ANTHI-AEBIC ACTIVITY OF DIOSGENIN ON NAEGLERIA FOWLERI TROPHOZOITES

Jundee Rabablert, Supathra Tiewcharoen, Prasert Aeewarakul, Thassanant Atithep, Natchagorn Lumlerdkij, Renu Vejaratpimol, and Virach Junnu

INTRODUCTION

Naegleria fowleri is a thermophilic free-living ameba found in freshwater environments worldwide. It can cause a rare, potentially fatal disease in humans known as primary amebic meningoencephalitis (PAM) (Tiewcharoen et al., 2009). The few surviving cases often have permanent neurological sequale (Schuster and Visvesvara, 2004). The current drug of choice to treat PAM is amphotericin B (AMB). AMB has significant toxicity; it is a hydrophobic molecule with negligible solubility in aqueous solutions (Kim et al., 2008; Brunton et al., 2011). There is an urgent need to develop other agents to treat PAM with fewer side-effects and more rapid onset of action. The World Health Organization (WHO) has encouraged countries to investigate traditional medicines to identify safe and effective remedies (WHO, 2000). Momordica charantia and its purified compound, diosgenin, has been found to have anti-viral (Wang

Correspondence: Supathra Tiewcharoen, Department of Parasiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok Noi, Bangkok 10700, Thailand.
E-mail: supathra.tie@mahidol.ac.th

Abstract. The aim of this study was to investigate the activity of diosgenin against Naegleria fowleri trophozoites at the cellular and molecular levels. Diosgenin (100 µg/ml; 241.2 µM) had a 100% inhibitory effect on N. fowleri trophozoites (5x10^5 cell/ml). Scanning electron micrograph revealed diosgenin decreased the number of sucker-like apparatuses and food cup formation among N. fowleri trophozoites at 3 and 6 hours post-exposure, respectively. Diosgenin down-regulated the nf cysteine protease gene expression of N. fowleri trophozoites at 6 and 12 hours post-exposure. The toxicity to mammalian cells caused by diosgenin at therapeutic dose was less than amphotericin B, the current drug used to treat N. fowleri infections. Our findings suggest diosgenin has activity against the surface membrane and the nf cysteine protease of N. fowleri trophozoites. However, the other mechanisms of action of diosgenin against N. fowleri trophozoites require further exploration.

Keywords: Naegleria fowleri, diosgenin, nf cysteine protease, scanning electron micrograph
et al., 2011), anti-fungal (Cho et al., 2013), anti-helminthic (Wang et al., 2010), and anti-malarial activity (Pabon et al., 2013). In a previous study, we found the crude methanolic extract of *M. charantia* (5 mg/ml) to have 100% activity against *N. fowleri* trophozoites and to have low cytotoxicity in SK-N-MC or LLC-MK2 cells (Luangboribun et al., 2014). In this study, we evaluated the anti-amebic activity of diosgenin against *N. fowleri* trophozoites at the cellular and molecular levels. The cytotoxicity of diosgenin against mammalian cells was also investigated.

**MATERIALS AND METHODS**

*N. fowleri* culture

*N. fowleri* (Siriraj-strain) was first isolated from a PAM patient at Siriraj Hospital, Bangkok, Thailand in 1986. Trophozoites were cultured in Nelson’s medium supplemented with 5% fetal bovine serum (FBS) without antibiotics at 37°C. The trophozoites were harvested during the logarithmic phase of growth and determined using the Trypan blue exclusion method (Tiewcharoen et al., 2008).

Mammalian cell culture

A rhesus monkey kidney LLC-MK2 cell line donated by the Late Professor Natth Bhamarapravati, Center for Vaccine Development, Institute of Molecular Bioscience, has been maintained in Dulbecco’s Modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA). A human neuroblastoma SK-N-MC cell line was purchased from Cell Line Service, Germany, in 2006. The cell has been maintained in DMEM; Ham’s F-12 (Cell-Line, Heidelberg, Germany). Ten percent of fetal bovine serum (FCS), 4 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin were added to both cell cultures and incubated at 37°C with 5% CO₂ (Tiewcharoen et al., 2014).

**Reagents**

Fifty grams of amphotericin B (AMB, C47H73NO17 MW: 924.07902) was purchased from Bharat Serums and Vaccines (Ambermath, India), and dissolved in 5 ml distilled water, giving a concentration of 10 mg/ml to be a stock solution, and then stored at -20°C until used. Diosgenin, a purified extract product of *M. charantia*, was donated by the Center for Applied Thai Traditional Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. Fifty milligrams of diosgenin was dissolved in 2 ml dimethyl sulfoxide (DMSO), (Sigma-Aldrich, St. Louis, MO), resulting in a concentration of 25 mg/ml to be a stock solution, and then stored at -20°C until used.

