Repurposing drugs: Ca^{2+} ion dependency that can be exploited to treat keratitis caused by *Acanthamoeba castellanii*

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Acanthamoeba castellanii is a free-living amoeba (FLA) and a protist pathogen that has persisted in the environment since the emergence of early unicellular eukaryotic life forms on this planet. Unlike other FLA, *Acanthamoeba* spp. are the causative agent of *Acanthamoeba* keratitis (AK), which is a vision-threatening corneal infection difficult to manage with present-day drug therapy. AK in humans has emerged as a challenging ocular infection to treat due to the prolonged treatment regimen and if left untreated, the disease causes blindness. Even in the patients who complete the treatment for over a year, residual corneal damage impacting the vision is seen. Though considered to be rare, epidemics have been recently reported in south-east England with an increased emergence of cases with AK [1]. As *Acanthamoeba* spp. survive in water and soil as FLA, use of contaminated water to clean contact lens has continued to be a source of acquiring AK. Factors like antimicrobial drug resistance and lack of specific drugs directed against discrete molecular targets in *Acanthamoeba* are the possible reasons that contribute toward treatment failures in AK.

Investigating the cardinal needs that are vital for the survival and growth of *Acanthamoeba* spp. is expected to reveal nutrients and minerals that can be depleted to target this protist pathogen. *Acanthamoeba* spp. have not been studied in greater details for its calcium influx regulating mechanisms and its dependency on calcium–calmodulin interaction. Mitochondrial calcium accumulation has been studied in *Acanthamoeba*, but a channel system that allows Ca^{2+} to get through the outer mitochondrial membrane in *A. castellanii* has not been explored previously [2] and the same is the case with our knowledge regarding the

movement of this ion across the cell membrane. With the rationale that Ca^{2+} ions play a vital role in *Acanthamoeba* spp., and the recently reported expression of human-like Ca^{2+} ion-channels and the effects of Ca^{2+} -channel blockers [3] in trophozoite forms of keratitis isolate of *Acanthamoeba castellanii*, we tested chelators of Ca^{2+} ions in trophozoite forms of this protist pathogen. Our intention was to elucidate the role of extracellular Ca^{2+} in the biology of *Acanthamoeba* spp. The eukaryotic cells have intracellular storage of Ca^{2+} ions but these storage sites get depleted if Ca^{2+} ions are not replenished from extracellular resources. Recent studies have reported that the depletion of cytosolic Ca^{2+} by blocking human-like voltage-gated Ca^{2+} channels and calmodulin in *Acanthamoeba castellanii* with the drugs loperamide [3], amlodipine and gabapentin [4] exert amoebistatic and amoebicidal effect in low and high doses respectively. However, the effects of Ca^{2+} chelation in extracellular space in *A. castellanii* have not been tested yet.

Herein, we show the effects of known Ca^{2+} chelating agents, Ethylenediaminetetraacetic acid (EDTA) and pirenixine eye drop, on growth and viability of the trophozoite forms of *Acanthamoeba* obtained from a patient with AK. Fura-2 AM staining was done to establish the effects of the Ca^{2+} chelating agents on intracellular levels of Ca^{2+} in trophozoites forms of *A. castellanii*. We show that both, EDTA and pirenixine, exert significant antiproliferative effects as compared with their solvent controls PYG (Fig. 1) with the induction of morphological changes within 20 min of exposure to the drugs. This is reflective of an acute cellular Ca^{2+} depletion stress (data not shown).

Fura-2 AM staining of EDTA exposed trophozoites showed reduced intracellular staining as compared with that of the controls, reflecting the effect of EDTA on the cytosolic Ca^{2+} (Fig. 2 first-row). Similar results were seen with pirenixine dissolved in its solvents (Fig. 2 middle-row). Muscarinic receptor agonist pilocarpine, that causes calcium influx upon binding these receptors (Fig. 2 last-row), was unable to revert the effects of EDTA (Fig. 2 last-row) that chelates Ca^{2+} ions in extracellular space.

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Fig. 1 Effects of Ethylenediaminetetraacetic acid (EDTA) (A1) and pirenoxine (A2) on the growth and proliferation of *A. castellanii* trophozoites. EDTA and Pirenoxine in a dose of 10 and 25 $\mu\text{g}/\text{ml}$ showed amoebicidal (Trypan blue positive) effects on the trophozoites.

A muscarinic receptor agonist, pilocarpine, which after binding on mAChR1 and mAChR3 induces Ca^{2+} influx in eukaryotic cells, failed to rescue the trophozoites exposed to EDTA and pirenoxine

Fig. 2 $\times 40$ images of Fura-2AM stained trophozoites of *A. castellanii*. Control without EDTA shows Fura-2AM staining which is similar to EDTA 5 $\mu\text{g}/\text{ml}$ (EDTA-1). Note that, EDTA 10 $\mu\text{g}/\text{ml}$ (EDTA-2) shows reduced trophozoite staining. Note the trophozoite turning spherical with EDTA-2. Pirenoxine dissolved in its solvent in a dose of 20 $\mu\text{g}/\text{ml}$ (Pirenoxine-1) and 15 $\mu\text{g}/\text{ml}$ (Pirenoxine-2) did not affect the intracellular

Fura-2 AM staining significantly as compared with when combined with EDTA (EDTA + Pirenoxine). Pilocarpine increased the Fura-2AM staining in a dose of 75 $\mu\text{g}/\text{ml}$ alone (Pilocarpine), reflecting its Ca^{2+} influx inducing effects. Note, pilocarpine 75 $\mu\text{g}/\text{ml}$ was not effective in inducing Ca-influx when given with 5 and 10 $\mu\text{g}/\text{ml}$ of EDTA (EDTA-1 + Pilocarpine 75 and EDTA-2 + Pilocarpine 75: last row), respectively

Exploiting the voltage-gated Ca²⁺ channels and calmodulin in unicellular microbes like *Acanthamoeba* spp. has proven to be effective in killing this microbe [3, 4]. Ca²⁺ ion chelation with EDTA and structural analogues can repurpose them for the treatment of AK and possibly granulomatous amoebic encephalitis. In-depth understanding of the dependencies of this protist pathogen on cardinal nutrients and vital minerals is expected to uncover mechanisms and pathways which can be targeted. In addition, the recently reported use of EDTA in the eye for breaking Ca²⁺ ion mediated adhesions in keratopathy and the use of pirenoxine in prevention of the progression of presbyopia [5, 6] reflect the safety of their use in the human eye.

In *Acanthamoeba* spp., Ca²⁺ influx, its passage through voltage-gated channels [3], and cardinal cellular events that occur subsequent to the binding of this divalent cation to calmodulin [4] are novel molecular targets that can be exploited to gain therapeutic benefits in AK. By inhibition of the influx of Ca²⁺ in the trophozoites of this protist pathogen, we show the vulnerability of *Acanthamoeba* spp. to this divalent cation, which could be implemented in human clinical trials to treat AK. To the best of our knowledge, this is the first report of drug-induced chelation of Ca²⁺ ion in trophozoites of *A. castellanii* that has shown antiproliferative and amoebicidal effects. We advocate that these two agents could be exploited in the management of AK. Firstly, adding to contact lens cleaning liquids may minimise the risk of acquiring AK infection in frequent contact lens users; and secondly, through drug development and translational clinical trials, repurposing of agents like EDTA and pirenoxine as an adjunctive topical therapy for patients with AK may aid in the management of this

disease. These measures could have substantial impact on the prevention of long-term corneal damage and sight loss in AK.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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