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Research paper

Topical semifluorinated alkane-based azithromycin suspension for the management of ocular infections

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ABSTRACT

The management of ocular infections is challenging due to poor drug bioavailability and vehicle related adverse effects associated with current antibiotic eye drops. Semifluorinated alkanes (SFAs) are reportedly well-tolerated on the ocular surface and can enhance ocular drug bioavailability. Therefore, an SFA-based azithromycin suspension (SFA-AZM) was prepared and its antibacterial efficacy was compared to that of marketed azithromycin eye drops by monitoring the growth of bioluminescent *Staphylococcus aureus* in *ex vivo* ocular tissues. Corneal and conjunctival distribution of hydrophobic fluorescent dye particles from an SFA suspension (SFA-BODIPY) resulted in preferential dye localisation in the epithelial layers of both tissues. However, corneal dye absorption was significantly lower than conjunctival absorption, likely due to limited adhesion of suspended dye particles to the corneal compared to the conjunctival epithelium. In line with the dye distribution results, bacterial colonisation in the conjunctiva reduced significantly upon application of SFA-AZM with the efficacy being greater than or at least equal to the marketed azithromycin eye drops. In the cornea, all tested azithromycin eye drops reduced the rate of bacterial growth with similar efficacy. Overall, the SFA-AZM suspension tested here may provide a safe and effective alternative for the management of ocular infections by enhancing conjunctival drug absorption and thus drug efficacy.

1. Introduction

Due to the vulnerable nature of the ocular surface, advancement in the field of ocular drug delivery has been restrained as formulation scientists face the challenge of circumventing the anatomical and physiological barriers of the eye while ensuring that there is no permanent damage to the ocular tissues. Therefore, eye drops are commonly formulated as viscous aqueous solutions, although the poor aqueous solubility of lipophilic drugs often necessitates emulsification or dissolution in oily vehicles [1–3], with corneal toxicity and adverse effects of oily ophthalmic solutions and emulsifiers being a significant concern [4,5]. The risk of toxicity may be even higher with novel colloidal drug delivery systems, such as micelles, micro- or nanoemulsions, liposomes and niosomes, which often contain a larger proportion of surfactants and co-surfactants than conventional formulations [6,7]. Finally, preservatives incorporated into multi-dose aqueous eye drops also present a significant toxicity risk [8]; therefore, preservative-free eye drops are often preferred.

Azithromycin (AZM) is a lipophilic macrolide antibiotic which inhibits bacterial growth by interfering with microbial protein synthesis [9,10]. Additionally, AZM has secondary anti-inflammatory properties due to its ability to block activation of pro-inflammatory cytokines in corneal epithelial cells [11,12]. Topical AZM eye drops have good conjunctival permeability and a relatively long elimination half-life [13,14]; however, they are difficult to formulate due to the poor aqueous solubility and large molecular size of AZM [15]. Topical AZM eye drops are currently approved in some markets for the treatment of conjunctivitis caused by susceptible strains; nevertheless, they are often used beyond their clinical indication for the treatment of other ocular surface diseases including blepharitis, dry eye disease, trachoma, and

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infectious keratitis [16,17]. At present, AzaSite® (InSite Vision Inc., USA), a 1% w/v aqueous ophthalmic solution of AZM based on the patented DuraSite® technology, is the only topical AZM eye drop approved in the USA [18,19]. However, there are some concerns regarding ocular surface toxicity due to formulation excipients including the preservative benzalkonium chloride in the DuraSite platform [20,21]. A preservative-free 1.5% w/v AZM eye drop (Azyter®, Laboratories Théa, France) based on medium chain triglycerides (MCT) is currently approved in Europe [22,23]. However, mild to moderate ocular discomfort upon drop instillation is a frequent adverse reaction to Azyter [24], which may be related to the MCT vehicle [25,26]. Hence, less irritating and more effective preservative-free vehicles are needed for topical delivery of AZM to improve therapeutic outcomes.

