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Novel Antiamoebic Tyrocidine-Derived Peptide against Brain-Eating Amoebae

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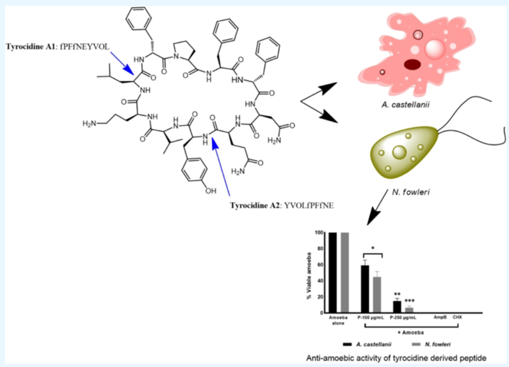
ABSTRACT: *Acanthamoeba castellanii* (*A. castellanii*) can cause *Acanthamoeba* keratitis, a sight-threatening infection, as well as a fatal brain infection termed granulomatous amoebic encephalitis, mostly in immunocompromised individuals. In contrast, *Naegleria fowleri* (*N. fowleri*) causes a deadly infection involving the central nervous system, recognized as primary amoebic encephalitis, mainly in individuals partaking in recreational water activities or those with nasal exposure to contaminated water. Worryingly, mortality rates due to these infections are more than 90%, suggesting the need to find alternative therapies. In this study, antiamoebic activity of a peptide based on the structure of the antibiotic tyrocidine was evaluated against *A. castellanii* and *N. fowleri*. The tyrocidine-derived peptide displayed significant amoebicidal efficacy against *A. castellanii* and *N. fowleri*. At 250 μg/mL, the peptide drastically reduced amoebae viability up to 13% and 21% after 2 h of incubation against *N. fowleri* and *A. castellanii*., whereas, after 24 h of incubation, the peptide showed 86% and 94% amoebicidal activity against *A. castellanii* and *N. fowleri*. Furthermore, amoebae pretreated with 100 μg/mL peptide inhibited 35% and 53% *A. castellanii* and *N. fowleri*, while, at 250 μg/mL, 84% and 94% *A. castellanii* and *N. fowleri* failed to adhere to human cells. Amoeba-mediated cell cytopathogenicity assays revealed 31% and 42% inhibition at 100 μg/mL, while at 250 μg/mL 75% and 86% *A. castellanii* and *N. fowleri* were inhibited. Assays revealed inhibition of encystation in both *A. castellanii* (58% and 93%) and *N. fowleri* (73% and 97%) at concentrations of 100 and 250 μg/mL respectively. Importantly, tyrocidine-derived peptide depicted minimal cytotoxicity to human cells and, thus, may be a potential candidate in the rational development of a treatment regimen against free-living amoebae infections. Future studies are necessary to elucidate the in vivo effects of tyrocidine-derived peptide against these and other pathogenic amoebae of importance.



■ INTRODUCTION

Free-living amoebae can cause life-threatening medical conditions. *Acanthamoeba castellanii* (*A. castellanii*) causes *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE), while *Naegleria fowleri* (*N. fowleri*) causes a fatal brain infection known as primary amoebic meningoencephalitis (PAM).[1−6] These parasites are present in the environment and exist as two or three biological stages.[7] *A. castellanii* has two forms: an infective trophozoite and difficult to treat cysts.[7−11] *N. fowleri* possess a trophozoite and cyst stage with an additional transient stage known as the flagellate form.[12−14] In the case of GAE, *A. castellanii* infects the central nervous system (CNS) and this results in a fatal brain infection with a high mortality rate (around 98%).[15] The infection causes necrotic lesions, which may lead to death within weeks to months upon the onset of clinical symptoms of the disease.[16] *Acanthamoeba* cross the blood−brain barrier (BBB) at the capillary endothelium utilizing one or more mechanisms comprising either paracellular transit or transcellular migration. Nonetheless, the exact mechanism by which *A. castellanii* invade the CNS is still not well understood.[17,18]

N. fowleri is associated with the CNS infection, PAM. This disease progresses rapidly with a mortality rate of more than 95%.[19−22] Humans are infected after contaminated water enters through the nasal route, either by partaking in recreational activities, such as swimming, or via nasal rinsing/irrigation practices and performing ablution with contaminated water.[19,23] Amoebae enter the CNS following attachment to the olfactory nerve and migrate to the olfactory bulbs (*bulbus olfactorius*) of the forebrain, invading the brain, causing widespread infections, hemorrhages, and necrosis which eventually leads to death.[24,25] Treating brain-eating

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amoebae is challenging, and the current recommended treatment consists of a combination of different drugs.[12,13,26] These drugs include broad-spectrum antifungals (amphotericin B), broad-spectrum disinfectants (chlorhexidine, propamidine), an anticancer drug (miltefosine), rifampicin, dexamethasone, fluconazole, phenytoin intravenous, and other antifungals and antibacterial drugs (sulfadiazine),[27−30] but the prognosis remains poor. The ineffectiveness (due to usage at high doses and cytotoxicity) of these drugs are major limitations, and treatment remains a "hit and miss" approach. Therefore, there is an urgent need for novel and potent antiamoebic drug(s) that can treat *N. fowleri* and *A. castellanii*.[27,31]

Tyrocidines are naturally occurring antimicrobial peptides that are a mixture of cyclic decapeptides produced by *Bacillus* spp. found in the soil.[32−34] These antibiotics have shown remarkable antimalarial, and antifungal efficacies in addition to antibacterial activity.[32,33] Furthermore, tyrocidine possesses significant antifungal properties versus plant fungal pathogens.[35] For the first time, here we assessed the antiamoebic activity of a tyrocidine-derived peptide against *A. castellanii* and *N. fowleri*, with an overall aim to develop novel chemotherapeutic therapy for these devastating and fatal infections.

