The effect of eye drop excipients against Acanthamoeba polyphaga by AlamarBlue™ assay

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Abstract

Objective: Based on the reduction of alamarBlueTM, we have therefore screened a variety of such eye drop excipients used for bacterial keratitis in order to identify any candidates that show inhibitory activity against *Acanthamoeba polyphaga*, one of the protozoal species responsible for the *Acanthamoeba* Keratitis.

Subjects and Methods: *Acanthamoeba* keratitis is a serious eye infection which is notoriously difficult to treat successfully. The currently employed drugs have significant disadvantages in that they have to be administered at hourly intervals for extended periods of time. The AlamarBlueTM assay has been optimized for determination of selected eye drop excipients efficacy against potentially pathogenic strain, *Acanthamoeba polyphaga*.

Results: The most effective agents were found to be fusidic acid and framycetin sulfate, with a combination of the two providing a reduction in *A. polyphaga* metabolic activity of around 75%.

Conclusion: These eye drop excipients can serve as new sources for the discovery and development of much needed new antimicrobials for both *Acanthamoeba* keratitis and bacterial keratitis.

Key words: AlamarBlue; *Acanthamoeba* keratitis; *Acanthamoeba polyphaga*

Introduction

Acanthamoeba keratitis is a serious eye infection caused by *Acanthamoeba* species of protozoa. These protozoa are present in the majority of water bodies, including sea water, sewage, soil and tap water. Previously a relatively rare condition, prevalence of *Acanthamoeba* keratitis is increasing [1]. This is mainly caused by the growing use of contact lenses, with approximately 85% of infections occurring in contact lens users [2, 4]. *Acanthamoeba polyphaga* is one of the two main protozoa species responsible for the condition. This microorganism has two stages to its life cycle, a rapidly reproducing trophozoite phase followed by encystation to form a robust double-layered cyst that allows survival under harsh conditions such as the presence of toxic chemicals [5]. This cyst stage provides a significant hurdle in the treatment of *Acanthamoeba* keratitis, with most drugs having demonstrated limited activity against it [6].

Chlorhexidine and polyhexamethylene biguanide (PHMB) are currently the standard treatments for the condition, being active against both the trophozoite and cyst phases of the organism. Diamidines such as hexamidine are sometimes used in conjunction with these; however, their use alone should be avoided owing to the development of resistance [4, 7]. A further issue associated with these agents is the necessity to apply them at hourly intervals for extended periods of time. Novel and more effective drugs for treating *Acanthamoeba* keratitis are therefore highly sought after. Phosphocholines have shown some promise, with inhibitory activity against Acanthamoeba and other parasites being demonstrated in vitro and in animal tests [8-11). A number of potential targets for new treatments have been identified. These include various components of the cell membrane, mitochondria, and protein synthesis pathways [12]. Such targets present a wide variety of agents that could be screened for activity against *A. polyphaga*. A selection of possible drugs of interest is already used in eye drop form; however, their efficacies for specifically treating *Acanthamoeba* keratitis have not yet been investigated.

In the present study, we have screened eight components of commercially available eye drop solutions in order to identify any agents with the potential for treating the condition. To allow for rapid analysis of all excipients simultaneously, we employed an alamarBlueTM microplate assay that has been previously verified for use in analysing the response of *A. polyphaga* to inhibitory drugs [13].

Materials and Methods

Culture of *A. polyphaga*

A. polyphaga (strain 1501/18) was obtained from Culture Collection of Algae and Protozoa (Lincoln, London). The cells were cultured in medium supplemented with 20% mycological peptone, 0.9% maltose, and 1% penicillin, streptomycin, and amphotericin B (all Sigma-Aldrich, Pennsylvania, USA). They were incubated in 75 cm^2 flasks at room temperature, when cultures reached 90-95% confluence.

Determination of optimal seeding densities for *Acanthamoeba*

After harvesting, *A. polyphaga* were diluted in culture medium to give a stock solution containing 8.0×10^5 cells/ml. Different concentrations of cells were used for tests of varying length. For the 24 hour test, 100 υl aliquots of a solution of 8.0×10^4 cells/ml were added to the wells of a 96-well plate. The seeding densities of A. polyphaga that attained close to 100% alamarBlue reduction were determined in assays conducted for total periods of 24 and 96 hours.