Semifluorinated alkanes (SFAs) are physically, chemically and biologically inert liquids that are well tolerated on the ocular surface [27,28]. The non-aqueous nature of SFAs enables the formulation of preservative-free eye drops without additional buffering or osmolarity regulating agents. Moreover, since SFAs are optically transparent and their refractive index is very similar to that of the tear fluid (1.336–1.338), the incidence of visual disturbances and patient discomfort on topical administration is reduced [29]. The pure SFA perfluorohexyloctane (NovaTears®; EvoTears®, Novaliq GmbH, Germany) reportedly has excellent spreading and tear film stabilising properties [28,30] rendering it particularly beneficial in the treatment of ocular surface disorders. Previous studies also suggest that hydrophobic drugs solubilized in SFAs exhibit enhanced ocular bioavailability and thus improved therapeutic outcomes [27,31].

Therefore, the SFA perfluorohexyloctane was used to formulate AZM eye drops for the treatment of ocular infections. Since the saturation solubility of AZM in this SFA is several-fold lower than the AZM concentration in currently marketed eye drop preparations, a 1.5% w/v suspension of AZM in SFA (SFA-AZM) was prepared. To compare corneal and conjunctival particle absorption, the distribution of a hydrophobic fluorescent dye (BODIPY) from an SFA based suspension (SFA-BODIPY) was evaluated using an *ex vivo* porcine eye model. The antibacterial activity of SFA-AZM against bioluminescent *Staphylococcus aureus* bacteria was initially evaluated *in vitro*. However, since *in vitro* tests make no attempt to replicate the intracellular environment, they are often not reliable in estimating the antibacterial potential of antibiotic agents in tissues [32,33]. Thus, a novel *ex vivo* model was developed to evaluate the antibacterial potential of the test formulations in real time by inoculating the cornea and tarsal conjunctiva with bioluminescent bacteria and observing bacterial proliferation in these tissues over 24 h. This model was then used to compare the efficacy of SFA-AZM to that of commercially available AZM formulations (AzaSite and Azyter) in treating bacterial ocular infections, with results suggesting that the SFA-AZM formulation is more, or at least as efficacious as, marketed azithromycin eye drops *ex vivo*.

2. Materials and methods

2.1. Materials

Bioluminescent *S. aureus* Xen36 (Bioware™ Microorganisms, Caliper Life Sciences Inc., USA) derived from the parental strain *S. aureus* ATCC 49525 (Wright), a clinical isolate from bacteraemia patients, was used for this study. Bacteria were cultured using BD Difco™ Müller-Hinton (MH) medium purchased from Fort Richard, New Zealand. Miglyol® 812 MCT was purchased from Caesar & Loretz GmbH, Germany, while AZM was purchased from Chemos GmbH, Germany. Pure SFA and SFA-AZM (1.5% w/v) were kindly provided by Novaliq GmbH, Germany. Dimethyl sulphoxide (DMSO) was purchased from BDH Laboratory Supplies, UK, while BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene) and Hank's balanced salt solution (HBSS) were purchased from Thermofisher Scientific, USA.

Table 1
List of test formulations used.

	Test Formulation	Vehicle	Drug/Dye concentration (% w/v)
Formulations without AZM	Negative Control	MH-medium	0%
	SFA	SFA	0%
	MCT	MCT	0%
Formulations with AZM	Positive Control	DMSO	1.5% AZM
	SFA-AZM	SFA	1.5% AZM
	Azyter	MCT	1.5% AZM
	AzaSite	DuraSite Platform	1.0% AZM
Formulation with Dye*	SFA-BODIPY	SFA	1.5% BODIPY

* Used only for visualisation of dye absorption from an SFA-based suspension.

2.2. Preparation of test formulations

Test formulations used for *in vitro* and *ex vivo* antibacterial studies are outlined in Table 1. All test formulations were used as received. The SFA-BODIPY suspension was prepared by adding 150 mg of BODIPY powder to 10 ml of SFA and mixing well.