■ MATERIALS AND METHODS

Peptide Synthesis. To synthesize tyrocidine-derived peptide a solid-phase peptide synthesis was conducted via fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry employing a Siro II synthesizer (Biotage, Uppsala Sweden), according to the manufacturer's protocol. Briefly, utilizing a lysing coupled quencher Dabcyl (Dbc), the peptides (FRET-labeled) were flanked at their C-termini with a fluorescent probe (FITC) and flanked at their N-termini with a lysine coupled quencher, Dabcyl. Next, purification of the peptide was performed to at least 95% purity via preparative reversed-phase high performance liquid chromatography (HPLC) with a Dionex Ultimate 3000 system (Thermo Scientific, Breda, The Netherlands). Authenticity was established via mass spectrometry utilizing Microflex LRF MALDI-TOF (Bruker Daltonik GmbH, Bremen, Germany) as communicated earlier.[36]

Protease Assay. Proteolytic assay was accomplished in 96-well plates (Corning, Lowell, USA), as described previously.[37,38] Briefly, 49 μL of *A. castellanii* culture supernatant was incubated with 1 μL of each peptide (800 μM) of the FRET-peptide substrate library or tyrocidine-derived peptide substrate (800 μM) at 37 °C. The final concentration of peptide substrates was 16 μM. The fluorescence was recorded for 60 min at 2 min intervals utilizing a fluorescence microplate reader (FLUOstar Galaxy, BMG Laboratories, Offenburg, Germany) comprising an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The increase in fluorescence is a measure for proteolytic activity.

Parasite Cultures. *Acanthamoeba castellanii* of the T4 genotype (clinical isolate) was acquired from the American Type Culture Collection (ATCC 50492) and cultivated in a 75 cm^3 tissue culture flask in media containing 0.75% protease peptone (Merck; CAS Reg. No. 91079-38-8), 1.5% glucose (Merck 1.08337.1000), and 0.75% yeast extract (Sigma-Aldrich 70161-500G) (PYG media). The flasks were kept at 30 °C as described earlier.[9] Amoebae adherent to the flasks represent the trophozoite stage. For the collection of amoebic culture supernatant, samples were taken after 8 h growth. Next, cultures were centrifuged and the supernatant was filter sterilized using a 0.22 μm filter (Corning PES membrane sterile filters, 28 mm). Adherent amoebae were detached by leaving the flask on ice for 10 min followed by moderate tapping. The detached cells were centrifuged for 5 min at 2500*g* and resuspended in Roswell Park Memorial Institute 1640 (RPMI-1640) for experiments. In addition, a clinical isolate of *N. fowleri* was obtained from ATCC (ATCC 30174), originally sourced from a primary amoebic meningoencephalitis patient, was used in the study. *N. fowleri* was cultured on HeLa cells as a feeder layer in 75 cm^3 tissue culture flasks kept at 37 °C, in a 5% CO_2 as stated earlier.[20] For experiments, 5×10^5 *A. castellanii* and *N. fowleri* amoebae were utilized for various assays, and the amoebae inoculum was ascertained using a hemocytometer, prior to various assays.

Amoebicidal Assays. Amoebicidal assays were performed by challenging 5×10^5 *A. castellanii* and *N. fowleri* trophozoites with two different concentrations of tyrocidine-related peptide (100 and 250 μg/mL) in 0.5 mL of RPMI-1640 in 24-well plates. Subsequently, plates were kept at 30 °C for 120 min and 24 h as previously described.[20,39] For negative controls, parasites were incubated in the absence of peptide. As positive controls, parasites were treated with 25 μM chlorhexidine and 25 μM amphotericin B (antiamoebic drugs). The peptide was dissolved in a deionized sterile water which was used as additional solvent control (data not shown). Following this incubation, trypan blue dye (0.5% final concentration) was incorporated and plates were incubated for 15 min. The trypan blue exclusion assay allows enumeration of dead cells (stained dark blue) with the use of a hemocytometer, and viable parasites are deduced accordingly. In some of the experiments, the peptide was tested at different concentrations (25, 50, 100, and 250 μg/mL) to determine 50% inhibitory concentration (IC_{50}) values.

Cultivation of Henrietta Lacks (HeLa) Cells. Human cervical adenocarcinoma (HeLa) cells (ATCC CCL-2) were cultivated in RPMI-1640 medium (Sigma-Aldrich), comprising 10% fetal bovine serum (PAN Biotech), 1% L-glutamine, 1% antibiotics (penicillin−streptomycin) (Gibco), and 1% minimum essential medium amino acids (Gibco) and kept at 37 °C in 5% CO_2 in a humidified incubator.

