EFFECT OF SYNTHETIC ANTIMICROBIAL PEPTIDES ON *NAEGLERIA FOWLERI* TROPHOZOITES

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Abstract. We evaluated the effect of tritrpticin, lactoferrin, killer decapeptide and scrambled peptide *in vitro* against *Naegleria fowleri* trophozoites compared with amphotericin B. Tritrpticin (100 µg/ml) caused apoptosis of *N. fowleri* trophozoites ($2x10^5$ cells/ml), while lactoferrin, killer decapeptide and scrambled peptide did not. On Gormori trichrome staining, tritrpticin affected the elasticity of the surface membrane and reduced the size of the nuclei of *N. fowleri* trophozoites. The ultrastructure surface membrane and food cup formation of the trophozoites were 100% inhibited. These results are consistent with inhibition of the *nfa1*, *Mp2CL5* of the treated trophozoite, which plays a role in food cup formation. Tritrpticin 100 µg/ml was not toxic against SK-N-MC cells. Our findings suggest tritrpticin has activity against the surface membrane and *nfa1*and *Mp2CL5* of *N. fowleri* trophozoites and could be developed as a potential therapeutic agent.

Keywords: *Naegleria fowleri,* antiamebic peptide, tritrpticin

INTRODUCTION

The free-living ameba *Naegleria fowleri* is a causative agent of primary amebic meningoencephalitis (PAM) in humans and animals (Madarova *et al*, 2010). PAM may occur, when an otherwise healthy person is exposed to contaminated water in the nose (Yoder *et al*, 2010). Clinical manifestations begin a few days after exposure (Visvesvara, 2010). Sources of contaminated water include environmental pools, inadequately chlorinated swimming pool water and heated and contaminated tap water (Yoder *et al*, 2012). Infection initially occurs when the *N. fowleri* ameba penetrates the mucous membranes of the nasal cavity and travels to the brain through the olfactory nerves (Visvesvara *et al*, 2005). The *nfa1* and *Mp2CL5* genes are found only in *N. fowleri* (Tiewcharoen *et al*, 2011). Nfa1 protein is expressed from the *nfa1* gene and is located in the pseudopodia and around food vacuoles (Kang *et al*, 2005). Nfa1 protein is localized

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in the food cups which are involved in phagocytic activity (Marciano-Cabral and Cabral, 2007; Tiewcharoen *et al*, 2011). The *Naegleria pore B* gene encodes Naegleria pore A and B proteins that display pore-forming activities and kill prokaryotic and eukaryotic target cells and the *nf actin* is a housekeeping gene (Tiewcharoen *et al*, 2012). The *ITS* is located on the 5.8S rRNA gene and species-specific chromosomal DNA *pB2.5* genes are used to identify pathogenic *N. fowleri* at the molecular level (Rabablert *et al*, 2011).

Patients with *N. fowleri* infection often have initial symptoms of high grade fever (38º-40ºC), sore throat, stuffy nose, severe headache and then rapidly progress to meningitis, encephalitis, ataxia, mental confusion and coma a few days prior to death (Yoder *et al*, 2012). Treatment includes amphotericin B (AMB) and multiple other drugs: rifampicin, fluconazole, dexamethasone and ceftriaxone (Vargas-Zepeda *et al*, 2005). Most patients with *N. fowleri* infection die; the rare cases who survive may have neurological sequel (Visvesvara *et al*, 2010).

Because of the poor treatment results, several medications such as AMB, miconazole, fluconazole, ketoconazole and rifampin had been used in combination (CDC, 2013) with the new drug, Miltefosine (Kim *et al*, 2008). However, the mortality of PAM is still high (Yoder *et al*, 2010). Treatment problems include limited availability of AMB and side effects of drugs (Kim *et al*, 2008). AMB resistance by *N. fowleri* has been reported (Donadio *et al*, 2010). Several studies have reported antifungal resistance to the azole group can be spread via resistant *Candida albicans* (Sterling and Merz, 1998; Barker and Rogers, 2006). *N. fowleri* resistance to AMB has been attributed to a virulent gene (Tiewcharoen *et al*, 2011).