2.3. Visualisation of dye absorption from an SFA-BODIPY suspension

Tissue absorption of dye particles suspended in SFA was evaluated using BODIPY (log P = 3.5) as the model molecule for AZM (log P = 3.03 and 2.44) due to its similar partition co-efficient. An *ex vivo* porcine eye model previously used to study corneal absorption [27] was further adapted to evaluate dye distribution in the tarsal conjunctiva. Freshly harvested porcine eyes (Fresh Pork, New Zealand) were collected and transported to the laboratory in phosphate buffered saline in an iced bin. The entire excised porcine eye was placed in a six-well microplate and a custom-made polycarbonate sleeve was affixed on top (Fig. 1). The cornea was covered with a section of porcine eyelid such that the tarsal conjunctival surface was in direct contact with the cornea. The model was incubated at 35 ± 3 °C for 10 min, after which the eyelids were carefully removed. A single 50 µl dose of SFA-BODIPY was then applied onto the cornea before repositioning the eyelid tissue. After 1 h, tissues were rinsed with HBSS and carefully excised. Ocular distribution of BODIPY in corneal and conjunctival tissues was qualitatively evaluated under a confocal laser scanning microscope (FV1000, Olympus, Japan). The amount of BODIPY in the cornea and tarsal conjunctiva after 1 h was also quantified as described previously [27].

2.4. Azithromycin susceptibility testing

Susceptibility and sensitivity of *S. aureus* Xen36 bacteria to AZM was evaluated by measuring the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) using the

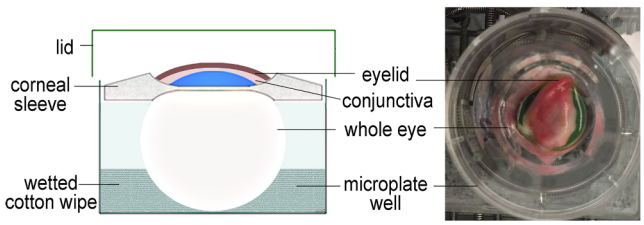


Fig. 1. *Ex vivo* whole-eye model used for studying corneal and conjunctival dye absorption. The model was set up by placing a section of porcine eyelid on top of the corneal sleeve with the conjunctival side facing the cornea.

broth microdilution method recommended by the National Committee for Clinical Laboratory Standards [34]. The MIC or the lowest concentration of AZM that caused at least 90% inhibition in bacterial growth was evaluated by observing the optical density at 600 nm (En-Spire® Multimodal Plate Reader, PerkinElmer, USA), as well as the bioluminescence with a 1 s exposure time (Victor X_1 Luminometer, PerkinElmer, USA). The MBC was determined by pipetting 20 µl of each MIC sample onto MH-agar plates and observing the minimum AZM concentration at which no colony forming units (CFU) could be observed after overnight incubation at 37 °C. Since DMSO was used to solubilise AZM for the positive control formulation, the MIC and MBC of a 1% v/v DMSO solution in MH medium was also determined. The median value of three technical replicates was computed to obtain the median MIC and MBC from three biological replicates performed on separate days.

