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

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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 (IC50) and 100% minimal concentrations (MIC100) were calculated for single drugs and the drugs combination with fixed combination ratios of IC50 of amphotericin B. Single drugs, amphotericin B had the best IC50 and MIC100 scores against N. fowleri trophozoites. Chlorpromazine, Artesunate and azithromycin had following IC50 and MIC100 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, chlorpromazine, artemesunate and azithromycin 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, pond 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; Marcno-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

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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; Proft 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, metragrass to N. fowleri had also been evaluated (Tiwcharoen et al., 2002; Goswick and Brenner, 2003; Schuster and Visvesvara, 2004; Vargas-Zepeda et al., 2005; Ondarza et al., 2006, 2007; Salotow 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 cm² tissue culture flasks (Coming, 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 (Tiwcharoen 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; C₃₀H₇₀N₄O₁₆). Fluconazole (Siam Bheasuch, Bangkok, Thailand; MW 306.3; C₂₁H₂₀F₂N₄O) and Voriconazole (Vifend (R); Roerig/Pfizer Inc., New York, NY; MW 349.3105; C₂₁H₂₂F₃N₄O₂), Group II, Antibacterial drugs; azithromycin (Ben Venue Laboratories, Ohio, USA; MW 748.984, C₃₅H₄₂N₁₂O₁₄), Gentamicin sulfate (T.P. Drug Laboratories, Bangkok, Thailand; MW 477.59; C₂₁H₂₃N₄O₁₂) and Ciprofloxacin (Ranbaxy laboratories limited, Secunderabad, India; MW 331.4; C₁₇H₁₄FN₅O₂). Group III, Antiprotozoal drugs; Artesunate (Guilin Pharmaceutical Organization, Guangxi, China; MW 384.42; C₁₅H₂₁O₃), Quinine dihydrochlorinate (Guangzhou Person Pharmaceutical Organization, China; MW 396.90; C₂₅H₂₅N₄O₇HCl•2H₂O) and Metronidazole (Biolab, Bangkok, Thailand; MW 171.16; C₇H₁₂N₂O₃). Group IV, Antiviral drug; acyclovir (Mayne Pharma Pty Ltd., Mulgrave, Australia; MW 225; C₁₀H₁₁N₄O₅). Group V, Antiphychotic drug; Chlorpromazine hydrochloride (The Government. Pharmaceutical Organization, Bangkok, Thailand; MW 355.3; C₂₀H₂₅ClN₂S). Group VI, Interferon-alpha
Morphology and Growth of *N. fowleri* Trophozoites

One milliliters of the trophozoites resuspended in Nelson medium containing 5% FCS (1×10⁵ 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 cm². Serial twofold dilutions of the single drugs, except Amphotericin B at conc. 0.976-1000 μg mL⁻¹ were prepared in Nelson medium containing 5% FCS. Amphotericin B had ranging of concentrations at conc. 0.01-0.1 μg mL⁻¹. Control wells received 500 μL of medium alone. Five hundred microliters of calibrated trophozoite suspension (1×10⁶ 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 IC₅₀ scores in 500 μL of Nelson medium containing 5% FCS. Control well was medium alone. The 500 μL of trophozoite suspension (1×10⁶ 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 mean±standard errors of the means (SEM). Student’s t test was used to determine the significance, which was taken as a value of p<0.05.

RESULTS

Morphology and Growth Kinetic of *N. fowleri* Trophozoites

Morphology, *N. fowleri* trophozoites growing in axenic medium exhibited eruptive movement by producing hemispherical 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) Microscopic 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 (5x10⁴ cells at started point) were detected at about 6 to 72 h interval with doubling times. The yields of trophozoites were detectable approximately 1x10⁴ 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-inoculation, the IC₅₀ and MIC₁₀₀ 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 IC₅₀ and MIC₁₀₀ scores at 0.0246±0.004 μg mL⁻¹ and 0.098±0.0037 μg mL⁻¹ (Fig. 2a). At MIC₁₀₀ scores of drug combination, treatment with fluconazole plus Amphotericin B (MIC₁₀₀ scores at 126.114±0.63 μg mL⁻¹) to N. fowleri trophozoites showed 3.99 fold-lower than treated the trophozoites with fluconazole alone (MIC₁₀₀ 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 IC₅₀ scores at 6.767±0.04, 15.662±0.03 and
Table 1: Mean (±SD) μg mL⁻¹ of IC₅₀ and MIC₅₀ scores of either single drugs alone or single drugs plus with Amphotericin B against Naegleria fowleri Sirinaj strain