AlamarBlue growth inhibition assay

A stock solution of A. polyphaga was prepared at 8.0×10^5 cells/ml in culture medium. Inhibition tests were carried out for two different time periods. For the 24 hour test, 100 υl aliquots of a solution of 8.0×10^4 cells/ml were added to the wells of a 96-well plate. For the 96 hour test, 100 υ l of a solution of 1.25×10^3 cells/ml were added to each well. Each test was carried out in triplicate, with the experiment carried out twice. The different eye drop excipients to be tested were added to the wells at the concentrations related to the compound solubility. Synergistic effects of the eye drop excipients were determined by using different combinations of the agents. The cultures were incubated at room temperature for the duration of the test period, after which 10 υl of alamarBlue reagent (Life Technologies, Renfrew, UK) was added to each well and the plates were incubated for a further 6 hours. The absorbance of the solutions was then measured at 570 nm and 600 nm using Gemini EM Microplate Reader (Molecular Devices, Sunnyvale, USA). The percentage reduction of alamarBlue was then calculated according to the following equation:

{[(εox) λ2Aλ1] - [(εred) λ1 Aλ2 of treated Acanthamoeba]
/ [(εox) λ2A^0λ1]
- [(εred) λ1 A0λ2 of untreated Acanthamoeba]} X 100

Where εred λ1 is 155,677 (molar extinction coefficient of reduced alamarBlue™ at 570 nm); εred λ2 is 14,652 (molar extinction coefficient of reduced alamarBlue™ at 600 nm); εoxλ1 is 80,586 (molar extinction coefficient of oxidised alamarBlue™ at 570nm); εoxλ2 is 117,216 (molar extinction coefficient of oxidised alamarBlue™ at 600nm); A λ_1 is the absorbance of treated wells at 570nm; A λ_2 is the absorbance of treated wells at 600nm; A^0 λ_1 is the absorbance of cells with medium only as control wells at 570nm; A^0 λ_2 is the absorbance of untreated control wells at 600nm. These absorbance values were multiplied by 100 to give percentage of alamarBlue™ reduction comparison to untreated trophozoites cultured. The results were expressed as a mean for each triplicate ± the standard error (SE) (AbD Serotec, alamarBlue™ Assay).

Statistics

All tests were carried out twice in triplicate. As the results of the two experiments were highly similar, statistical analysis was carried out on the data from one experiment. Values are expressed as the mean with the standard