2.5. Evaluation of the antibacterial activity of test formulations in vitro

The antibacterial potential of AZM formulations and vehicles was initially evaluated *in vitro* by adapting a previously described method [35]. The relationship between viable counts, measured as CFU, and bacterial bioluminescence, measured as relative light units (RLU) was studied by preparing two-fold dilutions of an overnight bacterial culture containing approximately 10^9 CFU/ml and plotting a calibration curve ($\log_{10}$ RLU/100 µl against $\log_{10}$ CFU/ml) to ascertain linearity, range and sensitivity of the bioluminescence measurements. For *in vitro* studies, inocula containing either 10^6 or 10^7 CFU/ml were prepared and a 10 µl dose of each test formulation was added to 90 µl of bacterial inoculum in a black 96-well microplate (Grenier Bio-One, Austria). Samples were shaken at 200 rpm and 37 °C before measuring the bioluminescence at 0, 0.5, 1, 2, 4, 6, 8 and 24 h. It should be noted that although the time 0 reading was taken immediately after sample addition, a lag time of few minutes is likely to have been introduced due to technical limitations including the time taken to transfer samples to the luminometer and read the plate. At the 24 h time point, samples from each well were plated on MH-agar plates and the number of CFU per ml of sample was counted after overnight incubation at 37 °C. Three technical replicates were performed on each occasion with the results being a representative of three biological replicates observed on separate days.

2.6. Optimisation of the ex vivo whole-eye model for antibacterial activity studies

The *ex vivo* whole-eye model described in Fig. 1 was adapted to evaluate the antibacterial activity of the test formulations by inoculating the cornea and tarsal conjunctiva with *S. aureus* Xen36 bacteria. The inoculation technique was optimised to consistently establish a detectable bacterial infection in the tissues. Three different inoculation methods were investigated. The first method involved wiping a 6.6 mm diameter section of the ocular tissue with 40% ethanol and scraping the epithelium with a surgical scalpel (Method 1). In the second method, the tissue was wounded by making three vertical and three horizontal incisions (Method 2), as previously described by Pinnock *et al.* [36]. Tissues prepared by Methods 1 and 2 were subsequently inoculated by applying 250 µl of a 10^7 CFU/ml *S. aureus* Xen36 inoculum inside the corneal sleeve and incubating the inoculated tissues at 37 °C. After 1 h, tissues were removed from the set-up and rinsed with HBSS. The third method involved injection of 50 µl of the 10^7 CFU/ml bacterial inoculum intra-stromally into the cornea or into the tarsal conjunctiva (Method 3). Bioluminescence in the cornea and conjunctiva was measured using the IVIS® Kinetic *In Vivo* Imaging System (Caliper Life Sciences, USA) in conjunction with Living Image® Software (Xenogen Corp., USA). Images were obtained using the photon-counting mode in which individual photons emitted by the bacterial cells were detected and integrated. A grey-scale background image was first created and a bioluminescence image displaying a graded rainbow pseudocolour scale ranging from red (most intense) to blue (least intense) was superimposed. The bioluminescence signal was accumulated over 1 min and images were recorded in auto bit-range mode. The total flux (photons/s) was used as a surrogate measure of bacterial viability in the region of interest (ROI), which was kept constant in all images.

2.7. Evaluation of the antibacterial activity of test formulations ex vivo

Ex vivo studies were performed using the adapted whole-eye model described above in which bacterial infection was established by injecting the inoculum intra-stromally into the cornea and the tarsal conjunctiva (Method 3) and bacterial bioluminescence in each tissue was observed immediately after inoculation (time 0). A single 50 µl dose of each test formulation (Table 1) was then applied to the corneal surface before placing the eyelid on top with the colonised tarsal conjunctiva facing the cornea. Eyes were incubated at 37 °C covered with a tissue culture dish to minimise excessive tissue dehydration and formulation evaporation. Bacterial bioluminescence was evaluated at 0.5, 1, 2, 4, 6, 8 and 24 h after carefully transferring the eyelid to a petri dish and imaging cornea and eyelid (with the conjunctival surface facing upwards), separately. Once bioluminescence measurements had been taken, the eyelid was placed back onto the cornea, with the epithelial surfaces adjacent, and the entire set-up was returned to the incubator.