Adhesion Assays. To ascertain the effect of tyrocidine-derived peptide on adhesion of *A. castellanii* and *N. fowleri* to human cells, adhesion assays were performed as reported earlier.[40] Briefly, 5×10^5 *A. castellanii* and *N. fowleri* trophozoites were treated with 100 or 250 μg/mL peptide at 30 and 37 °C, respectively, for 2 h in RPMI-1640 medium. Untreated amoebae in RPMI was considered as negative control, while chlorhexidine and amphotericin B treated amoebae were taken as positive control, respectively. Next, the total assay volume was adjusted (500 μL) containing parasites, plus the peptide was added to confluent HeLa cells monolayer grown in 24-well plates. The plates were incubated for 60 min at 95% humidity and 5% CO_2 at 37 °C. Following this incubation, unbound amoebae present in the supernatant were enumerated using a hemocytometer and percent bound amoebae was determined using the following calculation:

$$100\%\text{amoebae added} - \text{unbound amoeba trophozoites} = \%\text{bound amoebae}$$

Encystation Assays. Encystation assays were conducted as described previously.[41] Briefly, to determine the effect of tyrocidine-derived peptide on encystation, 5×10^5 *A. castellanii* and *N. fowleri* trophozoites were incubated with 100 or 250

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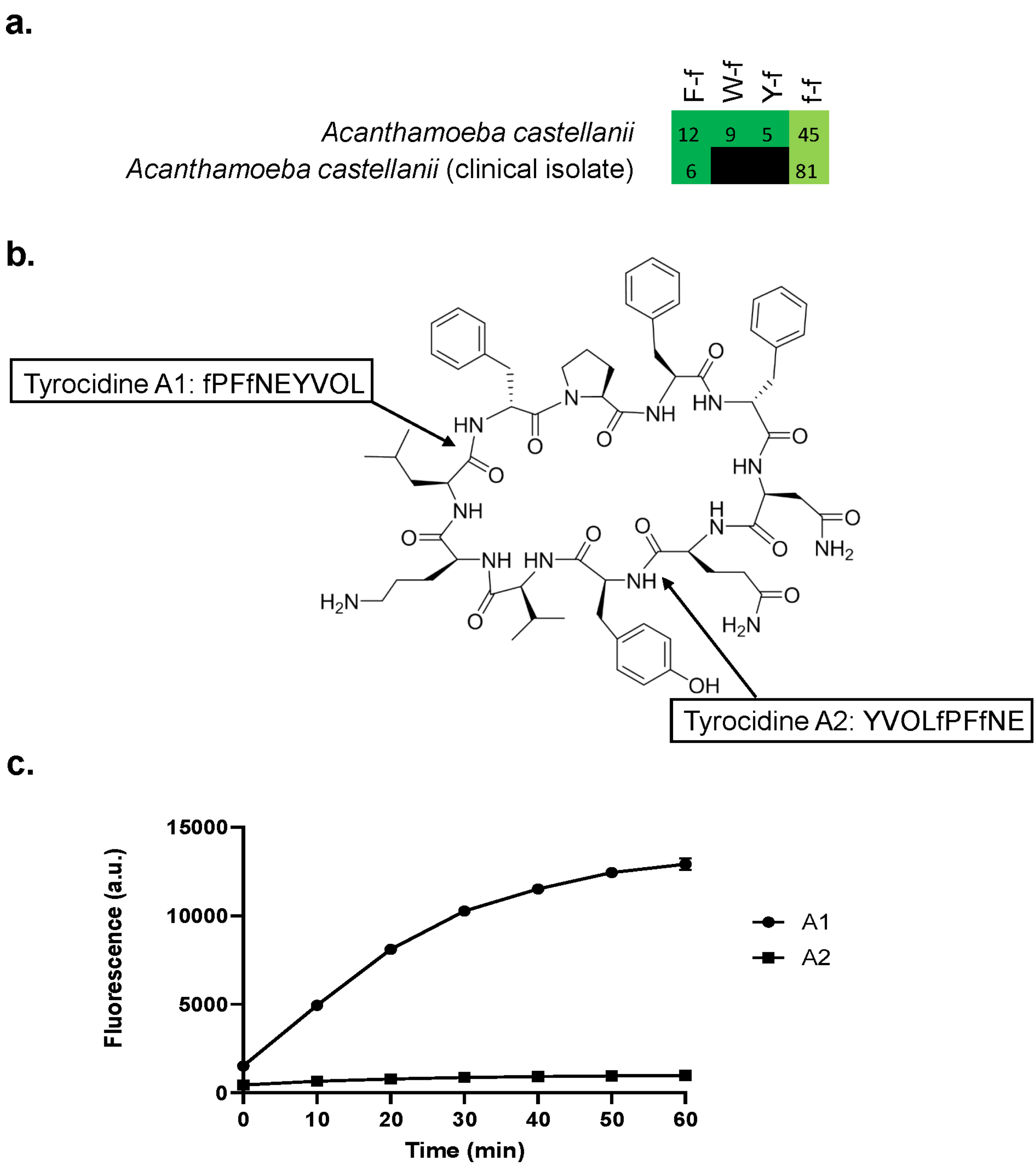