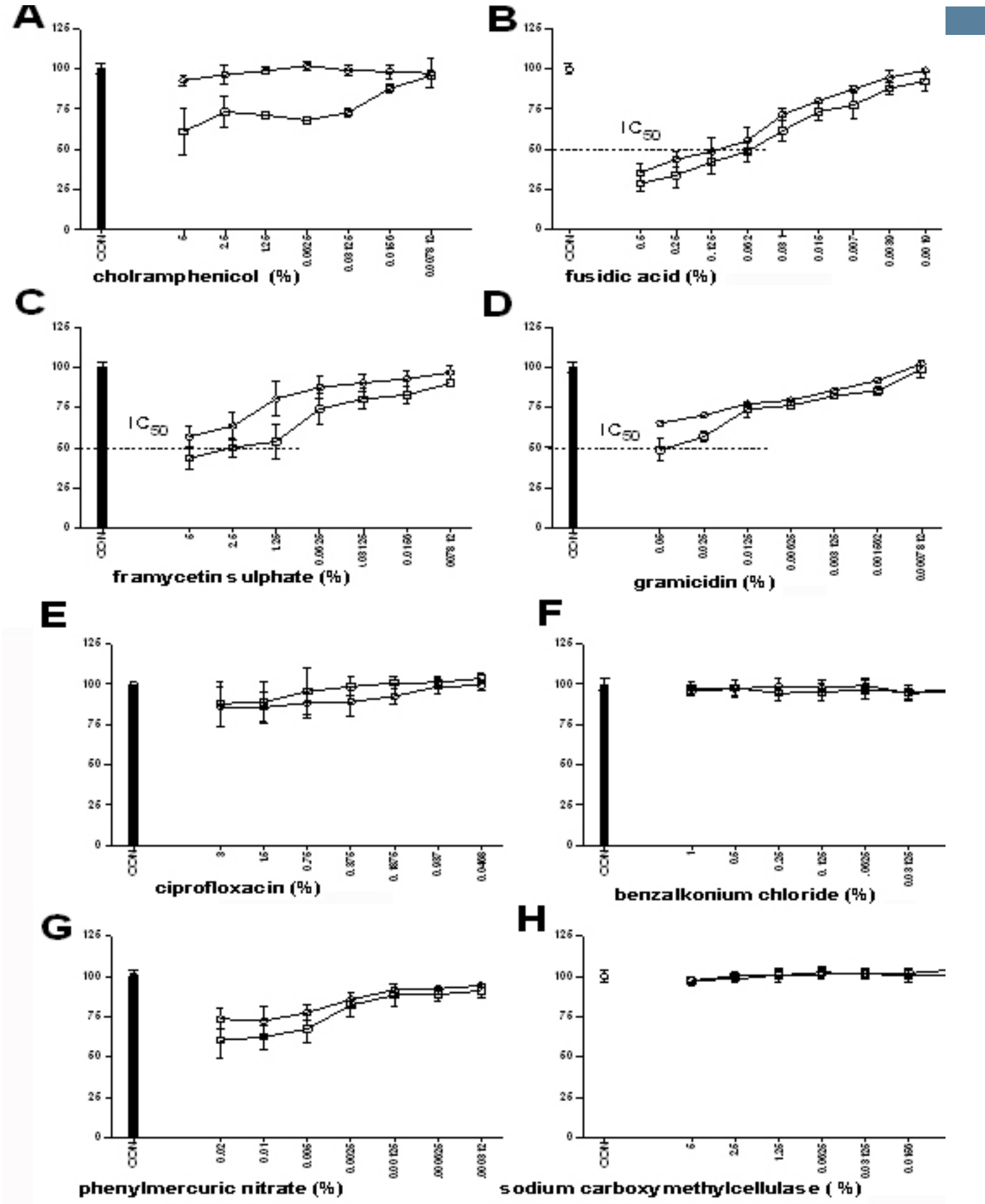

Figure 1: Relative susceptibilities of A. polyphaga (open circles 24 h) and (open squares 96 h) to the following: (A) cholamphenicol (no IC50 indicated), (B) fusidic acid (IC50 at 0.125 % for 24 h and 0.062 % for 96 h), (C) framycetin sulphate (IC50s at 2.5% for 96 h), (D) gramicidin (IC50 at 0.05 for 96 h), (E) ciprofloxacin (no IC50 indicated), (F) benzalkonium cholride (no IC50 indicated), (G) phenylmercuric nitrate (no IC50 indicated) and (H) sodium carboxymethylcellulase (no IC50 indicated). A. polyphaga to each of the eye drop excipients tested ($P < 0.05$). Acanthamoeba cell numbers were assessed by measuring the percent alamarBlue reduction relative to that for the untreated control cultures [CON] over 24 and 96 h. The results are expressed as the means for triplicate cultures ±SEs.

