Novel Applications of Elastin-like Polypeptides (ELPs) in the Treatment of Pathogenic Free-Living Amoeba
Avni Kalra
Novel Applications of Elastin-like Polypeptides (ELPs) in the Treatment of Pathogenic Free-Living Amoeba

Abstract

Primary amoebic meningoencephalitis (PAM) is a rare and acute yet fulminant infection caused by the amoeba Naegleria fowleri. PAM is characterized by headaches, fever, nausea, and stiff neck. Although rare, PAM is fatal, with a mortality rate of 98% and causes death within two weeks of exposure. There are several key factors involved in the high mortality rate including the ineffectiveness of common treatments such as amphotericin B, fluconazole, azithromycin, and Rifampin alongside poor penetration of the blood-brain barrier (BBB). Elastin-like polypeptides (ELPs) are biopolymeric nanoparticles that mimic the properties of natural elastin, a key component of the extracellular matrix found in connective tissue. ELPs are specifically characterized by their biocompatibility, targeted and controlled release, phase change behavior, and the ability to encapsulate multiple drugs. While ELPs have been extensively researched in the context of various diseases, their potential in treating PAM remains an unexplored area of interest. This papers therefore focuses on possible approaches in which ELPs might be leveraged to increase the efficacy of existing treatments for PAM. By imagining how ELP nanomedicines could be applied for novel therapeutic strategies against PAM, we hope to inspire future translational avenues for this rare disease to improve patient outcomes.

Primary Amoebic Meningoencephalitis

Primary amoebic meningoencephalitis, more commonly known as PAM, is a rare but fulminating amoebic infection caused by the free-living amoeba (FLA), Naegleria fowleri. N. fowleri is indigenous to freshwater environments such as freshwater habitats, still water lakes, rivers, and other aqueous bodies [1]. Although PAM is associated with numerous neurological manifestations, the avenue through which N. fowleri accesses the central nervous system (CNS) is singular: the nasal cavity. A person generally contracts N. fowleri by participating in recreational water activities, such as swimming or diving in which water inadvertently enters the nasal cavity [2].

The amoeba has a predilection for warm freshwater environments which are ideal for its growth and reproduction. The nasal cavity, to a certain degree, replicates the environment N. fowleri is found in, creating ideal conditions for the amoeba to thrive [3]. N. fowleri initiates its pathogenesis by breaching the nasal mucosa and cribriform plate. Subsequently, it utilizes the olfactory nerve bundle to make its way directly to the CNS where it corrodes brain tissue. This method of infection is highly beneficial for N. fowleri from an evolutionary standpoint due to the anatomical proximity of the olfactory nerve...
bundles to the CNS, thereby exacerbating the speed at which the infection progresses [1,4,5].

*N. fowleri* enters the nasal cavity in trophozoite form, which is the amoeba’s active infectious state as opposed to a cyst form — the dormant form of the amoeba adopted when environmental conditions are not suitable [6]. This trophozoite stage possesses food cups enabling the amoeba to destroy tissue in the CNS [7,8]. In tandem with the food cups for tissue destruction, *N. fowleri* releases cytolytic molecules which exacerbate nerve destruction [9,10]. These molecular invasions result in a constellation of neurological symptoms such as severe headaches, nausea, seizures, hallucinations and photophobia [1].

Prior to understanding treatment for alleviating symptoms of PAM, it is first imperative to understand the body’s innate immune responses to the amoebal assault. The primary line of defense against *N. fowleri* is the complement immune system — a part of the innate immune system that enhances the ability of antibodies to attack the pathogen’s cell membrane [11]. The complement system includes macrophages and neutrophils. Macrophages and neutrophils together orchestrate an immune response wherein neutrophils pinch and engulf the *N. fowleri* trophozoite, and macrophages release non-oxidative mediators such as TNF-α 6 [12,13]. In conjunction with the complement immune system, the mucosal epithelial tissues have innate responses to the pathogen. Collectively, they form a mucosal cell lining which acts as a barrier between the host’s nasal cavity and all structures located above it [14]. Additionally, the mucosal epithelial cells secrete chemical defensive compounds such as mucins, antibodies, defensins, protegrins, collectins, cathelicidins, lysozyme, histatins, and nitric oxide [15,10]. Despite this detailed and extensive immune response, the human body’s immune response often proves insufficient to halt *N. fowleri* infection and the rapid onset of PAM. Consequently, it is not surprising that PAM remains an insurmountable infection with a mortality rate of 97% [16].