Figure 1. Design and validation of the tyrocidine-derived peptide. (a) The FRET-peptide library was screened with *A. castellanii* culture supernatant. Key: black = lack of activity (*F*/min, <5); dark green = low activity (*F*/min, 5−25), and light green = moderate activity (*F*/min, 25−125). (b) Design of the tyrocidine-derived linear peptides based on the cyclic structure of the antibiotic compound of tyrocidine A. The arrows depict where the cyclic structure was opened. The names and sequences of the derived peptides are denoted within the box. L-amino acids are denoted as upper-case letters, D-amino acids as lower-case letters. (c) Proteolytic interaction between the tyrocidine-peptides and *A. castellanii.* Culture supernatant was treated with 16 μM FRET-peptide substrate. Fluorescence was measured for 1 h at 37 °C. Data are depicted as the mean ± standard error of three independent experiments.

μg/mL peptide in a 24-well plate containing 16% glucose (*A. castellanii*) and buffer containing 5 mM KCl, 95 mM NaCl, 0.4 mM $CaCl_2$, 8 mM $MgSO_4$, 20 mM Tris-HCl, and 1 mM $NaHCO_3$ (*N. fowleri*)[42] and plates were incubated for 72 h. After 72 h incubation, sodium dodecyl sulfate (0.15% final concentration) was added under agitation for 15−20 min. Finally, the remaining amoebae were counted using a hemocytometer. *A. castellanii* alone in 16% glucose and *N. fowleri* in the above-mentioned buffer were taken as negative control, whereas for positive control, chlorhexidine and amphotericin B were used.

Cell Cytotoxicity Assays. To determine the effect of tyrocidine-derived peptide on human cells 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT assay was employed.[43] Briefly, HeLa cells were grown in 96-well plates up to 80−90% confluency at 37 °C for 24 h in the presence of 5% CO_2 and 95% humidity. Next, the peptide was placed on HeLa cells at 100 and 250 μg/mL concentrations for 24 h and

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kept at 37 °C with 5% CO_2 and 95% humidity. The MTT solution was prepared by dissolving MTT powder (Sigma-Aldrich) in phosphate buffered saline at 5 mg/mL concentration. Following this incubation, 10 μL of freshly prepared MTT dye solution was incorporated to each well and incubated for 3−4 h at 37 °C. A 100 μL aliquot of dimethyl sulfoxide (DMSO) was added to dissolve formazan crystals formed by living cells. As negative control, the cell monolayers were treated with DMSO alone, and absorbance was measured at 540 nm. The percentage of viable cells was elucidated using the following calculation;

$$\%\ \text{cell cytotoxicity} = \frac{\text{mean OD of the test sample}}{\text{mean OD of the negative control}} \times 100$$

Cytopathogenicity Assays. To determine whether tyrocidine-derived peptide affects amoebae-mediated cytotoxic effects on human cells, cytopathogenicity assays were performed as described earlier.[44] *A. castellanii* and *N. fowleri* trophozoites were treated with 100 and 250 μg/mL peptide at 30 °C for 2 h. Pretreated amoebae were pelleted via centrifugation at 3000*g* for 5 min. Next, amoebae cell pellets were resuspended in RPMI-1640 and added to a confluent HeLa cell monolayer. Subsequently, the plates were incubated at 37 °C in the presence of 5% CO_2 and 95% humidity for 24 h. The amoebae-mediated cell death was estimated indirectly by measuring the liberated lactate dehydrogenase (LDH) enzyme into cell media.[20,45,46]

Statistical analysis. All statistical comparison was performed using the *t* test to highlight the effect of peptide on amoebae. The data are expressed as the mean ± standard error of three independent experiments performed in duplicate. Graph Pad Prism version 8.0.2 was used for all of the analyses and visualizations. The threshold for statistical significance was fixed at $p \leq 0.05$.

■ RESULTS

Design of Tyrocidine-Derived Peptide. Screening our FRET-peptide library[47] with *A. castellanii* culture supernatant revealed the discovery of a D-phenylalanine recognizing protease (Figure 1a). In nature, D-phenylalanines are rare but present in natural antibiotics produced by soil bacteria.[48] Groups of antibiotics containing D-phenylalanine in their structure are the tyrocidines, produced by the bacterium *Bacillus brevis*. The observation of D-phenylalanine recognizing proteolytic activity in *A. castellanii* culture supernatant led us to hypothesize that there is potential interference between *A. castellanii* and the cyclic peptide antibiotic tyrocidine. To study this hypothesis, two linear peptides were designed based on the structure of tyrocidine A (Figure 1b). Therefore, the cyclic antibiotic was "cut open" at two different positions, which led to two different linear peptides with the same physicochemical properties as tyrocidine A. The interaction between *A. castellanii* and the tyrocidine-derived peptides was studied using FRET-labeled analogs of the peptides. It was found that peptide A1 was degraded with high efficiency (Figure 1c). No degradation of peptide A2 could be observed (Figure 1c).