Antimicrobial peptides (AMPs) are recognized as an important component of the nonspecific host defense system against invading pathogens (Hancock and Chapple, 1999; Wilcox, 2004; McGregor, 2008). The characteristic of AMPs include small molecular size and cationic affinity (Dürr *et al*, 2006), it is usually non-immunogenic and has a short half-life. Their activity includes targeting the membrane, disrupting protein-protein interaction and the ability to penetrate tissues (McGregor, 2008). The activity of AMPs can be triggered by binding to negatively charged sites on the surface of brain capillary endothelial cells (Mahurkar *et al*, 2014). AMPs have a wide spectrum of activity, acting against gram-positive and gram-negative bacteria, protozoa, fungi, viruses, and mammalian cells (Brogden, 2005; Cirioni *et al*, 2006; Bagheri *et al*, 2011). The aim of the current study was to test for the first time the effectiveness of AMPs against *N. fowleri* trophozoites and to evaluate cell damage and alternations in luminescence by scanning electron microscopy.

MATERIALS AND METHODS

Culture of *Naegleria fowleri*

N. fowleri (Siriraj strain) was isolated in 1986 from a PAM patient at Siriraj Hospital, Bangkok, Thailand. The trophozoites were cultured in T 75-cm^2 flasks (Corning, Corning, NY) containing Nelson's medium supplemented with 5% fetal calf serum (FCS) without antibiotics at 37ºC. The trophozoites were incubated at 4ºC for 10 minutes, scraped and then centrifuged at 5,000 rpm for 2 minutes. The pellet was then dissolved in 2 ml of Nelson's medium. The number of cells was counted using the Trypan blue exclusion method: 10 µl of medium containing trophozoites was mixed with 90 µl of 0.4%

Table 1
List of antimicrobial peptide names and sequences.

Peptides	Amino acid sequences	Molecular weight
Tritrpticin	VRRFPWWWPFLRR	1902.30
Lactoferrin	RRWQWRMKKLG	1544.90
Killer decapeptide	AKVTMTCSAS	998.19
Scrambled peptide	MSTAVSKCAT	998.19

Trypan blue stain and then the cells were counted under a light microscope. The procedure was conducted in duplicate.

Human neuroblastoma cultivation

Human neuroblastoma SK-N-MC cells isolated from a female Caucasian patient with Askin's tumor were purchased from Cell Line Service (Eppelheim, Germany). The cells were maintained in Dulbecco's Modified Eagle Medium and HAM's F-12 (DMEM: HAM'S F-12) medium with 10% fetal bovine serum, 4 mM L-glutamine, 100 µl/ml penicillin and 100 µg/ml streptomycin and were grown in monolayer cultures at 37ºC in 5% CO_2 (Tiewcharoen *et al*, 2008).

Antimicrobial peptides

Tritrpticin (Trp; Infante *et al*, 2011), lactoferrin (LF; León-Sicairos *et al*, 2006), killer decapeptide (Kp) and scrambled peptide (Sp) (Fiori *et al*, 2006) are AMPs with properties against protozoa (Arrighi *et al*, 2002). The AMPs were obtained from China Peptides (Shanghai, China) (Table 1). The AMPs, which came as a powder, were dissolved in distilled water at a concentration of 1 mg/ml and then stored at -80ºC, until used.

Reagents

Caspase-Glo® 3/7 Substrate and Caspase-Glo® 3/7 Buffer obtained from Promega (Madison, WI), were mixed by inversion until the substrate was dissolved and then stored at -20ºC until used. Five milligrams of MTT (Invitrogen/ Molecular probes, Eugene, OR) was dissolved in 1 ml of phosphate buffer (PBS pH 7.4) and used immediately.