**Diagnosis**

Due to its rapid progression and rarity, a timely diagnosis of PAM remains a formidable obstacle. In fact, most diagnoses of PAM occur post-mortem, and autopsies typically reveal herniation in the frontal lobe alongside loss of grey matter in that region. PAM is officially diagnosed using a lumbar puncture for cerebrospinal fluid (CSF) analysis [17]. A definitive diagnosis can be made upon an observation of motile *N. fowleri* trophozoites on centrifuged CSF. PAM can also be diagnosed using laboratory testing for *Naegleria fowleri* nucleic acid in CSF, biopsy, tissue specimens, or *N. fowleri* antigen in CSF [2].
Current Treatments

Current treatments rely upon a timely diagnosis, broad spectrum anti-fungal drugs, and therapeutic hypothermia to manage the inflammation. According to the CDC, PAM is treated with a combination of drugs that will be briefly described below:

**Amphotericin B**

Amphotericin B (AmB) is a polyene anti-fungal that also has an amoebicidal effect. It functions by binding to the ergosterols in the cell membrane. This causes pores in the cellular membrane which facilitate permeability. The porous fungal cellular membrane subsequently causes lysis and breaks down the cell structure of the amoeba [18,19]. Amphotericin B, prescribed intravenously or intrathecally, has been used in all North American cases of PAM [2]. However, a high concentration of AmB is needed to reach the minimal inhibitory concentration (MIC); the MIC is the lowest dose that will inhibit growth of the microorganism [20]. A high concentration of AmB is required to reach the MIC and kill *N. fowleri* in the CNS because AmB demonstrates poor penetration of the BBB, is insoluble in aqueous solutions, and exhibits dose-limiting side effects including renal toxicity, anemia, chills, nausea, fever, vomiting, and headaches [21,1].

**Azithromycin**

Azithromycin is a macrolide antibiotic, and it works by inhibiting bacterial protein synthesis to prevent the transit of aminoacyl-tRNA and the growing protein through the ribosome [18]. In the case of PAM, a study by Goswick and Brenner discovered the potential synergistic effects of azithromycin and amphotericin B. Although azithromycin has a MIC 123 times higher than amphotericin B, it still demonstrates greater *in vivo* effects than amphotericin B due to its unique pharmacokinetic profile, which include a long-elimination half-life and high tissue accumulation levels [22].

**Fluconazole**

Fluconazole is an anti-fungal agent, and just like Azithromycin, has shown synergistic effects with amphotericin B in the treatment of PAM. It does so by inhibiting ergosterol synthesis [18]. Fluconazole’s synergistic effects can be attributed to its
ability to enhance the bactericidal effects of neutrophils. Beyond its primary antifungal function, fluconazole appears to have immunomodulatory effects by assisting neutrophils in the immune response [23].

**Rifampin**

Rifampin is typically used to treat bacterial infections but has also been administered in a select few cases of PAM. This is because it does not reach a sufficient concentration in the CNS at standard doses. Although it reaches an adequate concentration in the CSF, which bathes the CNS, compartmental concentrations in the CNS vary [2]. Another concern with Rifampin is that it induces certain liver enzymes. These liver enzymes hinder the pharmacokinetics of other drugs during combination therapy. As an example, rifampin increases fluconazole’s clearance rate and ultimately reduces the half-life of fluconazole [24].

**Miltefosine**

Miltefosine is an anti-neoplastic agent employed primarily for the treatment of breast cancer and leishmaniasis [18]. Its uses have also been explored for FLA. By 2013, the CDC reported the use of miltefosine for 26 cases of PAM [25]. It is categorized as a broad-spectrum phospholipid antimicrobial agent related to the signaling molecule lysophosphatidylcholine. Additionally, the phospholipid in miltefosine possesses an attached alkyl phosphocholine. The molecule is amphiphilic, having a polar phosphocholine head region and an aliphatic tail. It exists in a zwitterionic form with a permanently charged quaternary ammonium ion and anionic phosphate [1]. This is relevant because lysophosphatidylcholine’s permanently charged nature is responsible for its poor CNS penetration and results in a high MIC [26,1].