2.8. Data analysis

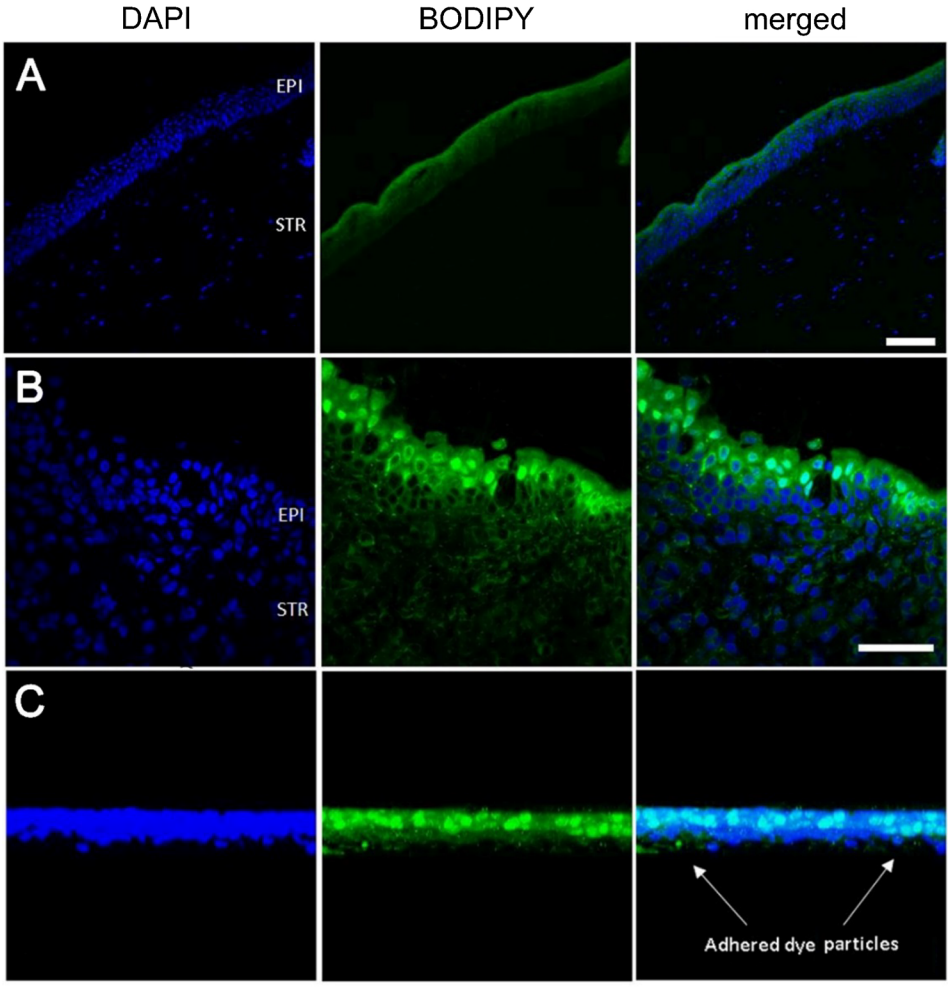
Statistical significance of difference in antibacterial activity between the test formulations *in vitro* and *ex vivo* was evaluated by plotting the bioluminescence observed at each time point as a percentage of the baseline (time 0) value against time and calculating the area under the curve (AUC), which was then compared by ordinary one-way ANOVA. Post-hoc multiple comparisons between SFA-AZM and the positive control, AzaSite and Azyter were performed as per the Holm-Sidak's method to maximise the power of the test. All data analysis was performed using GraphPad Prism V.7.00.

3. Results and discussion

3.1. Visualisation of dye absorption from an SFA-BODIPY suspension

An *ex vivo* porcine eye model was used due to the structural and morphological similarities between porcine and human eyes [37]. Preferential localisation of BODIPY within the superficial layers of the corneal and conjunctival epithelium was observed, although the intensity of BODIPY fluorescence was higher in the conjunctiva than in the cornea (Fig. 2A and B).