Caspase-Glo® 3/7 assay

N. fowleri trophozoites at a concentration of $1x10^6$ cells/ml, were dissolved in Nelson's medium. One hundred microliters of each studied peptide was then added to the trophozoites and incubated at 37ºC for 0.5, 1, 3, 6 or 12 hours. As a negative control, trophozoites in Nelson's medium were studied alone. The untreated and treated trophozoites were then harvested and centrifuged at 5,000*g* for 2 minutes. A pellet each of untreated and treated trophozoites were dissolved in PBS 7.4 (250 µl) and then 50 µl of Caspase-Glo® 3/7 reagent was added and incubated at room temperature (RT) for 30 minutes in darkness. Caspase activity was measured at a wavelength of 485 nm with an emission wavelength of 527 nm by a luminometer (luminescence-Octa AB-2270, Tokyo, Japan) (Renault *et al*, 2010).

Gormori trichrome staining

Untreated and treated trophozoies were fixed with Schaudinn's fixative at RT for 24 hours, stained with 2% iodine for 2 minutes, washed with 70% ethanol for 2 minutes, counterstained with Gormori trichrome for 10-15 minutes, washed with acid alcohol for 5 seconds, dehydrated

Table 2
Primers used for RT-PCR.

Genes	Primer sequences	bp	PCR product (bp)
nfa1	Forward: 5´ATGGCACTACTATTCCATCACCA 3´	23	360
	Reverse : 5´TTAAAGCACTCCCTTGTACTTCAT 3´	24	
Mp2CL5	Forward: 5´TCTAGAGATCCAACCAATGG 3´	20	166
	Reverse : 5´ATTCTATTCACTCCACAATCC 3´	21	
ITS	Forward: 5´GAACCTGCGTAGGGATCATTT 3´	21	450
	Reverse : 5´TTTCTTTTCCTCCCCTTATTA 3´	21	
pB2.5	Forward: 5´GTGAAAACCTTTTTTCCATTTACA 3´	24	310
	Reverse : 5´AAATAAAAGATTGACCATTTGAAA 3´	24	
Naegleria pore B	Forward: 5´TTGATGTCAATGCTGTCAAGC 3´	21	165
	Reverse : 5´CTTTGGGCAGACATCAACG 3´	19	
nf actin	Forward: 5´ACTCTGGTGATGGTGTCTCTCACAC 3´	25	170
	Reverse : 5´CTCTGACAATTTCTCTCTCAGTGG 3´	24	

bp, base pair.

using 90% and then 95% ethanol for 1 minute each xylene for 1 minute and then mounted on a glass slide and observed under a light microscope.

Scanning electron microscope

Untreated and treated trophozoites were pre-warmed in 2.5% glutaraldehyde and 0.1 M PBS at a pH of 7.3 at 37ºC for 30 minutes and then cooled to 4ºC for 24 hours. The trophozoites were then fixed in 1% osmium tetroxide (OsO_4) and 0.1 M PBS at RT for 90 minutes, rinsed with 0.1 M PBS, sequentially dehydrated in serial dilutions of ethanol, critical point dried, and then coated with gold-palladium (Au-Pd). Finally, the trophozoites were examined and photographed under a scanning electron microscope (SEM) (Hitachi S-51, Tokyo, Japan) at an accelerating voltage of 25 kV.

Total RNA extraction

Untreated and treated trophozoite pellets were extracted with a Favorprep™ Tissue total RNA mini kit (Favorgen, Ping-Tung,Taiwan) at 1, 3, 6, and 12 hours post-incubation. Briefly, approximately 1 x 10^6 trophozoites were added to 350 µl of FARB buffer and 3.5 µl β-ME and vortexed vigorously to lyse the cells. The sample was transferred to a filter column placed on a collection tube and centrifuged at 10,000*g* for 2 minutes. The supernatant from the collection tube was then transferred to a microcentrifuge tube. Ethanol (70%) was added to the lysate and mixed well by vortexing. The ethanol-treated sample was transferred to a FARB Mini column, centrifuged at 10,000*g* for 1 minute. Wash buffer 1 (500 µl) was added to wash the FARB Mini column and centrifuged at 10,000*g* for 1 minute. Wash buffer 2 (750 µl) was added to wash the FARB Mini column and centrifuged at 10,000*g* for 1 minute, twice. The FARB Mini column was centrifuged at 10,000*g* for 3 minutes to dry the column. RNase-free ddH_2O (45 µl) was added to the membrane center of the FARB Mini column for 1 minute and centrifuged at 10,000*g* for 2 minutes to elute the RNA. The RNA was stored at -80ºC until used.