**Granulomatous Amoebic Encephalitis**

Granulomatous amoebic encephalitis, better known as GAE, is an opportunistic infection with a staggering mortality rate of 97%-98%. Unlike PAM, GAE is caused by three different pathogens: *Acanthamoeba* spp, *Balamuthia mandrillaris*, and *Sappinia pedata*. GAE primarily affects immunocompromised individuals [27,28]. It is microscopically detected through a lung, sinus, brain tissue, or skin biopsy conducted postmortem. Diagnosing GAE can be challenging due to its rarity and nonspecific symptoms, which include headaches, fever, nausea, vomiting, and neurological deficits [29]. A definitive diagnosis involves cerebrospinal fluid analysis or neuroimaging studies such as MRI or CT scans. The optimal treatment for GAE involves polymicrobial microbial therapy coupled with the resection of lesions to better control the infection [30].
Acanthamoeba spp.

Being the primary etiological factor contributing to GAE, Acanthamoeba spp. are one of the most prevalent environmental protozoa because they are found in sea water, tap water, swimming pools, natural thermal water, soil, and dust [31]. Acanthamoeba are both free living and parasitic. Like N. fowleri, they can appear in both the actively feeding and dividing trophozoite stage or the dormant cyst form [32]. Acanthamoeba's amoeboid locomotion can be attributed to acanthopodia, which are spiny surface structures, alongside the formation of hyaline pseudopodia. They possess a central nucleus along with a nucleolus and a single pulsating vacuole [33].

There are direct and indirect factors contributing to the pathogenicity of Acanthamoeba; direct factors include phagocytosis and the ability to produce pre-forming toxins like acanthaporin which exhibit cytotoxicity for human neuronal cells and contribute to neural tissue damage [33,34]. Indirect factors, by contrast, don't directly contribute to virulence but can nevertheless affect pathogenicity. Some indirect factors include chemotaxis, the ability to interact with and/or form biofilms, the propensity to engage in encystation, and possible interactions with bacterial endosymbionts contributing to Acanthamoeba's pathogenesis [35].

Acanthamoeba exhibit multiple avenues of entry, the most prominent of which targets the nasal cavity. Infection occurs following inhalation of air or aspiration of water containing the trophozoite form of the amoeba [36,37]. The trophozoite subsequently makes its way to the CNS through the nasal mucosa and endothelium of the brain's capillaries [38]. Secondarily, trophozoites can also gain entry through ulcerated skin or oral mucosa, and once in the body, they enter the bloodstream and disseminate to different areas such as the [39].

Balamuthia Mandrillaris

B. mandrillaris is also considered free living and exhibits both cyst and trophozoite stages, allowing it to divide through binary fission [40]. Its ecological niche is not widely known; however, it is closely related to Acanthamoeba based on RNA sequencing and phylogenetic analysis [41, 42, 43]. B. mandrillaris was originally thought to infect the brain using the olfactory nerve bundles like N. fowleri. Histopathologic findings, though, do not show olfactory lobe involvement in the manner seen with N. fowleri [43]. Instead, B. mandrillaris enters the body through breaks in the skin or the respiratory tract by the inhalation of cysts. It primarily attacks two body systems: the brain, specifically the CNS, and the skin [44].
**Sappinia Pedata**

*S. pedata* is another FLA responsible for causing GAE. Similar to *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris*, *S. pedata* exists in both cyst and trophozoite forms [45]. Its mechanism of infection is unknown. However, based on *Acanthamoeba* and *B. mandrillaris*’s mechanisms of infection, it is thought to be through the nasopharynx or may be introduced into the bloodstream [46]. One person so far has been reported to have contracted GAE due to *S. pedata*. Due to this, generalized symptoms are unknown. However, this patient had a previous sinus infection and subsequently experienced nausea, vomiting, photophobia, blurry vision, and a loss of consciousness [47, 48]. The patient was subsequently treated with azithromycin, pentamidine, itraconazole, and flucytosine [49, 50].

**Treatment**

Taravoud, Fechtali-Moute, *et al* discussed the efficacy of drugs used in the treatment of GAE based on both *in vitro* and *in vivo* studies. Two Cotrimoxazoles were used. Trimethoprim and Sulfamethoxazole specifically. Cotrimoxazole was the most frequently used drug for GAE treatment. It demonstrated a relatively modest in vivo efficiency, only 47% of patients treated with Cotrimoxazole survived. *In vitro* studies demonstrated that Cotrimoxazole did not exhibit amoebicidal activity below 100 milligrams/ml [51].

Amphotericin B is another frequently used drug for the treatment of GAE. However, *in vitro* studies demonstrate a low efficacy and show a natural resilience of *Acanthamoeba* spp. to amphotericin B. Only 23% of patients treated with amphotericin B survived [52].