**Anti-amebic activity assay**

The *N. fowleri* trophozoites were seeded in 1.5 ml microcentrifuge tubes at a density of 5×10⁵ cells/ml. Two-fold serial dilutions of diosgenin (0, 25, 50, and 100 µg/ml) were then added to the trophozoites and incubated at 37°C for 6, 24, and 48 hours. AMB at 10 µg/ml was used as positive control, as previously described by Luangboribun et al. (2014). Nelson’s medium and 4% DMSO were used as negative control. At specified times, the untreated and treated trophozoites were then harvested and centrifuged at 5,000 g for 2 minutes. The cell pellets were then dissolved with 0.1 M phosphate-buffered saline (PBS), at a pH of 7.4. Trophozoite viability was determined using the Trypan blue exclusion method.

**Ultrastructure study**

*N. fowleri* trophozoites were placed in a 35 mm dish at a density of 5×10⁵ cells/ml. One hundred microliters of diosgenin
was added to the trophozoites and incubated at 37°C for 0, 3, and 6 hours. At the specified times, the untreated and treated trophozoites were then harvested and prepared for study as described previously (Tiewcharoen et al, 2014). Finally, the trophozoites were photographed under a scanning electron microscope (SEM, Hitachi S-510, Tokyo, Japan) at an accelerating voltage of 25 kV.

**Statistical analysis**

The results are expressed as means ± one standard deviation for each of the three independent experiments and were carried out in triplicate. A Student’s *t*-test was used for analysis. A *p*-value <0.05 was considered significant.

**RESULTS**

**Effect of diosgenin on *N. fowleri* trophozoite**

The cell viability of the tested trophozoites treated with diosgenin at 0, 50, and 100 µg/ml was determined using the Trypan blue exclusion method. Diosgenin at 100 µg/ml gave 98% inhibition 6 hours post-exposure, but diosgenin at 50 µg/ml did not cause any inhibition 6 hours post-exposure (Fig 1). Diosgenin at 100 µg/ml gave 100% inhibition 24 hours post-exposure but diosgenin at 50 µg/ml gave not significant inhibition 24 hours post-exposure. AMB (10 µg/ml) gave 100% inhibition at 6, 24, and 48 hours post-exposure (Fig 1). Four percent DMSO caused no inhibition (Fig 1), suggesting the diosgenin, not DMSO, caused inhibition. The effect of diosgenin on *N. fowleri* trophozoites was both dose- and time-dependent (*p*<0.05).

**Effect of diosgenin on the morphology of *N. fowleri* trophozoites**

On scanning electron micrographs of the *N. fowleri* trophozoites 3 and 6 hours post-diosgenin, the morphological characteristics of the *N. fowleri* trophozoites were studied. Untreated trophozoites had a sucker-like apparatus and a wrinkled membrane (Fig 2a). Diosgenin-treated amebae 3 hours post-exposure were smaller in size and had fewer sucker-like apparatuses (Fig 2b). The morphological characteristics of the treated trophozoites
Fig 1–Activity of diosgenin (50, and 100 µg/ml) and amphotericin B (AMB, 10 µg/ml) against *N. fowleri* trophozoites using the Trypan blue exclusion method. Nelson’s medium and DMSO (4%) were used as a negative control.

Fig 2–Scanning electron micrographs of *Naegleria* trophozoites: (a) untreated trophozoites with a sucker-like apparatus and wrinkled membrane; (b) diosgenin-treated (50 µg/ml) trophozoites 3 hours post-treatment, small in size; (c) diosgenin-treated (50 µg/ml) trophozoites 6 hours post-treatment, rounded small in size without pseudopodia or sucker-like apparati.

6 hours post-exposure: they were small in size, rounded up with cracking of the membrane, free of pseudopodia, and lack of sucker-like apparatus (Fig 2c).