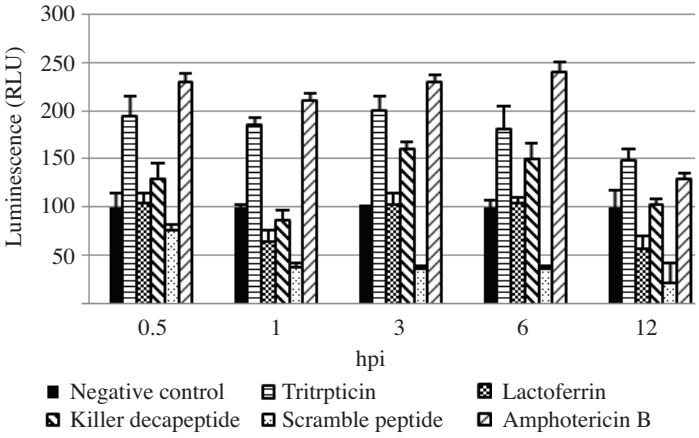

Fig 1–Apoptosis of *N. fowleri* trophozoites ($2x10^5$ cell/ml) treated with Trp, LF, Kp, Sp (100 µg/ml) and AMB (10 µg/ml) evaluated with the Caspase-Glo® 3/7 Assay at 0.5, 1, 3, 6 and 12 hours post-incubation. Data is expressed as relative light units (RLU). These experiments were preformed in triplicate. Error bars represent standard deviations ($p<0.05$). hpi, hours post incubation.

cDNA synthesis

Untreated and treated trophozoite pellets were extracted with the Favorprep™ Tissue total RNA mini kit (Favorgen, Ping-Tung,Taiwan). A total of 20 µl of RNA was used as a template to synthesize the first strand of the cDNA using the Maxime RT PreMix Kit (iNtRON Biotechnology, Gyeonggi-do, Korea), following the manufacturer's instructions. Briefly, template RNA and RNase-free water were added to the MaximeRT PreMix tubes (Random primer) to give a total volume of 20 µl; this was done in duplicate. The cDNA reaction was carried out at 45ºC for 60 minutes and then 95ºC for 5 minutes and then stored at -20ºC until used for PCR amplification.

Polymerase chain reaction

The PCR reaction was carried out in a volume of 20 µl, in the presence of 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of specific primers (Table 2), 2.5 µmol of *Taq* polymerase and 2 µl of single stranded cDNA. The cDNA templates were amplified at an initial incubation at 94ºC for 5 minutes followed by 35 cycles at 94ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 45 seconds in a Gene Amp PCR 2400 thermal cycle system (Perkin-Elmer, Cetus Crop, Waltham, MA). The PCR product was incubated at 72ºC for 10 minutes to ensure complete extension of all amplified molecules. Finally, the PCR products were subjected to 2% agarose Tris-borate-EDTA gel electrophoresis at 100 V for 30 minutes. The gel was stained with ethidium bromide and then visualized under ultraviolet light.

The effect of tritrpticin on morphology of SK-N-MC co-culture with *N. fowleri*

SK-N-MC cells ($1x10^6$ cells/ml) were cultured in Dulbecco's Modified Eagle Medium HAM's F-12 for 24 hours and then *N. fowleri* trophozoites were added to the SK-N-MC cells and incubated with or without Trp (100 µg/ml) at 37ºC for 0.5, 1, 2, or 3 hours. As a negative control, the SK-N-MC cells were treated with medium alone. The morphologies of the cultures were observed with a SEM. The preparation of SEM was described above.