Corneal observations were in agreement with previous studies demonstrating the tendency of hydrophobic drugs and dyes to accumulate in the hydrophobic epithelium with very slow transport into the hydrophilic stroma [27]. Studies evaluating corneal distribution of AZM by desorption electrospray ionisation imaging post-instillation of topical eye drops have shown similar results with preferential localisation of AZM in the corneal epithelium being observed for up to 2 h *in vivo* [38], thus demonstrating that BODIPY closely mimics the ocular distribution characteristics of AZM in this *ex vivo* model. Conjunctival absorption was several folds higher than that seen in the cornea on application of the SFA-BODIPY suspension (Fig. 2A and B), which may be attributed to the significantly larger intercellular spaces in the conjunctival epithelium [39]. It should be noted that, despite repeatedly rinsing the tissue before fixation, undissolved BODIPY particles could be seen adhering to the conjunctival surface without entirely penetrating it (Fig. 2C), which may also have contributed to the 500-fold higher dye concentrations observed in the conjunctival tissue in comparison to the cornea.



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BODIPY concentration	Cornea	Conjunctiva	Aqueous humour
µg/g of tissue	2.232 ± 0.297	1112.084 ± 47.462	0.040 ± 0.008
µg/ tissue	0.464 ± 0.122	39.407 ± 1.709	0.007 ± 0.004
% dose penetrated	0.062 ± 0.016	5.254 ± 0.228	0.001 ± 0.001

Fig. 2. Ocular absorption of a hydrophobic dye from a SFA-BODIPY suspension. BODIPY (green) localisation was observed in the A. corneal and B. conjunctival epithelium (EPI; nuclei stained with DAPI in blue) with limited absorption into the stroma (STR). C. Rotated z-stack images showing the suspended dye particles adhering to the superficial conjunctival epithelial surface; scale bar = 100 µm. D. BODIPY concentrations determined in ocular tissues after dye extraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

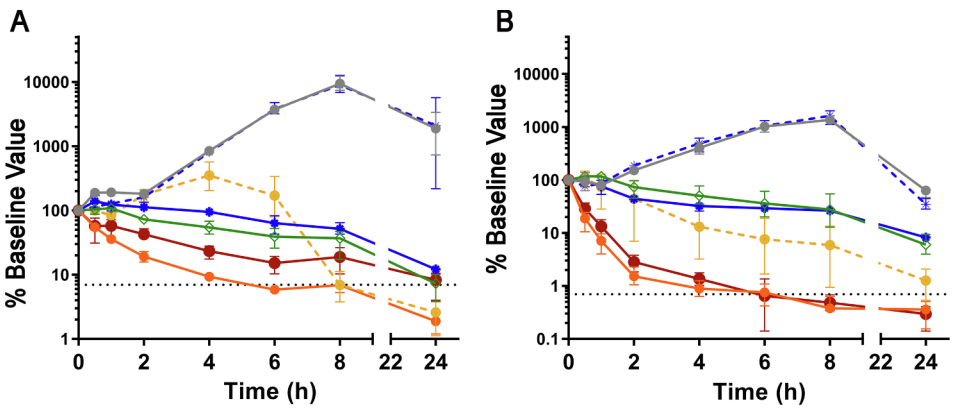
3.2. Azithromycin susceptibility testing

Susceptibility and sensitivity testing of *S. aureus* Xen36 bacteria to AZM in 1% v/v DMSO in MH medium was performed by measuring bacterial bioluminescence and optical density. The MIC of AZM against *S. aureus* ranged between 1.56 and 3.125 µg/ml (median 1.56 µg/ml) using both methods, which is comparable to the MIC reported previously for 89 AZM-susceptible *S. aureus* ocular isolates collected from 35 different institutions in the USA [40]. The MBC of AZM was 16-fold greater and ranged between 25 and 50 µg/ml with a median value of 25 µg/ml. The MIC, MBC and MIC/MBC ratio observed were also in close agreement with previous studies performed on *S. aureus* strains [40,41]. No inhibition of bacterial growth was observed with the negative control (MH medium) or with 1% v/v DMSO in MH medium, which was used to solubilise AZM in the positive control.