Tyrocidine-Derived Peptide Exhibits Amoebicidal Effects against *A. castellanii* and *N. fowleri*. To determine the antiamoebic effects of tyrocidine-derived peptide against *A. castellanii* and *N. fowleri*, amoebicidal assays were employed. Results revealed that upon 2 h of treatment, tyrocidine-derived peptide showed noteworthy amoebicidal effects. Incubation of 100 μg/mL peptide inhibited the number of viable amoebae to only 14% for *A. castellanii* and 29% for *N. fowleri*. At 250 μg/mL the percent amoebicidal effect was significantly augmented and the peptide reduced amoebae viability up to 21% and 13% against *A. castellanii* and *N. fowleri*, respectively ($P < 0.05$) (Figure 2a). Additionally, the amoebae were challenged with

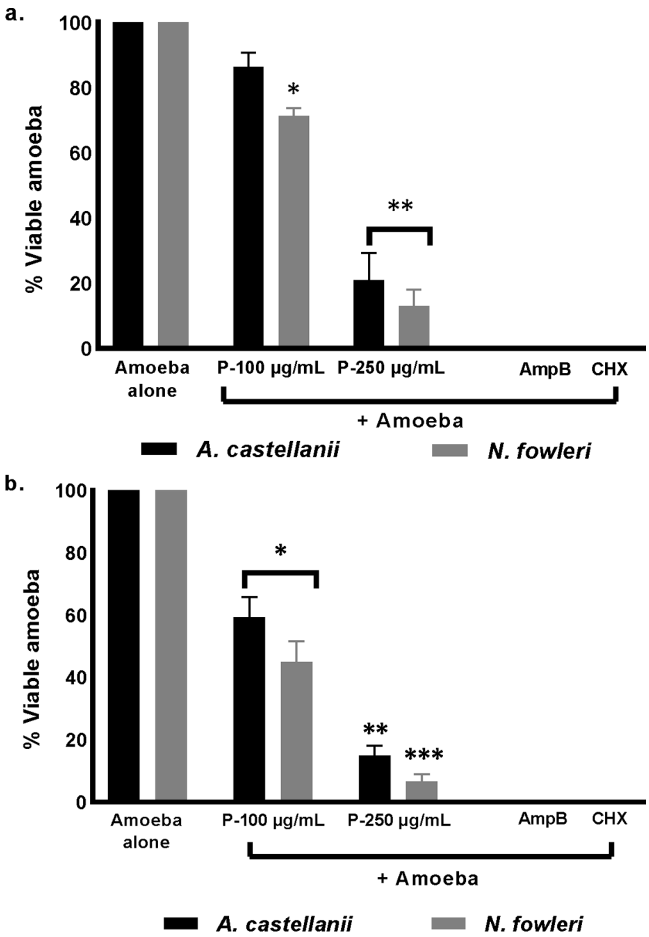

Figure 2. Tyrocidine-derived peptide exhibiting significant amoebicidal activities against *A. castellanii* and *N. fowleri*. (a) Amoebae were treated with the peptide for 2 h and (b) challenged with peptide for 24 h. The data are representative of three independent experiments and presented as the mean ± standard error. AmpB and chlorhexidine were used as positive control, while for negative control amoebae were cultured in RMPI alone. *P*-values were established utilizing the two-sample *t* test, two-tailed distribution; (*) is $P \leq 0.05$, (**) is $P \leq 0.01$, and (***) is $P \leq 0.001$.

the peptide at both concentrations for 24 h and these results showed that peptide exhibited significant antiamoebic activity ($P < 0.05$) (Figure 2b). The overnight incubation of peptide with amoebae resulted in further reduction in the viability of amoebae. Approximately, at 100 μg/mL, 41% reduction was observed for *A. castellanii* and a reduction of 55% was observed in the case of *N. fowleri*, and an even more drastic inhibition was observed at 250 μg/mL. At this concentration, the peptide had 86% and 94% amoebicidal activities against both *A. castellanii* and *N. fowleri* ($P < 0.05$) (Figure 2b). Overall, tyrocidine-derived peptide presented remarkable antiamoebic effects against viability of *A. castellanii* and *N. fowleri* trophozoites. Results from IC_{50} indicated that tyrocidine-

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derived peptide exhibited IC_{50} against *A. castellanii* at 111.8 μg/mL while 81.84 μg/mL against *N. fowleri*.

Tyrocidine-Derived Peptide Repressed Amoebae Binding to Human Cells. Adhesion assays were carried out to study the effects of tyrocidine-derived peptide on binding capability of both amoebae to HeLa cell lines. The results revealed that the peptide prevented binding of the amoebae to HeLa cells (Figure 3). At 100 μg/mL, binding of *A. castellanii* amoebae to human cells was inhibited up to 35%. Adherence of *N. fowleri* amoebae to human cells was inhibited up to 53% and 47% amoebae being bound to HeLa cells ($P < 0.05$). When the amount of peptide was increased to 250 μg/mL, 84% and 94% inhibition was found for *A. castellanii* and *N. fowleri*, respectively ($P < 0.05$) (Figure 3).