Statistical analysis

The results are expressed as means ± standard deviations for the three experiments carried out in triplicate. A Student's *t*-test was used for analysis; a *p*-value <0.05 was considered significant.

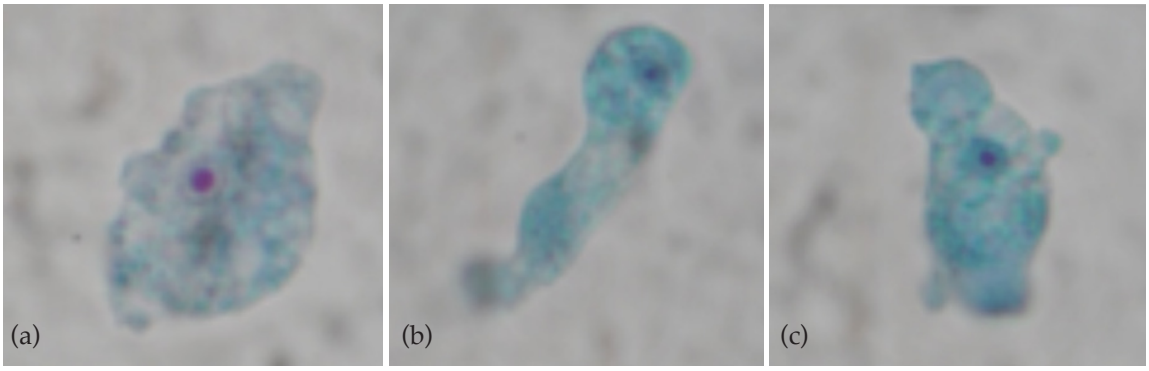

Fig 2–Photomicrograph of *N. fowleri* trophozoites in the absence or presence of Trp (100 µg/ml) in Gormori's trichrome stain at 1 and 3 hours post-exposure: (a) control trophozoites exposed to medium alone showing a vesicular nucleus and small granules in the cytoplasm; (b) 1 hour post-exposure showing a small nucleolus; (c) 3 hours post-exposure appears the same as control (x 400).

RESULTS

Effect of antimicrobial peptides on apoptosis of *N. fowleri* trophozoites

As shown in Fig 1, Trp induced apoptosis in *N. fowleri* trophozoites after a 30-minute exposure period at a concentration of 100 µg /ml. The activity of Trp is also comparable to AMB (10 µg /ml). In contrast, LF, Kp and Sp did not induce apoptosis. Trp (100 µg/ml) did not damage human neuroblastroma SK-N-MC cells at indicated times (data not shown).

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

The morphological characteristics of *N. fowleri* trophozoites exposed to Trp (100 µg/ml) and stained with Gormori trichrome at 0.5, 1, 2 and 3 hours post-exposure were compared to untreated trophozoites. Untreated trophozoites had homogenous cytoplasm with a normal elastic membrane and a large nucleolus in the central nucleus surrounded by a complete nuclear membrane (Fig 2a). Trp-treated trophozoites had an elongated shape with a loose elastic membrane and a small nucleolus 1 hour post-exposure (Fig 2b). However, treated trophozoites had a similar morphology to untreated trophozoites 3 hours after exposure (Fig 2c). A SEM micrograph of *N. fowleri* trophozoites showed a sucker like apparatus and wrinkled membrane (Fig 3a). Trp-treated trophozoites were small in size and few in number. In addition, the ultrastructural surface membrane and food cup formation were 100% inhibited at 1 hour post-exposure (Fig 3b). However, the abnormalities of the surface membrane recovered by 3 hours post-exposure (Fig 3c).

Effect of tritrpticin on *N. fowleri* trophozoites at the gene level

Trp inhibited the *nfa1* and *Mp2CL5* genes of *N. fowleri* trophozoites 1 hour post -incubation, but did not inhibit the *nfa1* or *Mp2CL5* genes at 3, 6 and 12 hours post-incubation. This indicates Trp had time-dependent activity against *N. fowleri* trophozoites. Trp did not inhibit the *ITS, pB2.5, Naegleria pore B* or *nf actin* genes of *Naegleria* trophozoites at 1, 3, 6 or 12 hours post-incubation (Fig 4).

