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Research brief

Effect of non-steroidal anti-inflammatory drugs on biological properties of *Acanthamoeba castellanii* belonging to the T4 genotype

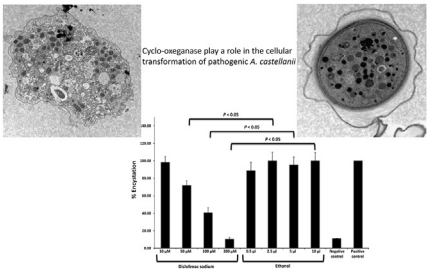
Ruqaiyyah Siddiqui [a, 1], Sahreena Lakhundi [b, 1], Junaid Iqbal [c], Naveed Ahmed Khan [a, *]

[a] Department of Biological Sciences, Faculty of Science and Technology, Sunway University, Selangor, Malaysia
[b] Department of Biological and Biomedical Sciences, Aga Khan University, Karachi, Pakistan
[c] Division of Pediatric Infectious Diseases, Vanderbilt University School of Medicine, Nashville, USA

HIGHLIGHTS

- Non-steroidal anti-inflammatory drug (NSAIDs), Diclofenac, targeting COX were tested against *Acanthamoeba.*
- NSAIDs affected growth but not the viability of *Acanthamoeba castellanii.*
- Importantly, NSAIDs abolished *A. castellanii* encystation.
- Cyclooxygenases (COX-1 and COX-2) and prostaglandins play significant role(s) in *Acanthamoeba* biology.
- NSAIDs in combination with other anti-amoebic drugs may help design improved preventative and/or therapeutic strategies.

GRAPHICAL ABSTRACT



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ABSTRACT

Non-steroidal anti-inflammatory drug, Diclofenac, targeting COX have shown promise in the treatment of *Acanthamoeba* keratitis, but the underlying mechanisms remain unknown. Using various NSAIDs, Diclofenac sodium, Indomethacin, and Acetaminophen, here we determined the effects of NSAIDs on the biological properties of *Acanthamoeba castellanii* belonging to the T4 genotype. Using amoebicidal assays, the results revealed that Diclofenac sodium, and Indomethacin affected growth of *A. castellanii*. In contrast, none of the compounds tested had any effect on the viability of *A. castellanii*. Importantly, all NSAIDs tested abolished *A. castellanii* encystation. This is a significant finding as the ability of amoebae to transform into the dormant cyst form presents a significant challenge in the successful treatment of infection. The NSAIDs inhibit production of cyclo-oxegenase, which regulates the synthesis of prostaglandins suggesting that cyclooxygenases (COX-1 and COX-2) and prostaglandins play significant role(s) in *Acanthamoeba* biology. As NSAIDs are routinely used in the clinical practice, these findings may help design improved preventative strategies and/or of therapeutic value to improve prognosis, when used in combination with other anti-amoebic drugs.

* Corresponding author. Department of Biological Sciences, Faculty of Science and Technology, Sunway University, Selangor, 47500, Malaysia.
E-mail address: naveed5438@gmail.com (N.A. Khan).
[1] Both authors contributed equally to this manuscript.

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1. Introduction

Acanthamoeba keratitis is a painful infection with blinding consequences. Characteristic clinical presentation of *Acanthamoeba* keratitis includes excruciating pain due to radial neuritis, inflammation with redness and photophobia (Perez-Santonja et al., 2003; Clarke and Niederkorn, 1999; Panjwani, 2010). The cascade of events involves corneal abrasion followed by the attachment of the parasite leading to breakdown of the epithelial barrier, stromal invasion, loss of keratocytes, induction of an intense inflammatory response and stromal necrosis (Garner, 1993; Vemuganti et al., 1976; Clarke and Niederkorn, 1999; Panjwani, 2010). If not promptly diagnosed, and treated aggressively, the cornea becomes ulcerated leading to perforation, ring infiltrate, stromal abscess formation, loss of visual acuity and eventually blindness (Niederkorn et al., 1999). Treatment however is problematic and best outcomes are expected only when, diagnosed early and treated adequately with a high level of patient compliance. However, the current treatment options are aggressive, lengthy and not fully effective against all strains resulting in recurrence of the disease (Perez-Santonja et al., 2003; Turner et al., 2004). The current treatment often lasts several months and consists of topical administration of several drugs, and patient compliance is often an issue. Besides, the chronic use of these drugs is often associated with significant ocular toxicity (Varga et al., 1993). A key challenge in the successful treatment of *Acanthamoeba* infection is the phenotypic switching of the organism into a quiescent cyst form that is hardy and unaffected by harsh environmental conditions and available pharmacological therapies, leading to infection recurrence (Khunkitti et al., 1998; Turner et al., 2000; Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007; Khan, 2006). Hence, there is a need for improved preventative and therapeutic strategies.