Rifampicin is frequently used in GAE treatment, but it does not show any anti-acanthamoebal activity *in vitro*. Nevertheless, it has lipophilic properties which allow it to penetrate the blood-brain barrier and contributes to its efficacy *in vivo* [53].

Among theazole-based drugs (e.g., fluconazole, ketoconazole, voriconazole, itraconazole), ketoconazole was the most efficientazole with an EDU of 46%. The most effective combination GAE treatment aligning with the recommendations from the Infectious Disease Society of America (IDSA) involves co-administration of rifampicin, cotrimoxazole, and ketoconazole. It is recognized and successful in 83% of cases [51].
A similar treatment has been used for GAE and may vary due to the pathogen causing it. For example, in a survival case of GAE caused by Balamuthia Mandrillaris, the patient was treated with sulfamethoxazole, azithromycin, flucytosine, and amphotericin B [54]. In another surviving case of GAE caused by Balamuthia Mandrillaris, a non-conventional treatment of nitroxoline was used. Nitroxoline is primarily used to treat Urinary Tract Infections (UTI). Nitroxoline had demonstrated amoebicidal activity against B. mandrillaris in vitro. This patient’s combination treatment involved nitroxoline, miltefosine, azithromycin, albendazole, fluconazole, and dose reduced flucytosine [55].

Nanoparticles as a Novel Therapeutic Strategy

Nanoparticles typically range in size from one to 100 nanometers (nm) and have garnered significant attention for their versatile applications in the diagnosis, treatment, and prevention of various diseases [56]. Their small size and large surface area to volume ratio particularly enable efficient drug delivery by: a) encapsulating therapeutic agents such as drugs; b) protecting the therapeutic payload from degradation; and c) enhancing their bioavailability [57,58].

Nanoparticles can also be designed to exhibit specific behaviors including controlled release and active targeting of specific tissues and/or cells [59]. This essentially optimizes their therapeutic efficacy across various diseases such as cancer, infectious diseases, and neurological disorders [60,61].

Nanoparticles can also be effectively utilized within the context of combating FLA the conjugation of nanoparticles with drugs specifically holds significant promise for treating diseases caused by FLA by more effectively addressing the challenges seen with more conventional treatment methods [62,63]. By leveraging the unique properties of nanoparticles including their customizable surface drug conjugations, ability to improve drug solubility, enhanced stability, and potential for targeted delivery, nanoparticles can overcome current limitations associated with standard-of-care treatments [64, 65]. The following is a survey of the literature highlighting various nanoparticle-based approaches to treating amoebal infections:

Metronidazole conjugated magnetic nanoparticles loaded with amphotericin B

A study by Abdelnasir, S., Anwar, A, et.al introduces metronidazole-modified iron oxide nanoparticles loaded with amphotericin B as a therapeutic avenue for combating
infections due to FLA. It focuses specifically on A. Castellani, a potential cause of GAE.

The use of metronidazole conjugated magnetic nanoparticles provides multifaceted advantages. These nanoparticles demonstrate excellent drug entrapment efficiency, and they ensured the effective delivery of both metronidazole and amphotericin B. Moreover, their biocompatibility and minimal hemolytic activity render them suitable drug carrier candidates. The in vitro experiments demonstrated potent synergistic effects alongside dose-dependent amoebicidal and cytoidal activities against both A. Castellani trophozoites and cysts.

These drug-laden nanoparticles outperformed the control groups (individual drugs alone and nanoparticles alone). Additionally, the incorporation of iron oxide nanoparticles introduced magnetic properties, effectively enabling enhanced imaging sensitivity via MRI or magnetic resonance imaging for multifunctional theragnostic applications [66].

**Gold Nanoparticles**

A study by Mungroo, et. all focuses on the therapeutic potential of curcumin, a bioactive small molecule compound derived from turmeric. Known for its diverse biological properties, curcumin was specifically explored for its amoebicidal effects. Curcumin was conjugated with gold nanoparticles as a treatment strategy against infections caused by two FLA B. mandrillaris and N. fowleri.

The synthesis of curcumin and gold nanoparticles was confirmed through dynamic light scattering (DLS), a technique used to measure the size distribution of particles in a solution. It indicated an average particle size of 53 nanometers. Curcumin exhibited substantial activity against both the amoeba with concentration-dependent effects showcasing an AC50 of 172 μM for B. mandrillaris and 74 μM for N. fowleri. An AC50 of 172 μM and 74 μM for B. mandrillaris and N. fowleri respectively represents the concentrations at which the inhibitory effect of amoebic growth is at 50% of its maximum. Once curcumin was conjugated with gold nanoparticles to enhance its amoebicidal activity, the resulting nanoparticle showed a remarkable increase of up to 78 in amoebicidal activity against B. mandrillaris and 69% against N. fowleri.