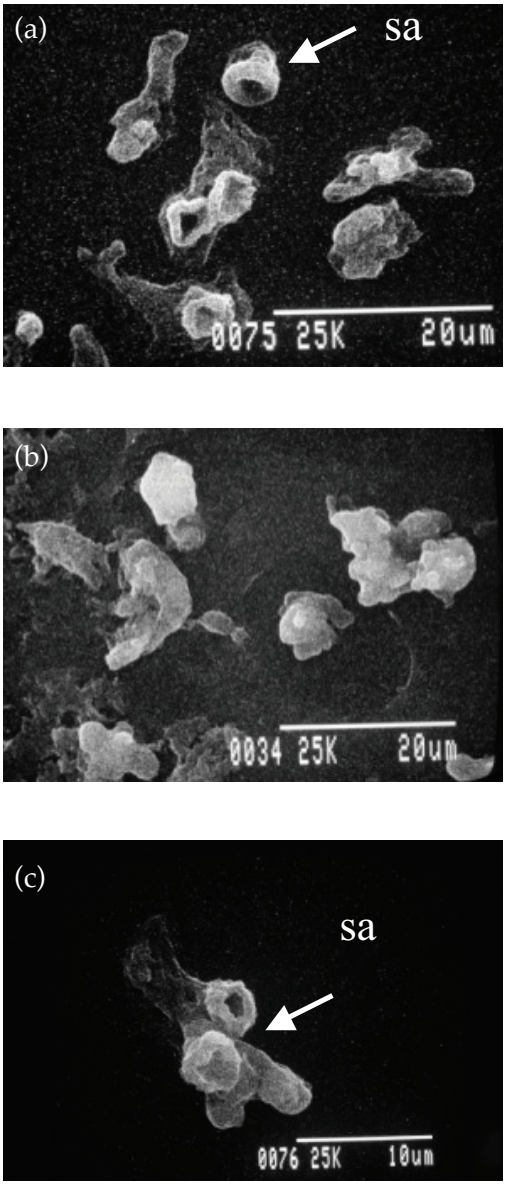

Fig 3–Scanning electron micrograph of *Naegleria* trophozoites; (a) control trophozoites exposed to medium alone showing a sucker like apparatus and wrinkled membrane; (b) Trp (100 µg/ml) treated amebae are small in size and few in number; (c) 3 hours post-exposure is similar to control. Bars represent 10, 20 µm. Sa, sucker apparatus.

The effect of tritrpticin on the morphology of the SK-N-MC/*N. fowleri* co-culture

A SEM micrograph of the SK-N-MC cells not exposed to *N. fowleri* trophozoites showed an elongated shape with dendrites and axons. The SK-N-MC cells cultured with *N. fowleri* trophozoites had trophozoites attached to the surface membrane of the SK-N-MC cells 1 hour post-incubation. Pre-incubation, the tritrpticin treated SK-N-MC/*N. fowleri* co-culture had a reduction in the size and number of trophozoites. This suggests Trp may prevent phagocytosis of *N. fowleri* by SK-N-MC cells.

DISCUSSION

Free living *N. fowleri* ameba can cause acute, fulminent, necrotizing, hemorrhagic PAM leading to death (Budge *et al*, 2013). AMB is the only medication with proven clinical efficacy in treating PAM (Brunton *et al*, 2006). However, AMB is not always successful in treating PAM and is associated with severe adverse effects (Soltow and Brenner, 2007).