Despite the aforementioned challenges, Agahan et al. (2009) reported three cases of *Acanthamoeba* keratitis treated successfully with non-steroidal anti-inflammatory drugs (NSAIDs) eye drops. NSAIDs inhibit production of cyclo-oxegenase (COX), an enzyme involved in the synthesis of prostaglandins (PGs), which are potent immunomodulating agents. Increased production of PGs may aggravate inflammation and down-regulate immune response and cytokine production (IL-1, IL-2, IFN-γ and TNF-α), key determinants in controlling the severity and outcome of the disease (Kunkel et al., 1986; Dey et al., 2003). Stewart et al. (1992) demonstrated the killing of *Acanthamoeba castellanii* by macrophages when incubated in the presence of IFN-γ and immune rat serum showing that exposure of macrophages to IFN-γ increased their amoeba-lytic potential. However, the effects of NSAID on the biology of *Acanthamoeba* remains unknown and it is the subject of the present study.

2. Material and methods

All chemicals were purchased from Sigma (Poole, Dorset, UK), unless otherwise stated. Non-steroidal anti-inflammatory drugs, Diclofenac sodium, Indomethacin, and Acetaminophen were purchased from a local pharmacy. Both drugs were dissolved in ethanol and stored at −20 °C until tested.

2.1. Cultures of Acanthamoeba castellanii

A clinical isolate of *A. castellanii* belonging to the T4 genotype, isolated from a keratitis patient (American Type Culture Collection, ATCC 50492) was used in the present study. Amoebae were grown without shaking in 15-mL of PYG medium [0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract, and 1.5% (w/v) glucose] in T-75 tissue culture flasks at 30 °C as previously described (Sissons et al., 2005). To obtain vegetative trophozoites, the media were refreshed 17–20 h prior to experimentation, which resulted in more than 99% amoebae in the trophozoite forms.

2.2. Amoebicidal assays

To determine the killing effects of Diclofenac sodium, Indomethacin, and Acetaminophen against *A. castellanii*, amoebicidal assays were performed. Briefly, 5×10^5 *A. castellanii* trophozoites were incubated with different concentrations of drugs in PBS in a 24-well plate. For physiological relevance, plates were incubated at 37 °C for 24 h, subsequently, parasite viability was quantified using a haemocytometer via Trypan blue exclusion assay. Amoebae stained blue were considered dead, while non-stained amoebae were considered viable. The number of amoebae were counted using a haemocytometer. Amoebae incubated with the solvent alone were used as negative control, whereas for positive control, Chlorhexidine (final concentration 25 μM) was used, which exhibited 100% killing.

2.3. Amoebistatic assays

To determine the effect of Diclofenac sodium, Indomethacin, and Acetaminophen on *A. castellanii* growth, amoebistatic assays were performed by inoculating 5×10^5 trophozoites in growth medium, i.e., PYG, in a 24-well plate along with different concentrations of inhibitors. Next the plate was incubated at 30 °C for 48 h. For controls, 5×10^5 trophozoites were inoculated in growth medium (i.e., PYG medium), and non-nutritive PBS and respective amounts of solvents plus PYG medium. Following this incubation, the number of amoebae was determined by haemocytometer counting.

2.4. Encystation assays

Encystation assays were performed as described previously (Dudley et al., 2005). Briefly, 2×10^6 amoebae were incubated in PBS in the presence of 50 mM $MgCl_2$ and 10% glucose (i.e., encystation trigger) in a 24-well tissue culture plates without shaking at 30 °C for 72 h. After this incubation, amoebae viability was quantified using a haemocytometer via Trypan blue exclusion assay. Next, SDS (sodium dodecyl sulphate, 0.5% final concentration) was added for 10 min to solubilize trophozoites and cysts were enumerated using a haemocytometer. To ensure that 0.5% SDS does not affect cyst viability, mature cysts were used. To achieve this, cysts were prepared using solid agar, i.e., by inoculating *A. castellanii* trophozoites on non-nutrient agar plates and incubating at 30 °C for 14 days. Next, 10 mL of dH_2O was added to each plate. Cysts were then scraped off the agar surface using a cell scraper and enumerated using a haemocytometer and used for assays.

To determine the effects of Diclofenac sodium, Indomethacin, and Acetaminophen on encystation, assays (PBS plus 50 mM $MgCl_2$ and 10% glucose) were performed in the presence of different concentrations of both drugs. Briefly 2×10^6 amoebae were incubated in PBS with both drugs in the presence of 50 mM $MgCl_2$ and incubated at room temperature for 20 min. Following this, 10% glucose was added as a trigger for encystation and plates were incubated at 30 °C for 72 h. Encystation in wells without inhibitors and encystation trigger was used as positive and negative controls, respectively. The respective amounts of solvents were used as solvent controls. Amoebae counts were determined using a haemocytometer.