<table>
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<tr>
<th>Drugs</th>
<th>IC₅₀</th>
<th>MIC₅₀</th>
<th>IC₅₀</th>
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<td>Azithromycin</td>
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<td>15.637±9±1.600</td>
<td>1001.360±9±0.659</td>
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<td>Ciprofloxacin</td>
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<td>Artesunate</td>
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<td>Quinine sulphate</td>
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<td>Metronidazole</td>
<td>25.12229±9±0.7100</td>
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<tr>
<td>Acyclovir</td>
<td>31.312±9±0.0300</td>
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<td>8.823±9±0.020</td>
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<td>Antifolate</td>
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63.52±±0.071 μg mL⁻¹, respectively. Azithromycin was effective to the trophozoites MIC₅₀ at 510.308±±0.62 μg mL⁻¹, while gentamicin sulphate or ciprofloxacin was ineffective to the trophozoites at concentrations of MIC₅₀ 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 trophozoites. In contrast, we observed the sensitivity of combined gentamicin sulphate was not different from gentamicin sulphate alone at MIC₅₀ 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, artemesunate was effective to the N. fowleri trophozoites than quinine sulphate and metronidazole at IC₅₀ and MIC₅₀ scores, respectively. The sensitivities of combined artemesunate with amphotericin B to the trophozoites were 2-fold less than artemesunate alone at IC₅₀ and MIC₅₀ scores. In contrast, no susceptible activity of trophozoites to quinine sulphate alone or combined quinine sulphate was observed. Metronidazole alone or drug combination with amphotericin B had no also detectable effect on the trophozoites. It was suggested that artemesunate and amphotericin B had the synergistic effects on N. fowleri trophozoites.

Antiviral Drugs

As shown in Table 1 and Fig. 2c, the IC₅₀ of combined acyclovir (8.824±±0.02 μg mL⁻¹) to the N. fowleri trophozoites was 3.548 fold than acyclovir alone (31.312±±0.06 μg mL⁻¹). The MIC₅₀ of combined acyclovir to the N. fowleri trophozoites was concentrations of 504.79±±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) antipsychotic 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 IC₅₀ score at 0.980±0.01 μg mL⁻¹ with a MICₙₐ₀ of 7.96±0.01 μg mL⁻¹, thereby combined Chlorpromazine had an IC₅₀ score at 0.4937±0.01 μg mL⁻¹ with a MICₙₐ₀ of 1.9739±0.01 μg mL⁻¹. The MICₙₐ₀ scores of combined chlorpromazine to the trophozoites were 4 fold lower than chlorpromazine alone.

Interferon-Alpha

IFN-α had an IC₅₀ score at 5.8 log 10 IU with a MICₙₐ₀ of ≥7 log 10 IU to N. fowleri trophozoites (data not shown). The trophozoites were susceptible to IFN-α in combination with amphotericin B (MICₙₐ₀ of 6.18 log 101 IU mL⁻¹), while they were 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 (azithromycin, gentamicin sulphate and ciprofloxacin) antiprotozoa (artesunate) and antiviral (anticyclovir) and antipsychotic (chlorpropramide) 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_mean scores of fluconazole alone and combined fluconazole were 50.3.75±0.35 and 126.11±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, disseminated candidiasis and invasive candidal 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 amoebicidal effect against *N. fowleri*. Trophozoites (Tiewcharoen *et al.*, 2002). These 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-demethytransferase (P450 14DM) (Bachmann *et al.*, 2002). The MIC_mean scores of voriconazole in combination with amphotericin B to the trophozoites was 126.07±0.62 μg mL⁻¹. Our group did not observe detectable effect of voriconazole 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-demethytransferase (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.*, 1997). Antibacterial agents, the MIC_mean scores of azithromycin (510.30±0.62 μg mL⁻¹) were 3.89 times greater than those of combined azithromycin (131.20±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_mean 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 lead 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_90. 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_{90} (8.1705±0.06 µg mL^{-1}). Artesunate is one of semi-synthetic derivatives of artemisin isolated from decoctions of traditional Chinese medicine Artemisia annua L. (qingho, 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 dihydroartemisinin, one of artemisinin derivatives, on elevated NF-κB 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-artemisininoyl) phenoxyl succinic acid (SM735), a novel artesinin derivative, was also demonstrated to play a potential immunosuppressive role (Zhou et al., 2005). Furthermore, artesinin 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_{90} = 62.479±0.03 µg mL^{-1} and MIC_{90} = 1000.78±0.61 µg mL^{-1}, respectively. Combined-quinine showed high potency at IC_{90} (15.8798±0.35 µg mL^{-1}) and MIC_{90} (1000.67±0.92 µg mL^{-1}) to N. fowleri trophozoites. This data revealed the sensitivity of amphotericin B-combined quinin at IC_{90} scores were 3.934 times to N. fowleri than Quinin alone, suggesting that both drugs were synergistic drugs to amoeba. 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 causativaly 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 (Lefievre et al., 2007).
Metronidazole had IC₅₀ and MIC₉₀ effects against *Naegleria* trophozoites at 251.222±0.61 and 1012.105±0.74 µg mL⁻¹. Combined metronidazole against the trophozoites expressed as IC₅₀ and MIC₉₀ 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 MIC₉₀ 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 Thymidic 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 MIC₉₀ 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 MIC₉₀ 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 decrease 80% trophozoites during the 24 h incubation (Kim *et al.*, 2008) while as the amphotericin B and micamicozole 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 log₁₀ IU mL⁻¹ of IC₅₀ scores and >6.18 log₁₀ IU mL⁻¹ of MIC₉₀ scores compared with one of amphotericin B-combined IFN-α was 5.1 log₁₀ IU mL⁻¹ of IC₅₀ and 6.18 log₁₀ IU mL⁻¹ of MIC₉₀, 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 B with 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.

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