trophozoites was 5.6 log$_{10}$ IU mL^{-1} of IC_{50} scores and >6.18 log$_{10}$ IU mL^{-1} of MIC_{100} scores compared with one of amphotericin B-combined IFN-α was 5.1 log$_{10}$ IU mL^{-1} of IC_{50} and 6.18 log$_{10}$ IU mL^{-1} of MIC_{100}, respectively. Thyrell *et al.* (2002) found that apoptosis induced by IFN-α was associated with activation of caspases-1, -2, -3, -8 and -9 and that this activation was a critical event. Caspase-3 activation was dependent on activity of caspases-8 and -9, moreover, activation of caspase-8 seems to be the upstream event in IFN-α-induced caspase cascade. Thyrell *et al.* (2002) also found loss of mitochondrial membrane potential as well as release of cytochrome c post IFN-treatment, clearly implicating the involvement of mitochondria in IFN-mediated apoptosis. Furthermore, IFNα-induced apoptosis was found to be independent on interactions between the Fas-receptor and its ligand.

CONCLUSION

In the present study, we evaluated additional synergy studies of amphotericin Bwith other agents in order to improve drug selection and treatment of *N. fowleri* infection. Present results indicated that amphotericin B plus chlorpromazine were new synergistic drugs against *N. fowleri* trophozoites. Moreover, we first reported that acyclovir or IFN-α in combination with amphotericin B had effect on *N. fowleri* trophozoites. It was suggested that the combined use of these agents might provide a useful regimen for treating human infections with this organism. In Further interesting topic, the drug mechanisms and the cytopathic effect in mouse model against *N. fowleri* should be studied.

ACKNOWLEDGMENTS

REFERENCES

Aldieri, E., D. Atragene, L. Bergandi, C. Riganti, C. Costamagna, A. Bosia and D. Ghigo, 2003. Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-B activation. FEBS Lett., 552: 141-144.

Anand, B.S. and A.K. Mitra, 2009. Mechanism of corneal permeation of L-Valyl Ester of acyclovir: Targeting the oligopeptide transporter on the rabbit cornea. Pharm Res., 19: 1194-1202.

Auslander, D.E., A. Felmeister, B.J. Sciarrone and A.B. Segelman, 1973. Proposed mechanism for gentamicin transport. J. Pharm. Sci., 62: 167-168.

Bachmann, S.P., T.F. Patterson and T.F. Lopez-Ribot, 2002. *In vitro* activity of caspofungin MK-0991) against *Candida albicans* clinical isolates displaying different mechanisms of azole resistance. J. Clin. Microbiol., 40: 2228-2230.

Balfour, H.H. Jr., 1999. Antiviral drugs. N. Engl. J. Med., 340: 1255-1268.

Church, D.L., R.D. Bryant, V. Sim and E.J. Laishley, 2006. Metronidazole susceptibility and the presence of hydrogenase in pathogenic bacteria. Anaerobe, 2: 147-153.

Clark, A.B., B.L. Lobo and M.S. Gelfand, 1996. Fluconazole and amphotericin B for cryptococcaln meningitis. Ann. Pharm., 30: 1408-1410.

Cursons, R., J. Sleigh, D. Hood and D. Pullon, 2003. A case of primary amoebic meningoencephalitis: North Island, New Zealand. N. Z. Med. J., 116: 712-712.

Dinos, G.P., M. Michelinaki and D.L. Kalpaxis, 2001. Insights into the mechanism of azithromycin interaction with an *Escherichia coli* functional ribosomal complex. Mol. Pharm., 59: 1441-1445.

Goodman, J.S. and M.G. Koenig, 1970. Amphotericin B-specifics of administration. Mod. Treat., 7: 581-595.

Goswick, S.M. and G.M. Brenner, 2003. Activities of azithromycin and amphotericin B against *Naegleria fowleri in vitro* and in a mouse model of primary amebic meningoencephalitis. Antimicrob. Agents Chemother., 17: 524-528.

Griggs, D.J., M.M. Johnson, J.A. Frost, T. Humphrey, F. Jørgensen and L.J.V. Piddock, 2005. Incidence and mechanism of ciprofloxacin resistance in *Campylobacter* spp. Isolated from commercial poultry flocks in the United Kingdom before, during and after fluoroquinolone treatment. Antimicrob. Agents Chemother., 49: 699-707.

Haynes, R.K., 2001. Artemisinin and derivatives: The future for malaria treatment? Curr. Opin. Infect. Dis., 14: 719-726.