AMPs are important components of the nonspecific host defense system against invading pathogens (Brogden, 2005). Typically, these peptides are relatively short, positively charged, and amphiphilic (Reddy *et al*, 2004). Previous publications have reported AMP activity against bacteria, fungi, viruses, and protozoa (Cirioni *et al*, 2006; Bagheri *et al*, 2011). It has been believed AMPs act at the membrane level by permeabilization of the cytoplasmic membrane of the microorganism (Franco *et al*, 2006; Jaeyong *et al*, 2012).

In our study, Trp at 100 µg/ml reduced *N. fowleri* trophozoite viability (Fig 1). Our findings are consistent with other reports in which Trp at 100 µg/ml reduced

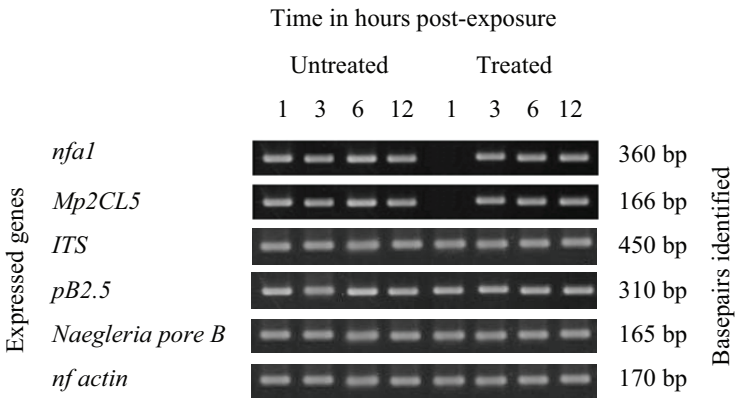

Fig 4–Expression of *nfa1*, *Mp2CL5*, *ITS*, *pB2.5*, *Naegleria pore B* and *nf actin* genes in untreated and Trp-treated *N. fowleri* trophozoites at 1, 3, 6 and 12 hours post-exposure using RT-PCR.

viability of *Trichomonas vaginalis* (Infante *et al*, 2011). Trp has also been shown to lyse *Bacillus subtilis* and *Escherichia coli* membranes (Bagheri *et al*, 2011). Trp caused *N. fowleri* trophozoites to have abnormal membranes and damaged food cups in our study (Figs 2 and 3). AMB and chlorpromazine damage *N. fowleri* trophozoites, causing bleb formation and disappearance of suckers and pseudopodia (Tiewcharoen *et al*, 2011). Trp activity is comparable to AMB and chlorpromazine, but it appears to be ameba cestatic.

Previous studies have found *nfa1* and *Mp2CL5* genes of *N. fowleri* trophozoites to be pathogenic (Kang *et al*, 2005). Nfa1 protein, expressed by the *nfa1* gene, is located in the pseudopodia and around food vacuoles (Shin *et al*, 2001). Nfa1 protein is specifically localized to food cups, which are involved in phagocytic activity (Kang *et al*, 2005). In our study, Trp inhibited the *nfa1* gene which can cause the amebae to lose their phagocytic activity. Réveiller *et al* (2002) found expression of *Mp2CL5* protein in *N. fowleri* during the growth phase to be regulated. Mp2CL5 protein is increased in expression during the stationary phase of growth, when the cells are experiencing nutrients. In our study, Trp inhibited the *Mp2CL5* gene, suggesting the activity of Trp may cause a loss of cell recognition, sensing the environment and growth of the ameba (Fig 4).

Tritrpticin (>150 µg/ml) retains most of its antimicrobial activity, but has enhanced hemolytic and membrane-disruption activity (Yang *et al*, 2002). Trp (>150 µg/ml) has an inhibitory effect against human MDA-MB-361 and A549 cells (Yang *et al*, 2009). In our study, Trp (100 µg/ml) did not damage human neuroblastroma SK-N-MC cells. In contrast, AMP (10 µg/ml) decreased cell viability by 40% 12 hours post-incubation (data not shown). Trp (100 µg/ml) had an effect on *N. fowleri* trophozoites at both the cellular and molecular levels (Figs 1 and 4). We believe Trp may be a good candidate for development as a potential drug against *N. fowleri* trophozoites.

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