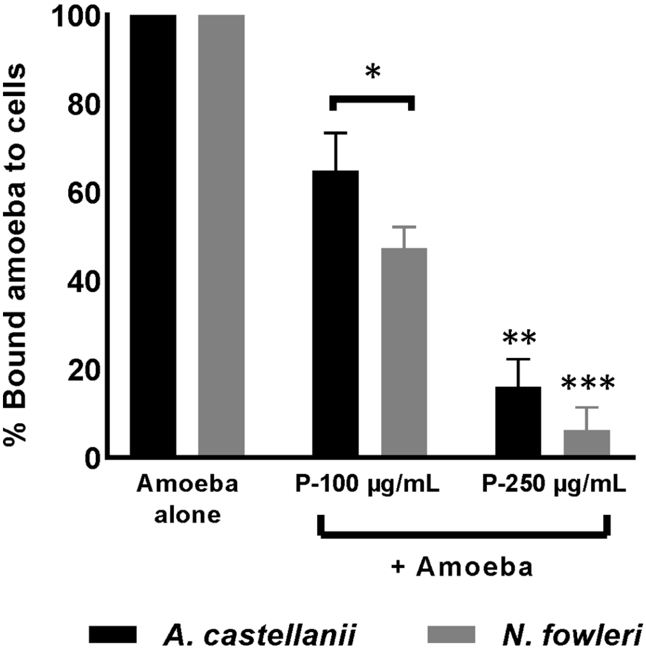

Figure 3. Tyrocidine-derived peptide inhibiting binding of *A. castellanii* and *N. fowleri* to human cells. In brief, *A. castellanii* and *N. fowleri* (5×10^5 amoebae) were pretreated with peptide for 2 h at 30 °C followed by a binding assay to HeLa cells. The data are representative of three independent experiments and presented as the mean ± standard error. *P*-values were established via the two-sample *t* test, two-tailed distribution; (*) is $P \leq 0.05$, (**) is $P \leq 0.01$, and (***) is $P \leq 0.001$.

Tyrocidine-Derived Peptide Inhibited Encystation of Amoebae. Encystation assays revealed that tyrocidine-derived peptide prevented the transformation of trophozoites into cysts. These results are comparable to the results of the amoebicidal assays (Figure 4). As a negative control, amoebae trophozoites were incubated in encystation media alone and, following the formation of cysts (approximately after incubation 72 h), were considered as 100%. The results obtained from the peptide are presented as relative to the control. At 100 μg/mL, only 42% *A. castellanii* and 27% *N. fowleri* cysts were detected ($P < 0.05$) (Figure 4). When the amount of peptide was increased to 250 μg/mL, only 6% *A. castellanii* and 3% *N. fowleri* cysts were found.

Tyrocidine-Derived Peptide Had Minimal Cytotoxic Effects on Human Cell Lines and Inhibited Amoebae-Mediated Host Cell Death. MTT assays were accomplished to evaluate the cytotoxicity of the peptide on HeLa cells. The

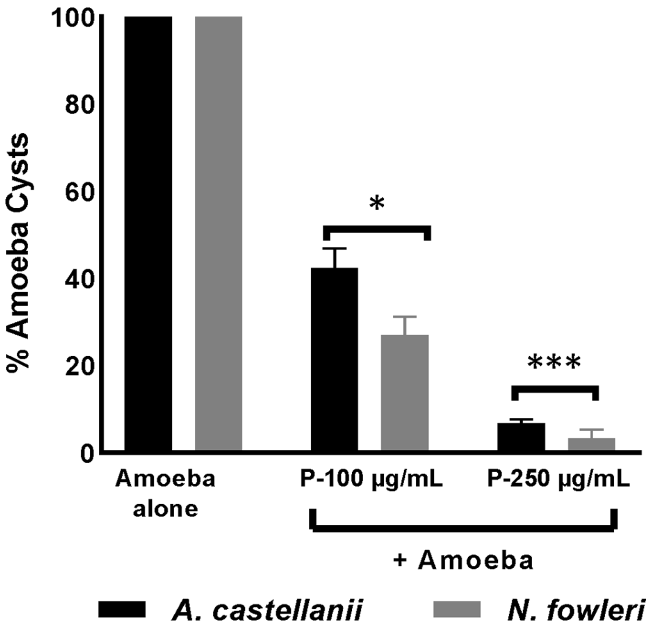

Figure 4. Effect of the tyrocidine-derived peptide on amoebae trophozoites encystation. The results revealed that peptide significantly inhibited the encystation process as compared to the negative control. The data are representative of three independent experiments and presented as the mean ± standard error. *P*-values were ascertained via two-sample *t* test, two-tailed distribution; (*) is $P \leq 0.05$ and (***) is $P \leq 0.001$.

peptide exhibited minimal cytotoxicity against HeLa cells at all concentrations tested (Figure 5). The peptide revealed only 7% and 17% cytotoxic effects at 100 and 250 μg/mL concentrations (Figure 5). Cell cytotoxicity less than 20% is non-cytotoxic, 20−40% is weak, 40−60% is moderate, and greater than 60% is potently cytotoxic.[49] In order to investigate the effect of tyrocidine-derived peptide on amoebae-cytopathogenicity, HeLa cells were challenged with *A. castellanii* and *N. fowleri* trophozoites pretreated with the