**Effects of diosgenin on trophozoite at the genetic level**

To study the activity of diosgenin on *N. fowleri* trophozoite genes, we used diosgenin at 50 µg/ml and evaluated 8 genes: *nfa1, Mp2CL5, pB2.5, Naegleria pore B, nf cysteine protease, Nf314, and nf phospholipase* of *N. fowleri* trophozoites at 1, 3, 6, and 12 hours post-exposure using reverse-transcriptase (RT)-PCR. The *nf actin* was used as house-keeping gene. Diosgenin down-regulated the *nf cysteine protease* gene expression of *N. fowleri* trophozoites at 6 and 12 hours, post-incubation (Fig 3). Diosgenin at the dose tested, did not affect the other genes of *N. fowleri* trophozoites.
Activity of Diosgenin on N. fowleri Trophozoites

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nf cysteine protease (187 bp)

nf actin (170 bp)

Fig 3–Expression of nf cysteine protease in untreated and diosgenin-treated N. fowleri trophozoites at 1, 3, 6, and 12 hours post-exposure using RT-PCR. The nf actin was used as a house-keeping gene.

at 1, 3, 6, and 12 hours, post-incubation (data not shown).

Effect of diosgenin on mammalian cell viability

Diosgenin at 200 µg/ml, similar to AMB at 10 µg/ml resulted in 63%, 39%, and 24% of SK-N-MC cells being viable at days 1, 2, and 3 post-exposure, respectively (Fig 4a). Diosgenin at concentrations of 50, and 100 µg/ml caused less damage to SK-N-MC cells 3 days post-exposure. Diosgenin (200 µg/ml), similar to AMB (10 µg/ml), resulted in 56%, 45%, and 46% of LLC-MK2 cells being viable 1, 2, and 3 days post-exposure, respectively (Fig 4b). Diosgenin at concentrations of 50, and 100 µg/ml caused less damage to LLC-MK2 cells 3 days post-exposure. Diosgenin had a dose- and time-dependent affect on the tested cells.

DISCUSSION

The free-living ameba Naegleria fowleri can cause an acute, fulminant, necrotizing, hemorrhagic meningoencephalitis (PAM) that can cause serious morbidity and mortality (Budge et al, 2013). AMB is the current drug of choice to treat PAM, but it has significant renal toxicity (Kim et al, 2008). Diosgenin [(25R)-spirost-5-en-3b-ol] is a steroidal saponin found in M. charantia (Danial et al, 2014). It can cause changes in membrane permeability and pore formation among chloroquine-resistant Plasmodium falciparum (Pabon et al, 2013). Diosgenin also causes increased permeability of the cell membrane, disrupts the membrane potential and changes the osmolarity leading to cell shrinkage in Candida albicans, resulting in cell death (Nelson, 2009). In our current study, diosgenin at 100 µg/ml (241.2 µM) reduced N. fowleri trophozoite viability (Fig 1), and was 50 times more effective than the methanol extract of M. charantia (5 mg/ml) (Luangboribun et al, 2014). In our current study, diosgenin caused N. fowleri trophozoites to develop abnormal membranes and damaged food cups (Fig 2). AMB damages N. fowleri trophozoites, causing bleb formation and the disappearance of suckers and pseudopodia (Tiewcharoen et al, 2009). Diosgenin, like AMB, appears to be amebicidal.

In our current study, diosgenin at 50 µg/ml (120.6 µM) inhibits nf cysteine protease (Fig 3). Aldape et al (1994) purified a 30 kDa secreted cysteine protease of N. fowleri that had a cytopathic effect on BHK cells. Amin (2004) isolated and characterized the 128 and 170 kDa cysteine protease in cell lysates and purposed these proteases were involved in tissue destruction...
and pathogenesis. Rojas-Hernandez et al (2004) found *N. fowleri* trophozoites do not damage epithelial nasopharyngeal cells or their extracellular matrix during the initial stages of meningoencephalitis in mice. Tissue damage apparently occurs only when a host inflammatory reaction appears at the site of infection. In addition to amebic proteases, other factors may be contributing to the pathogenesis of PAM.
*N. fowleri* lysate and excretory-secretory proteins include various pathogenic proteins, such as secreting effect proteins, cysteine proteases (including cathepsin B and cathepsin B-like proteases), secretory lipase, peroxiredoxins, and thrombin receptors, which play a role in the entry of the ameba into the host cell and function as various dominant antigenic proteins; which are involved in pathology (Kim *et al*, 2009). In our study, the diosgenin inhibited the *nf* cysteine protease gene, which prevents the ameba from entering host cells, leading to an aberration in amebic pathogenesis. Therefore, diosgenin had an effect on *N. fowleri* trophozoites at both the cellular and molecular levels (Figs 1-3). Diosgenin at 100 µg/ml caused less damage to SK-N-MC cells and LLC-MK2 cells than AMB. These findings suggest diosgenin may be a good candidate for development as drug against *N. fowleri* trophozoites.

In conclusion, diosgenin had activity against *N. fowleri* at the cellular and molecular levels. The anti-amebic effect of diosgenin needs to be further investigated to better understand the mechanism of action.

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