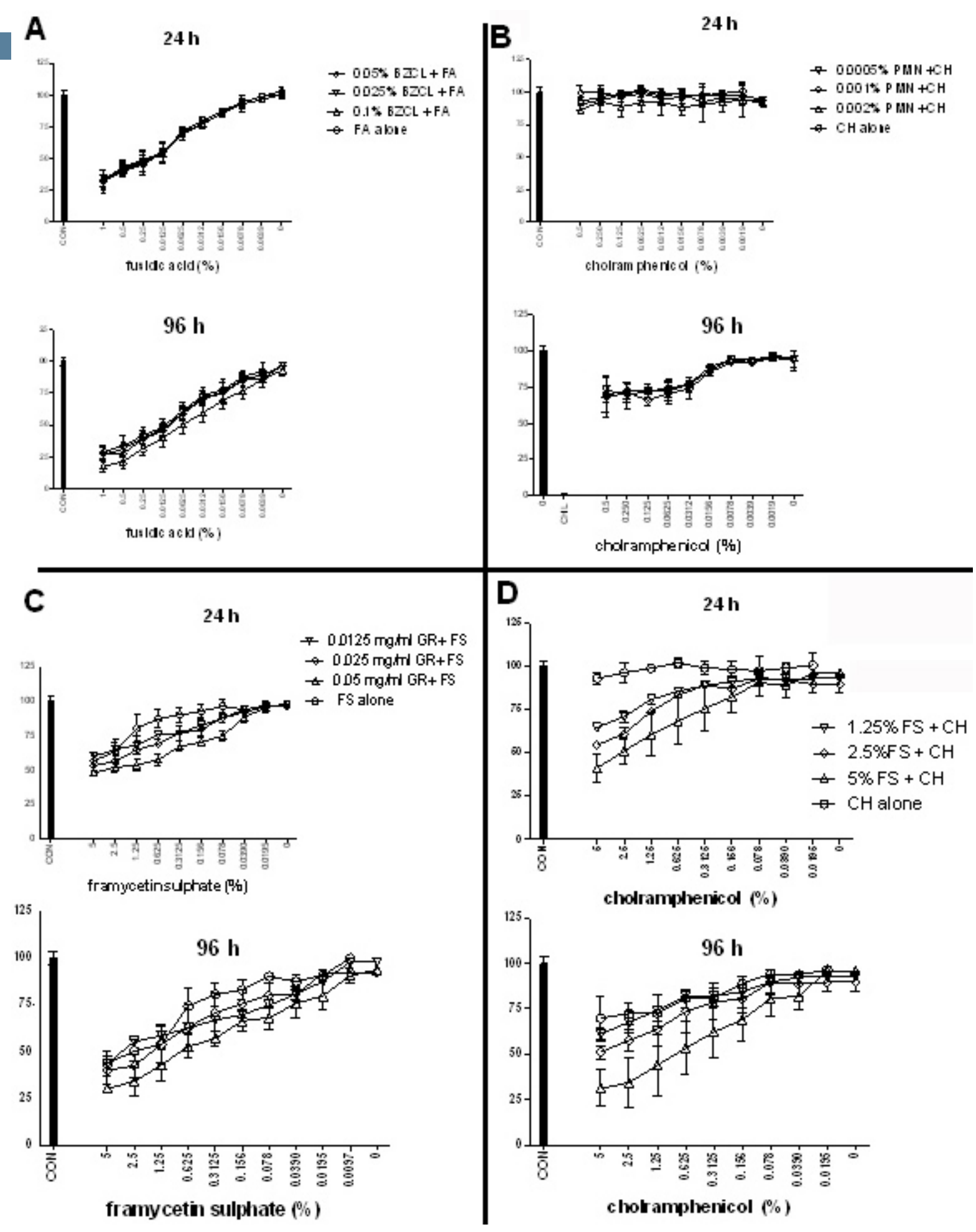

Figure 2: Percentage reduction of alamarBlue by A. polyphaga in the presence of combinations of excipients
Legend: A) fusidic acid and benzalkonium chloride, B) chloramphenicol and phenylmercuric nitrate, C) gramicidin and **framycetin sulfate, D) framycetin sulfate and chloramphenicol. Cultures carried out for a) 24 h and b) 96 h.**

error (SE). Statistical significance was calculated using the Mann-Whitney U test, with a p-value of <0.05 considered to be significant. Statistical analysis was performed using the GRAPH PAD PRISM 5 software.

Results

The addition of chloramphenicol to the A. polyphaga cultures resulted in a significant dose-dependent decrease in alamarBlue reduction by the cells, but only for the 96 hour test (Figure 1A). No inhibitory activity was found for the 24 hour culture. On the other hand, fusidic acid had a large inhibitory effect for both culture lengths, with the concentrations at which 50% inhibition was achieved (IC50 values) being 0.125% and 0.062% for the 24 hour and 96 hour experiments, respectively (Figure 1B).

Framycetin sulfate also displayed inhibitory activity, although this was only significant enough to calculate an IC50 when the culture was carried out for 96 hours (IC50: 5.0%; (Figure 1C). Gramicidin caused a level of inhibition for both culture durations, with an IC50 of 5.0% calculated for the 96 hour experiments (Figure 1D). Ciprofloxacin showed some activity at the highest concentrations, but this reached no more than a percentage reduction of alamarBlue of 85% (Figure 1E).

Neither benzalkonium chloride nor sodium carboxymethyl cellulose displayed any inhibitory activity against A. polyphaga at any concentration for either culture length (Figure 1 F and H). Phenylmercuric nitrate on the other hand, displayed a level of activity at the higher concentrations, with the effect being more pronounced for the 96 hour culture (Figure 1G). The effect was not significant enough for an IC50 to be calculated, however.