The observed enhancement in amoebicidal activity post-conjugation was attributed to the biological activity of gold nanoparticles as they have been reported to induce reactive oxygen species (ROS) formation; this, in turn, leads to apoptosis and influences many cellular processes. The intricate interplay between curcumin and gold nanoparticles therefore holds promise for improving the treatment of infections caused by these amoebae [67].
Elastin-like polypeptide (ELP) nanoparticles

Elastin-like polypeptide (ELP) nanoparticles are biopolymers derived from the structural protein elastin. Elastin is a polymeric extracellular matrix protein integral to the complex macromolecular network which provides a structural framework outside of cells within tissues and organs alongside contributing to the stretchable nature of vertebrate tissues [69]. ELP nanoparticles are inspired by tropoelastin, the soluble precursor of elastin that contains hydrophobic motifs; a hydrophobic motif is a specific pattern of amino acids (e.g., valine, isoleucine) within a protein that exhibits hydrophobic properties [70]. Specifically, the hydrophobic motifs in ELP nanoparticles are (Valine-Proline-Glycine-Xaa-Glycine)_n, where Xaa is any amino acid and n specifies the number of times this pentapeptide motif repeats in the polymer chain [71]. ELPs uniquely exhibit temperature-sensitive behavior by transitioning from a soluble state below their transition temperature (T_t) into a cloudy coacervate comprised of insoluble microparticles beyond their T_t; unlike most polymers, however, this process is reversible, which permits novel applications as a ‘smart’ biomaterial within the drug delivery field [72,73].

1) Passive Targeting

The passive targeting capability of ELP nanoparticles could potentially prove invaluable in the treatment of PAM and GAE. Considering the predilection of these diseases for specific anatomical sites such as the CNS, ELPs can be engineered to carry drugs (e.g, amphotericin B, miltefosine) targeting both diseases.

Specifically, ELP nanoparticles loaded with amphotericin B might exploit the thermoresponsive behavior of ELPs to accumulate within the CNS, where GAE and PAM manifest. This passive delivery approach would nevertheless ensure that a high concentration of amphotericin B or miltefosine reaches the infection site, thereby enhancing therapeutic efficacy against the amoeba. In other words, this method could lower the minimum inhibitory concentrations required and therefore remove the obstacle of dose-limiting toxicities during administration of these drugs.

2) Controlled Release

ELP nanoparticles potentially open the possibility of leveraging sophisticated controlled release strategies suitable for novel treatments targeting both PAM and GAE. In the case of GAE, where prolonged therapeutic intervention is required, ELPs encapsulating miltefosine with thin cholesterol layers or hydrogels present a unique method for
sustained drug release. This approach would ensure that encapsulated miltefosine is released gradually over time to maintain a consistent therapeutic concentration within the affected tissues, thereby improving efficacy against the amoebic pathogens causing GAE relative to existing treatment options.

3) Active targeting

Active targeting using ELP nanoparticles might prove pivotal in addressing the challenges of treating both GAE and PAM. By fusing an ELP with cell penetrating peptides (CPPs), for instance, the drug delivery system gains the ability to efficiently traverse the blood brain barrier (BBB). For example, ELPs loaded with amphotericin B, when modified with cell penetrating peptides, can effectively cross the blood brain barrier thus ensuring that the drug reaches the CNS.

4) Multifunctional approaches

The multifunctional attributes of ELP nanoparticles, particularly in micellar structures with coronal modifications, could play a critical role in optimizing treatments against PAM and GAE. For instance, designing micellar ELPs capable of encapsulating miltefosine within a core while cell penetrating peptides are displayed on the corona could theoretically present a novel means of simultaneously achieving passive and active targeting to ensure that the drug is delivered directly to the site of amoebal infection. The synergy of these elements within a putative ELP nanoparticle design would contribute significantly to potentiating a multifaceted approach required for the effective treatment of both diseases.

Possible ELP nanoparticle design

ELPs have several tailorable properties such as coacervate formation at a transition temperature ($T_t$), an amphiphilic nature, and — most importantly — the ability to provide surface modifications (e.g., ligands, peptides, or other targeting molecules) that can be utilized for the treatment of FLA and their respective indications.