Huan-Huan, C., Y. Li-Li and L. Shang-Bin, 2004. Artesunate reduces chicken chorioallantoic membrane neovascularisation and exhibits antiangiogenic and apoptotic activity on human microvascular dermal endothelial cell. Cancer Lett., 211: 163-173.

Jacobs, S., E.D.A. Price, M. Tariq and N.F. Al-Omar, 2003. Fluconazole improves survival in septic shock: A randomized double-blind prospective study. Crit. Care Med., 31: 1938-1946.

John, D.T., 1982. Primary amebic meningoencephalitis and the biology of *Naegleria fowleri*. Ann. Rev. Microbiol., 36: 101-123.

Kim, J.H., S.Y. Jung, Y.J. Lee, K.J. Song and D. Kwon., 2008. Effect of therapeutic chemical agents *in vitro* and on experimental meningo-encephalitis due to *Naegleria fowleri*. Antimicrob. Agents Chemother., 52: 4010-4016.

Lebel, M., 1988. Ciprofloxacin: Chemistry, mechanism of action, resistance, antimicrobial spectrum, pharmacokinetics, clinical trials and adverse reactions. Pharmacotherapy, 8: 3-33.

Lelievre, J., A. Berry and F. Benoit-vical, 2007. Artemisinin and chloroquine: Do mode of action and mechanism of resistance involve the same protagonists? Curr. Opin. Investig. Drugs, 8: 117-124.

Li, W.D., Y.J. Dong, Y.Y. Tu and Z.B. Lin, 2006. Dihydroarteannuin ameliorates lupus symptom of BXSB mice by inhibiting production of TNF- and blocking the signaling pathway NF-kappa B translocation. Int. Immunopharmacol., 6: 1243-1250.

Liu, Y.J., 2005. IPC: Professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu. Rev. Immunol., 23: 275-306.

Ma, P., G.S. Visvesvara, A.J. Martinez, F.H. Theordore, P.M. Daggett and T.K. Sawyer, 1990. *Naegleria* and *Acanthamoeba* infections. Rev. Infect. Dis., 12: 490-513.

Marciano-Cabral, F., 1988. Biology of *Naegleria* spp. Microbiol. Rev., 52: 114-133.

McCurdy, D.K., M. Frederic and J.R. Elkinton, 1968. Renal tubular acidosis due to amphotericin B. N. Engl. J. Med., 278: 124-130.

Meshnick, S.R. and S. Kamchonwongpaisan, 1996. The mode of action of the antimalarial artemisinin and its derivatives. General Pharmacol., 27: 587-592.

Ondarza, R.N. and E.M. Tamayo, 2004. Characterization of two long vesicle-associated membrane proteins or longins genes from Entamoeba histolytica. Arch. Med. Res., 35: 378-384.

Ondarza, R.N., A. Iturbe and E. Hernαndez, 2006. *In vitro* antiproliferative effects of neuroleptics, antimycotics and antibiotics on the human pathogens *Acanthamoeba polyphaga* and *Naegleria fowleri*. Arch. Med. Res., 37: 723-729.

Ondarza, R.N., A. Iturbe and E. Hernαndez, 2007. The effects by neuroleptics, antimycotics and antibiotics on disulfide reducing enzymes from the human pathogens *Acanthamoeba polyphaga* and *Naegleria fowleri*. Exp. Parasitol., 115: 41-47.

Proffitt, R.T., A. Satorious, S.M. Chiang, L. Sullivan and J.P. Adler-Moore, 1991. Pharmacology and toxicology of a liposomal formulation of amphotericin B (AmBisome) in rodents. J. Antimicrob. Chemother., 28: 49-61.

Sanati, H., P. Belanger, R. Fratti and M. Ghanom, 1997. A new trizole, voriconazole blocks sterol biosynthesis in *Candida albicans* and *Candida krusi*. Antimicrob. Agents Chemother., 41: 2492-2496.

Sanchez, C.P., W.D. Stein and M. Lanzer, 2008. Dissecting the components of quinine accumulation in Plasmodium falciparum. Mol. Microbiol., 67: 1081-1093.

Schuster, F.L. and E. Rechthand, 1975. *In vitro* effects of amphotericin B on growth and ultrastructure of the amoeboflagellates *Naegleria gruberi* and *Naegleria fowleri*. Antimicrob. Agents Chemother., 8: 591-605.