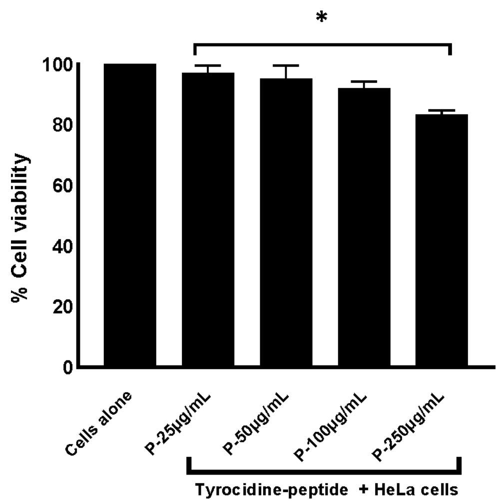

Figure 5. Tyrocidine-derived peptide showing minimal cytotoxicity. A confluent monolayer of human cells (HeLa) were challenged with varying amounts of tyrocidine-derived peptide (25, 50, 100, and 250 μg/mL). At all tested concentrations, the peptide showed limited cytotoxicity toward human cell lines. Data are depicted as mean ± standard error. Experiments were accomplished in duplicates.

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peptide. At 100 μg/mL, the pretreated amoebae showed moderate to potent cytotoxicity (*A. castellanii* 74% and *N. fowleri* 65%) (Figure 6). When the amoebae were pretreated with 250 μg/mL peptide, no amoebae cytopathogenicity could be observed ($P < 0.05$) (Figure 6).

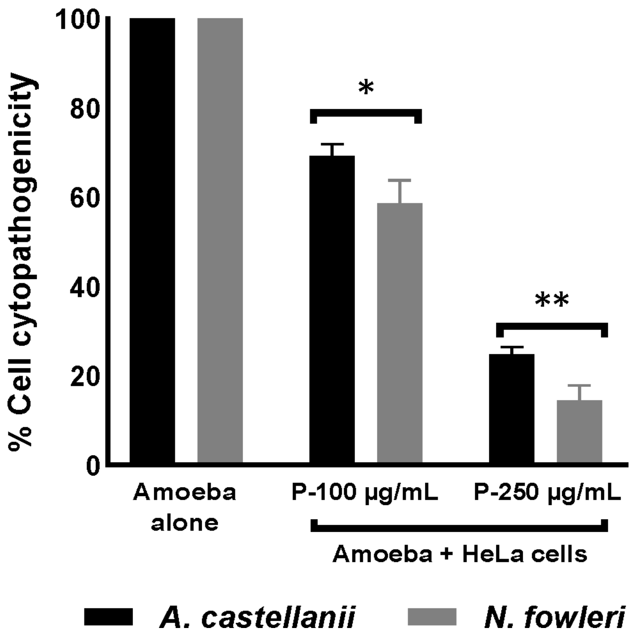

Figure 6. Tyrocidine-derived peptide inhibiting amoebae-mediated cytotoxicity against human cells. Briefly, 5×10^5 amoebae were treated with 100 and 250 μg/mL peptide at 30 °C for 2 h. Pretreated amoebae were then transferred to HeLa cells and kept overnight at 5% CO_2 at 37 °C. The results revealed inhibition of amoebae-mediated host cytotoxicity when compared to amoeba alone (untreated). The data are representative of three independent experiments and presented as the mean ± standard error. *P*-values were ascertained with the sample *t* test, two-tailed distribution; (*) is $P \leq 0.05$ and (**) is $P \leq 0.01$.

DISCUSSION AND CONCLUSION

With increased urbanization and climate change, it is likely that infections due to ubiquitously distributed pathogenic amoebae will likely rise.[5] Moreover, with a mortality rate of more than 95%, innovative and novel antiamoebic drugs with potent effects, high bioavailability, and properties to cross the BBB are urgently needed to combat these fatal infections.[19,21,50] Current compounds available have damaging systemic side effects and are administered via the intravenous route; thus, large doses are required to achieve minimum inhibitory concentration at the infection sites.[51] Herein, we synthesized and evaluated the antiamoebic activity of a novel antiamoebic tyrocidine-derived peptide against *A. castellanii* and *N. fowleri*. The antiamoebic effects were determined via amoebicidal, binding, and encystation as well as cytopathogenicity assays. The results revealed that the novel tyrocidine-derived peptide displayed potent antiamoebic effects against both pathogenic amoebae *Acanthamoeba and Naegleria* without affecting host cells.

Previously, it was considered that peptides are unable to traverse through the BBB; however, later it was established that gastrointestinal peptides may communicate with the brain.[52] Recently it has become apparent that they can cross the BBB;[53] hence, the novel peptide was synthesized, as it may have the ability to cross the BBB, yet this needs to be confirmed and should be the subject of future studies. Another prior study reported that amphotericin B and clotrimazole were the most effective drugs in growth inhibition of *Naegleria*[54] and tetracycline was moderately active in inhibiting growth of *N. fowleri*. Furthermore, *N. fowleri* cultured in Nelson's medium showed greater resistance to several antimicrobial agents such as mithramycin, sulfamethoxazole, tyrocidine, and D, daunomycin.[54] The aim of the study conducted by Cline et al. was to compare the growth of *Naegleria fowleri* and *Naegleria gruberi* in different nutrient mediums, rather than evaluate the antiamoebic effects of drugs.[54]