3.3. Evaluation of the antibacterial activity of test formulations in vitro

Although antibacterial efficacy often differs considerably under *in vitro* and *ex vivo* conditions, *in vitro* studies were initially performed as a developmental tool for optimisation of the more relevant *ex vivo* model and to test the antibacterial potential of other formulation excipients. The density of metabolically active bacteria was estimated by measuring the bioluminescence, which was found to increase linearly in the range of 2.52×10^6 to 9.67×10^8 CFU/ml ($R^2 = 0.9915$). Therefore, both 10^6 CFU/ml and 10^7 CFU/ ml inocula were used to improve the test sensitivity (Fig. 3).

Among the vehicles tested, pure SFA did not show any antibacterial activity *in vitro* with the bioluminescence curve similar to that of the negative control (MH medium). The bacterial density, evaluated by plating samples after 24 h, was in the order of 10^9 CFU/ml for pure SFA and the negative control, irrespective of the initial bacterial density. However, MCT, which is the vehicle in Azyter eye drops, showed a



C

Activity of SFA-AZM *vs.*	inoculum = 10^6 CFU/ml	inoculum = 10^7 CFU/ml
Positive Control	ns	ns
Azyter	* *a*	* *a*
AzaSite	* *a*	* *a*

Fig. 3. Bacterial bioluminescence observed after exposure to the test formulations for 24 h *in vitro*. The bioluminescence after application of the test formulations to inocula containing either A. 10^6 or B. 10^7 CFU/ml was calculated as a percentage of the baseline (time 0) value (measured in RLU) at each time point and plotted against time (n = 3; median ± range). The dotted black line represents the sensitivity threshold. C. The AUC of SFA-AZM was statistically compared to the positive control (1.5% AZM solution in DMSO), Azyter and AzaSite. ns: no significant difference; *a: antibacterial activity of SFA-AZM is significantly lower.

concentration-dependent antibacterial activity with a bactericidal effect being evident against the 10^6 CFU/ml inoculum, while only a bacteriostatic effect was observed at the higher bacterial density of 10^7 CFU/ml.

A bactericidal effect was observed 24 h after application of all AZM containing test formulations with no CFUs being observed in plated samples suggesting that the available AZM concentration was above the MBC. The antibacterial activity of SFA-AZM was comparable to that of the positive control (1.5% AZM solution in DMSO) with only a bacteriostatic effect being evident for the first 8 h. At the 24 h time point, although no CFUs were observed on plating samples, some bioluminescence within the calibration range could still be observed. The reason for this residual bioluminescence in the absence of bacterial growth is not definitively known. Antibacterial activity of AZM has previously been evaluated either by conventional colony counting methods *in vitro* [41,42], or by measuring the bioluminescence *in vivo* alone [43], thus leaving an obvious void in our knowledge of the correlation between bacterial bioluminescence and colony counts on prolonged exposure to macrolide antibiotics *in vitro*. However, it may be speculated that due to its unique mechanism of action involving time-dependent inhibition of protein synthesis [9], exposure of bacteria to AZM for up to 24 h only injures or weakens the bacteria. These injured bacteria, although incapable of proliferation, may still be metabolically active and retain functional intracellular biochemistry leading to the weak bioluminescence signal [44].

The *in vitro* antibacterial activity of Azyter and AzaSite was significantly greater than that of the positive control and SFA-AZM with a log two-fold reduction in bioluminescence being evident within the first 2 to 4 h. The bioluminescence curves of Azyter and its vehicle MCT closely paralleled each other suggesting that the *in vitro* antibacterial effect of Azyter may largely be dependent on the vehicle. This is in agreement with previous *in vitro* studies suggesting synergistic enhancement of the antibacterial efficacy of antibiotics in the presence of MCT [45–47]. Similarly, the DuraSite vehicle in AzaSite may have also enhanced its antibacterial activity *in vitro*. Since the DuraSite platform technology is not commercially available, and its detailed composition is proprietary information, its antibacterial activity could not be determined in the present study. However, previous studies have shown that the DuraSite vehicle possesses a significant bacteriostatic effect of its own, which is further enhanced by benzalkonium chloride present in the formulation as a preservative [48].