When different combinations of the eye drop excipients were tested for activity against A. polyphaga, the combination of fusidic acid and benzalkonium chloride (Figure 2A) appeared to provide much the same response to that of fusidic acid alone (Figure 1B). Again, high inhibitory activity was found for both culture lengths, with perhaps a slight increase in activity when the highest concentration of benzalkonium chloride was present for the 96 hour experiment. The combination of chloramphenicol and phenylmercuric nitrate produced no inhibition of the microorganism for the 24 hour culture (Figure 2B). For the 96 hour culture, however, some activity was evident at the higher chloramphenicol concentrations. The shape of the curves closely mirrored that of when phenylmercuric nitrate was tested alone (Figure 1G), but the concentration of this excipient had no effect on the level of inhibition.

Another combination of excipients that was tested was gramicidin with framycetin sulfate. When tested alone, both of these agents demonstrated inhibitory activity against A. polyphaga in both the 24 hour and 96 hour cultures (Figure 1C and D). Together, a small additive effect can be seen, with the level of inhibition being greatest when the highest concentrations of each excipient were used in combination (Figure 2C).

While chloramphenicol alone only showed inhibitory activity for the 96 hour culture, in combination with framycetin sulfate, activity was evident for both lengths of experiment (Figure 2D). For the shorter of the two cultures, the inhibitory activity was higher for the combination of excipients than that when framycetin was tested alone. The maximum level of alamarBlue reduction for the highest concentration of framycetin sulphate (5 mg/ml) was approximately 55%, while this decreased to around 40% when combined with 5% chloramphenicol. For the 96 hour culture, alamarBlue reduction reached a low of approximately 25% for the combination of the highest concentrations of the two excipients. This was much lower than the 70% and 45% found for chloramphenicol and framycetin sulfate alone, respectively (Figure 1A and C).

Discussion

There is an increasing need to develop novel agents against *A. polyphaga*, one of the protozoal species responsible for Acanthamoeba keratitis. The task of identifying such compounds is ongoing; however, to date, no study has evaluated the efficacy of agents already used in commercial eye drops. Having already been demonstrated to be safe for ophthalmologic use, such compounds could rapidly gain regulatory approval for the treatment of this potentially blinding condition.

Chloramphenicol displays broad bacteriostatic activity against both gram positive and gram negative bacteria by inhibiting protein synthesis via irreversible binding to the 50S subunit of the ribosome. It has long been used to treat bacterial conjunctivitis [14], and has recently demonstrated anti-yeast properties [15]. We found that alamarBlue reduction by *A. polyphaga* was inhibited by the compound, but only when the cells were cultured in its presence for 96 hours. This indicates that chloramphenicol works slowly against the organism, requiring a certain length of time in order to achieve an effect.

Fusidic acid is another bacteriostatic compound, displaying activity against gram positive bacteria. It works by inhibiting protein synthesis via prevention of turnover of elongation factor G from the ribosome [16], and is used in the treatment of bacterial conjunctivitis [17]. We found that the agent produced significant inhibitory activity against *A. polyphaga* in both the 24 hour and 96 hour cultures. This demonstrates that despite its narrow scope as an antibacterial, it is a promising candidate for the treatment of *Acanthamoeba* keratitis.

Framycetin sulfate is a broad spectrum aminoglycoside antibacterial that works by inhibiting protein synthesis via ribosomal binding. It is active against gram negative and some gram positive bacteria, but has not been demonstrated to have any antifungal activity. Whilst there are no reports on the effect of this agent on any protozoal species, another aminoglycoside antibacterial, paromomycin, has demonstrated activity against the *Leishmania* species of protozoa [18-20]. We found that framycetin sulfate inhibited alamarBlue reduction by

A. polyphaga, with a more significant effect evident for the longer 96 hour culture. The data therefore suggest that this compound is another potential therapy for *Acanthamoeba* keratitis, and warrants further study.

Gramicidin is an antibacterial that causes cell death by increasing the permeability of the cell membrane leading to leakage of small molecules such as monovalent ions and amino acids. To date, reports of the activity of gramicidin have been limited to gram positive bacteria, with no evidence of antifungal or antiprotozoal activity when used alone. Here, we found that the compound had a level of activity against *A. polyphaga*, with this being greater for the 96 hour culture in comparison with the 24 hour.

Ciprofloxacin is a fluoroquinolone broad spectrum antibacterial that is used to treat conjunctivitis, keratitis, and corneal ulcers [21]. It works by hindering cell division via inhibition of DNA topoisomerases [22]. The compound has also displayed activity against *Leishmania* or topoisomerases present in this organism [23, 24]. Here, we found that ciprofloxacin demonstrated a low level of activity against *A. polyphaga*, with similar activity profiles for the two different culture lengths. Such limited activity indicates that this compound would not be useful for the treatment of *Acanthamoeba* keratitis.