Schuster, F.L. and N. Mandel, 1984. Phenothiazine compounds inhibit *in vitro* growth of pathogenic free-living amoebae. Antimicrob. Agents Chemother., 25: 109-112.

Schuster, F.L. and G.S. Visvesvara, 2004. Opportunistic amoebae: Challenges in prophylaxis and treatment. Drug Resist. Update, 7: 41-51.

Schuster, F., J.B. Gudlielmo and G.S. Visvesvara, 2006. *In vitro* Activity of Miltefosine and Voriconazole on Clinical Isolates of Free-Living Amebas: *Balamuthia mandrillaris*, *Acanthamoeba* spp. and *Naegleria fowleri*. J. Eukar. Microbiol., 53: 121-126.

Shin, N.J. and I. Ki, 2004. Pathogenic free-living amoebae in Korea. Korean J. Parasitol., 42: 93-119.

Soltow, S.M. and G.M. Brenner, 2007. Synergistic activities of azithromycin and amphotericin B against *Naegleria fowleri*, *in vitro* and in a mouse model of primary amebic meningoencephalitis. Antimicrob. Agents Chemother., 51: 23-27.

Taylor, J.L., P. Tom and O.W.J. Brien, 1998. Combined effects of interferon-α and acyclovir on herpes simplex virus type 1 DNA polymerase and alkaline DNase. Antiviral Res., 38: 95-106.

Thyrell, L., S. Erickson, B. Zhivotovsky, K. Pokrovskaja and O. Sangfelt., 2002. Mechanisms of Interferon-alpha induced apoptosis in malignant cells. Oncogene., 21: 1251-1262.

Tiewcharoen, S., V. Junnu and P. Chinabut, 2002. *In vitro* effect of antifungal drugs on pathogenic *Naegleria* sp. Southeast Asian J. Trop. Med. Public Health, 33: 38-41.

Tiewcharoen, S., N. Malainual, V. Junnu, P. Chetanachan and J. Rabablert, 2008. Cytopathogenesis of *Naegleria fowleri* Thai strains for cultured human neuroblastoma cells. Parasitol. Res., 102: 997-1000.

Vargas-Zepeda, J., A.V. Gomez-Alcala, J.A. Vazquez-Morales, L. Licea-Amaya, J.F. Jonckheere and F. Lares-Villa, 2005. Successful treatment of *Naegleria fowleri* meningoencephalitis by using intravenous amphotericin B, fluconazole and rifampicin. Arch. Med. Res., 36: 83-86.

Visvesvara, G.S., J.F. de Jonckheere, R. Sriram and B. Sriram, 2005. Isolation and molecular typing of Naegleria fowleri from the brain of a cow that died of primary amebic meningoencephalitis. J. Clin. Microbiol., 43: 4203-4204.

Wang, J., H. Zhou, J. Zheng, J. Cheng and W. Liu., 2006. The antimalarial artemisinin synergizes with antibiotics to protect against Lethal live *Escherichia* challenge by decreasing proinflammatory cytokine release. Antimicrob. Agents Chemother., 50: 2420-2427.

Wartenberg, M., S. Wolf, P. Budde, F. Grunheck and H. Acker., 2003. The antimalaria agent artemisinin exerts antiangiogenic effects in mouse embryonic stem cell-derived embryoid bodies. Lab. Invest., 83: 1647-1655.

Wassmann, C., A. Hellberg, E. Tannich and I. Bruchhaus, 1999. Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase. J. Biol. Chem., 274: 26051-26056.

Xu, H., Y. He, X. Yang, L. Liang and Z. Zhan., 2007. Anti-malarial agent artesunate inhibits TNF-α-induced production of proinflammatory cytokines via inhibition of NF-κB and PI3 kinase-Akt signal pathway in human rheumatoid arthritis fibroblast-like synoviocytes. Rheumatology, 1: 1-7.

Yang, H., Q. Wang and W.F. Elmquist, 1996. Fluconazole distribution to the brain: A crossover study in freely-moving rats using *in vivo* microdialysis. Pharm. Res., 13: 1570-1575.

Zhou, W.L., J.M. Wu, Q.L. Wu, J.X. Wang and Y. Zhou., 2005. A novel artemisinin derivative, 3-(12-beta-artemisininoxy) phenoxyl succinic acid (SM735), mediates immunosuppressive effects *in vitro* and *in vivo*. Acta Pharmacol. Sin., 26: 1352-1358.