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 meningitis (PAM).\(^7\)–\(^\text{10}\) 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 cyst stage with an additional transient stage known as the flagellate form.\(^\text{11} \text{–} \text{14}\) 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%).\(^\text{15}\) The infection causes necrotic lesions, which may lead to death within weeks to months upon the onset of clinical symptoms of the disease.\(^\text{16}\) *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.\(^\text{17,18}\)

*N. fowleri* is associated with the CNS infection, PAM. This disease progresses rapidly with a mortality rate of more than 95%.\(^\text{19} \text{–} \text{22}\) 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.\(^\text{19} \text{,} \text{23}\) 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.\(^\text{24} \text{,} \text{25}\) Treating brain-eating...
amoebae is challenging, and the current recommended treatment consists of a combination of different drugs.\textsuperscript{13,19,26} These drugs include broad-spectrum antifungals (amphotericin B), broad-spectrum disinfectants (chlorhexidine, propamidine), an anticancer drug (mifliferine), rifampicin, dexmethasone, fluconazole, phenytoin intravenous, and other antifungals and antibacterial drugs (sulfadiazine),\textsuperscript{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 \(A.\ castellanii\).\textsuperscript{31}

Tyrocidines are naturally occurring antimicrobial peptides that are a mixture of cyclic decapeptides produced by \(Bacillus\) spp. found in the soil.\textsuperscript{32–34} These antibiotics have shown remarkable antimalarial, and antifungal efficacies in addition to antibacterial activity.\textsuperscript{32,33} Furthermore, tyrocidine possesses significant antifungal properties versus plant fungal pathogens.\textsuperscript{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.

\section*{MATERIALS AND METHODS}

\textbf{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.\textsuperscript{36}

\textbf{Protease Assay.} Proteolytic assay was accomplished in 96-well plates (Corning, Lowell, USA), as described previously.\textsuperscript{27,34} Briefly, 49 \(\mu\)L of \(A.\ castellanii\) culture supernatant was incubated with 1 \(\mu\)L of each peptide (800 \(\mu\)M) of the FRET-peptide substrate library or tyrocidine-derived peptide substrate (800 \(\mu\)M) at 37 °C. The final concentration of peptide substrates was 16 \(\mu\)M. 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.

\textbf{Parasite Cultures.} \(Acanthamoeba\ castellanii\) of the T4 genotype (clinical isolate) was acquired from the American Type Culture Collection (ATCC \#S0492) and cultivated in a 75 cm\(^2\) 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) (PVG media). The flasks were kept at 30 °C as described earlier.\textsuperscript{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 filtered sterilized using a 0.22 \(\mu\)m 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 2500g 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) originially 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\(^2\) tissue culture flasks kept at 37 °C in a 5% CO\(_2\) as stated earlier.\textsuperscript{30} For experiments, 5 \(\times\) \(10^5\) \(A.\ castellanii\) and \(N.\ fowleri\) amoebae were utilized for various assays, and the amoeba inoculum was ascertainment using a hemocytometer, prior to various assays.

\textbf{Amoebicidal Assays.} Amoebicidal assays were performed by challenging 5 \(\times\) \(10^5\) \(A.\ castellanii\) and \(N.\ fowleri\) trophozoites with two different concentrations of tyrocidine-related peptide (100 and 250 \(\mu\)g/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.\textsuperscript{25,39} For negative controls, parasites were incubated in the absence of peptide. As positive controls, parasites were treated with 25 \(\mu\)M chlorhexidine and 25 \(\mu\)M 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 trypsin 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 \(\mu\)g/mL) to determine 50% inhibitory concentration (IC\(_{50}\)) values.

\textbf{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.

\textbf{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.\textsuperscript{25} Briefly, 5 \(\times\) \(10^5\) \(A.\ castellanii\) and \(N.\ fowleri\) trophozoites were treated with 100 or 250 \(\mu\)g/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 \(\mu\)L) 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:

\[
\text{100\% amoebae added} - \text{unbound amoebae trophozoites} = \% \text{bound amoebae}
\]
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 CaCl₂, 8 mM MgSO₄, 20 mM Tris-HCl, and 1 mM NaHCO₃ (N. fowleri) 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-y1)-2,5-diphenyltetrazolium bromide or MTT assay was employed. 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% CO₂ and 95% humidity. Next, the peptide was placed on HeLa cells at 100 and 250 μg/mL concentrations for 24 h and

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.
kept at 37 °C with 5% CO₂ 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;

\[
\% \text{ cell cytotoxicity} = \left( \frac{\text{mean OD of the test sample}}{\text{mean OD of the negative control}} \right) \times 100
\]

**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% CO₂ 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 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 amoebicidal 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 amoeba viability up to 21% and 13% against A. castellanii and N. fowleri, respectively (\( P < 0.05 \)) (Figure 2a). Additionally, the amoebae were challenged with 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 amoebicidal effects against viability of A. castellanii and N. fowleri trophozoites. Results from IC₅₀ indicated that tyrocidine-
derived peptide exhibited IC\textsubscript{50} against \textit{A. castellanii} at 111.8 μg/mL while 81.84 μg/mL against \textit{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 \textit{A. castellanii} amoebae to human cells was inhibited up to 35%. Adherence of \textit{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 \textit{A. castellanii} and \textit{N. fowleri}, respectively (\( P < 0.05 \)) (Figure 3).

**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 were presented as relative to the control. At 100 μg/mL, only 42% \textit{A. castellanii} and 27% \textit{N. fowleri} cysts were detected (\( P < 0.05 \)) (Figure 3). When the amount of peptide was increased to 250 μg/mL, only 6% \textit{A. castellanii} and 3% \textit{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 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.\(^6\) In order to investigate the effect of tyrocidine-derived peptide on amoeba-cytotoxicity, HeLa cells were challenged with \textit{A. castellanii} and \textit{N. fowleri} trophozoites pretreated with the...
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 cytotoxicity could be observed (P < 0.05) (Figure 6).

![Graph showing cytotoxicity of peptides](image)

**Figure 6.** Tyrocidine-derived peptide inhibiting amoebae-mediated cytotoxicity against human cells. Briefly, 5 × 10^9 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% CO₂ 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 ≤ 0.05 and (**) is P ≤ 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. 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. Current compounds available have damaging systemic side effects, high bioavailability, and properties to cross the BBB are urgently needed to combat these fatal infections. Another prior study reported that amphotericin B and clotrimazole were the most effective drugs in growth inhibition of *Naegleria* 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. 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.

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. It was found that the peptides exhibited antiamoebic effects 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. Nonetheless, these peptides were found to be cytotoxic to murine cells. 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. 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. 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.

Another study revealed that the peptides gramicidin and polymyxin B were able to successfully eliminate eye infections caused by *A. castellanii* in combination. Similarly, insect-derived antimicrobial peptides such as gomesin and trislysin showed remarkable antiamoebic activity against both the trophozoites and cysts form of *A. castellanii* as well as magainins (derived from the African clawed frog) which depicted amoebicidal and amoebastatic activities against both cyst and trophozoites of *Acanthamoeba polyphaga*. 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.

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. 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...
encystation, these results are exciting and the peptide may be able to inhibit the formation of the cyst stage, which is crucial as Acanthamoeba encysts deep within the corneal stroma and the brain tissue. 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. 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.

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 amoeba 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. 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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Notes
The authors declare no competing financial interest.

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