Benzalkonium chloride is a quaternary ammonium salt preservative used in many forms of eye drops and artificial tears. It works as an antibacterial by binding to the negatively charged cell membrane and increasing its permeability, resulting in leakage of monovalent ions and subsequently, cell death. No inhibitory activity against *A. polyphaga* was found in the present study, for either culture length. This is in contrast to the data published by Tu et al., who demonstrated significant in vitro activity of benzalkonium chloride against three species of Acanthamoeba, among them *A. polyphaga* [25). Activity was high after just an hour, even at concentrations much lower than those used in the present work. Zanetti et al. reported activity of the compound against *A. castellanii,* another organism responsible for *Acanthamoeba* keratitis, both in trophozoite form and cyst form [26]. These conflicting results suggest that further tests should be carried out before benzalkonium chloride is discounted as a treatment for the condition.

Phenylmercuric nitrate is another preservative used in eye drops. It displays both antibacterial and antifungal activity as a result of increasing the permeability of the cell membrane [27, 29]. We found that the compound was active against *A. polyphaga* at the higher concentrations, with a greater effect for the 96 hour culture. This indicates that phenylmercuric nitrate may be useful in the treatment of *Acanthamoeba* keratitis if the dosing can be sustained over a number of days.

The final eye drop excipient that we tested was the viscosity modifier, sodium carboxymethyl cellulose. As expected, this compound demonstrated no inhibitory activity against *A. polyphaga.*

Fusidic acid eye drops often contain benzalkonium chloride as a preservative; we therefore tested these two agents in combination in order to determine if there was an additive effect. The activity of the combination was generally the same as that of fusidic acid alone. The only exception to this was a slightly greater activity when the highest concentration of benzalkonium chloride was used in the 96 hour culture. Importantly, no detrimental effect was found, indicating that commercially available fusidic acid eye drops may be a potential treatment option for *Acanthamoeba* keratitis.

Another commonly found combination of agents in eye drops is chloramphenicol and phenylmercuric nitrate. For the 24 hour culture, this combination appeared to have no inhibitory activity against *A. polyphaga*, despite phenylmercuric nitrate alone showing some activity in the earlier experiments. This is likely due to the low concentrations of this compound that were added to the chloramphenicol for this particular test. The activity of the phenylmercuric nitrate was only found at the higher concentrations that were tested in the single compound experiment. It is also possible that the presence of chloramphenicol lowered the activity of the phenylmercuric nitrate. The data taken from the 96 hour culture also point to this possibility as the level of A. polyphaga inhibition was almost the same as that seen for the chloramphenicol alone, with no additional effect found on the addition of the phenylmercuric nitrate. This combination of excipients does not appear to be a potentially effective treatment for *Acanthamoeba* keratitis.

Gramicidin and framycetin sulfate are often used in combination for treating eye infections. We found that the inhibitory activity of this combination was greater than that found for either agent alone. For the 96 hour culture in particular, alamarBlue reduction decreased to approximately 25%, one of the lowest values found in the present study. The data therefore suggest that the commercially available eye drops that utilise this combination could be effectively used for the treatment of *Acanthamoeba*-based infections.

We also investigated the combination of framycetin sulfate and chloramphenicol. For the 24 hour culture, while chloramphenicol alone had not demonstrated any activity in the prior tests, on the addition of even a small amount of framycetin sulfate, there was an increase in A. polyphaga inhibition. This additive effect was even more evident for the 96 hour culture, with alamarBlue reduction decreasing to around 25% when the highest concentrations of the two compounds were used together. This combination of agents appears to be a promising treatment option for Acanthamoeba keratitis and warrants further evaluation.

Conclusions

We have demonstrated significant activity against *A. polyphaga* of a number of compounds that are currently used as active ingredients of preservatives in commercially available eye drops. These preliminary data indicate that

further investigation into some of these agents may lead to additional treatment options for *Acanthamoeba* keratitis. As these excipients have already been approved for ocular use, there is potential for new combinations of them to be rapidly introduced to the market.

Conflict Of Interest

The author of this publication receives research support from Public Authority for Agriculture Affairs and Fish Resources - Al-rabia, Kuwait City.. The terms of this arrangement have been reviewed and approved by the University of Kuwait in accordance with its policy on objectivity in research.

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