An ELP nanoparticle, for example, can be tailored to enhance its stability and efficacy. In the case of PAM, *N. fowleri* has a G-protein coupled receptor (GPCR) on its amoebal surface [74]. Specialized ligands targeting the GPCR on *N. fowleri* can be covalently bonded to ELP nanoparticles to enhance the ELP nanoparticles’ targeted delivery.

Several methods can be used to develop or find ligands specific to *N. fowleri* GPCRs. Molecular docking work involves evaluating factors such as binding affinities, hydrophobicity, and hydrogen bonding; pharmacophore modeling, by contrast, works by identifying common structural features and chemical properties among ligands with
similar pharmacophoric elements.

Additionally, a quantity structure-activity relationship (QSAR) analysis might be leveraged to identify potential ligands by correlating the chemical structure of a ligand under investigation with its biological activity. Bioinformatics and homology modeling can also be employed to identify potential ligands as well. Bioinformatics analysis, for example, could be performed on the known sequence of the GPCR while homology modeling would subsequently be used to predict that GPCR’s three-dimensional structure; this would be helpful in isolating the specific structures targeted by any potential ligands identified.

Lastly, high-throughput screening (HTS) — involving the screening of hundreds to thousands of compounds — would be employed to identify those exhibiting a strong affinity for the GPCR being targeted.

Apart from specialized targeting ligands, there are several surface modifications that can be made to enhance the properties of the ELP nanoparticle including the addition of cationic polymers covalently bonded to the ELP nanoparticle. Cationic polymers might potentially enhance the affinity of the ELP nanoparticles when targeting negatively charged amoebal cell surfaces based on physicochemical principles; such improved cellular adhesion and uptake might possibly enhance therapeutic efficiency.

**Conclusion**

ELP nanoparticles can be leveraged as an advanced yet adaptable drug delivery system for the design of novel therapeutics at the intersection of neurology and infectious diseases. ELP nanoparticles' biocompatibility, temperature responsive behavior, and capacity for finely tuned drug release kinetics therefore render them optimal candidates for the treatment of two high-mortality illnesses: PAM and GAE. According to Stahl and Olson, as surface water temperatures increase due to anthropogenic climate change, it is likely that *N. fowleri* will become a more significant threat to human health, and this might easily be extrapolated to other FLA. Therefore, this paper's emphasis on developing ELP-based nanoparticles as prophylactic measures against pathogenic FLA offer new avenues of investigation for future investigators.
References


activation. *Microbiology*, 155(11), 3739–3747.  
https://doi.org/10.1099/mic.0.030635-0

https://doi.org/10.15585/mmwr.mm6836a3

https://www.ncbi.nlm.nih.gov/books/NBK430754/

https://doi.org/10.1021/acschemneuro.6b00232

https://www.ncbi.nlm.nih.gov/books/NBK482327/

https://doi.org/10.1093/jac/48.suppl_1.5

https://doi.org/10.1021/acschemneuro.7b00430


https://doi.org/10.1016/b978-0-12-415846-7.00014-7


https://doi.org/10.1016/b978-0-7234-3515-0.00018-0


https://doi.org/10.4103/tp.tp_36_21
41. *Balamuthia mandrillaris* | *Acanthamoeba* and free-living amoebae. (n.d.).

https://u.osu.edu/acanthamoeba/the-history-of-acanthamoeba-at-osu/balamuthia-mandrillaris/#:~:text=Results%20from%20this%20phylogenetic%20analysis.the%20Amebozoa%20and%20the%20Acanthamoebidae.


https://doi.org/10.1038/s41598-021-99903-8


https://doi.org/10.1093/cid/ciy813


https://doi.org/10.1155/2009/251406

https://doi.org/10.1155/2009/251406

47. CDC - DPDX - Free Living Amebic infections. (n.d.).

https://www.cdc.gov/dpdx/freelivingamebic/index.html


https://doi.org/10.1086/597473


https://med-chem.com/sappinia-spp/


https://doi.org/10.1086/597473


https://doi.org/10.1111/cts.12955


   https://doi.org/10.3389/fbioe.2023.1177151


   https://doi.org/10.1038/s41573-020-0090-8


   https://doi.org/10.1186/s13568-020-01061-z


discovery for the deadly, pathogenic free-living amoeba. *PLOS ONE, 16*(3), e0241738.  https://doi.org/10.1371/journal.pone.0241738