In another study the hydrophobic peptides (amoebicins d13-A, d13-B, and d13-C) isolated from *Bacillus licheniformis* D-13 were tested against species of *Naegleria* as well as *Acanthamoeba* for their antiamoebic properties.[55] It was found that the peptides exhibited antiamoebic affects against *Naegleria* but not against *Acanthamoeba*. Furthermore, on the basis of electron micrographs, the authors concluded that lysis of amoebae was most likely via disruption of the cell membrane.[55] Nonetheless, these peptides were found to be cytotoxic to murine cells.[55] Previous studies have revealed that antimicrobial peptides may play a role in the elimination of *A. castellanii*. Antimicrobial peptides are a part of the innate immune response and represent the first line of defense of many organisms.[56,57] The gene expression of the ocular antimicrobial peptides in human corneal epithelial cells stimulated with *Acanthamoeba* were studied. In response to amoeba infection of the eye, antimicrobial peptides resulted in the upregulation of gene expression, namely, human β defensin 3 (hBD3), which depicted significant upregulation in exposed cells as well as ribonuclease-7 (RNase-7). Human β defensin 1 (hBD1) was downregulated.[58] This study signified the potential role of antimicrobial peptides in fighting amoebic infection at the ocular surface and concluded that the use of such peptides may be a viable strategy in the treatment of *Acanthamoeba* keratitis.[58]

Another study revealed that the peptides gramicidin and polymyxin B were able to successfully eliminate eye infections caused by *A. castellanii* in combination.[59] Similarly, insect-derived antimicrobial peptides such as gomesin and trialysin showed remarkable antiamoebic activity against both the trophozoites and cysts form of *A. castellanii*[60−62] as well as magainins (derived from the African clawed frog) which depicted amoebicidal and ameobistatic activities against both cyst and trophozoites of *Acanthamoeba polyphaga*.[63] Of note, our results depicted that tyrocidine-derived peptide significantly inhibited *A. castellanii* and *N. fowleri* from binding to human cell lines; this is a substantial result, as binding is a critical step that parasites use to cause infection and was not investigated in prior studies investigating the antiamoebic effects of tyrocidine.[54]

Of note, the novel tyrocidine-derived peptide inhibited the encystation process in *A. castellanii* and *N. fowleri*, respectively. The encystation process is one of the key reasons that *A. castellanii* infections are difficult to manage and treat effectively.[64] Currently, available drugs target various functional aspects of microorganisms, for example DNA and RNA synthesis, cell wall, or metabolic activities. However, the cysts of amoebae are dormant and thus are mostly unharmed by therapy. As the novel peptide from our study inhibits

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encystation, these results are exciting and the peptide may be able to inhibit the formation of the cyst stage, which is crucial as *Acanthamoeba* encyst deep within the corneal stroma and the brain tissue.[2] Nonetheless, future studies in vivo models of *Acanthamoeba* keratitis are necessitated, as well as in animal models of *Naegleria fowleri* infection, to examine the amoebicidal activity of the novel peptide further.

A recent study highlighted the potent antimicrobial activity of gramicidin A, the natural antibiotic which is produced by *Bacillus brevis* simultaneously with tyrocidine. However, its prolonged application resulted in high host cytotoxicity.[65] In the present study, tyrocidine-derived peptide presented minimal cytotoxic effects against human cell lines (HeLa cells) and pretreated amoebae were able to restrict amoeba-mediated host cell cytopathogenicity for *Acanthamoeba* and *Naegleria*. The results from our peptide are motivating, as our data are indicative of minimal cytotoxicity. However, future work on human corneal epithelial cells as well as in vivo models is warranted to further assess the cytotoxic effects. An alternative approach may be the delivery of the novel peptide utilizing nanovehicles to minimize any cytotoxic effects even further, and these can be utilized for targeted drug delivery across the BBB.[66]

In conclusion, we synthesized and examined the antiamoebic activity of a tyrocidine-derived peptide against pathogenic *A. castellanii* and *N. fowleri*. Our results revealed substantial and potent effects on amoebae viability, encystation, and the ability of amoebae to bind to the host cells. Furthermore, the novel antiamoebic tyrocidine-derived peptide revealed minimal cytotoxic effects toward human cells. As the peptide depicts potent activities against both amoebae, it is a potential candidate to include in the treatment regimen against free-living amoeba infections in general and should also be evaluated against *Balamuthia mandrillaris*, *Vermamoeba vermiformis*, and other potentially pathogenic amoebae that cause debilitating infections.[67,68] Our findings show that the tyrocidine-derived peptide is an anticipated chemotherapeutic compound against pathogenic amoebae. Future studies are needed to unravel the exact mechanism of action as well as the in vivo effects of the novel tyrocidine-derived peptide against these pathogenic amoebae, as well as perform in vivo experimentation with peptide in models for both *Acanthamoeba* keratitis and CNS infection caused by both *Acanthamoeba* and *Naegleria*.

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Author Contributions

W.E.K., F.J.B., and K.N. provided the materials. M.S. performed the pilot experimentations under the supervision of W.E.K. N.A. performed all of the experimentations under the supervision of R.S. and N.A.K. and wrote the first draft of the manuscript. R.S., W.E.K., and N.A.K. conceived the idea. R.S., N.A.K., W.E.K., F.J.B., and M.S. corrected the manuscript.

Notes

The authors declare no competing financial interest.

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