3.4. Optimisation of the ex vivo whole-eye model for antibacterial activity studies

The *ex vivo* whole-eye model was optimised to identify the most appropriate method of tissue inoculation to establish a traceable infection in a consistent manner. Bacterial bioluminescence in corneas inoculated with the bacterial culture after epithelial scraping (Method 1, Fig. 4A) or incision (Method 2, Fig. 4B) was too low to be reliably detected by the IVIS system. Adherence of bacteria to the corneal tissue was minimal with preferential accumulation being demonstrated towards the surrounding bulbar conjunctiva, despite repeated rinsing. Intrastromal injection of the bacterial inoculum (Method 3, Fig. 4C)

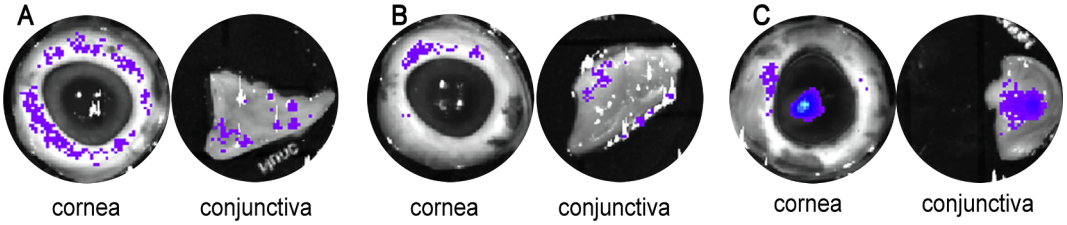


Fig. 4. Optimisation of the *ex vivo* whole-eye model to compare the antibacterial activity of the test formulations. Representative images of corneas and conjunctivas inoculated with bacteria either by A. scraping off the epithelium (Method 1), B. making three vertical and three horizontal incisions (Method 2), or C. injecting 50 µl of the inoculum into the stroma of the tissues (Method 3).

significantly increased the bioluminescence signal, which was consistently observed in all six corneal samples. Method 3 also ensured that bacteria remained localised within the corneal tissue.

In the tarsal conjunctiva, bacterial bioluminescence was evident in all samples irrespective of the inoculation technique; however, bacterial colonisation was most reproducibly obtained after conjunctival injection (Fig. 4C) with bacteria remaining localised within the conjunctiva, thus minimising contamination of adjacent areas. Intrastromal injections yielded a bacterial density of approximately 10^6–10^7 CFU/g of cornea and 10^7–10^8 CFU/g of conjunctiva, which is comparable to that previously observed in ocular surface diseases such as bacterial keratitis, conjunctivitis and blepharitis [49,50].

It should be noted, however, that while *S. aureus* bacteria have a historical association in ocular surface disorders, several different bacteria make up the ocular surface microbiome [51]. Thus, additional studies with other bacterial strains prevalent in ocular surface disorders may be beneficial. Furthermore, while the developed *ex vivo* model is able to mimic physiological conditions more accurately than *in vitro* studies, correlation with *in vivo* animal models may need to be established, especially considering the lack of a tear clearance mechanism in the studies performed here.

3.5. Evaluation of the antibacterial activity of test formulations ex vivo

Bioluminescent bacteria appeared to proliferate rapidly in corneal and conjunctival tissues (Fig. 5) with an exponential increase in bacterial bioluminescence evident over 24 h in the presence of MH medium (negative control).

Bacteria appeared to have spread across the entire tissue by the 24 h time point (Fig. 6) although the bioluminescence intensity was significantly lower in corneas treated with AZM-containing test formulations (Fig. 6D–G) compared to the vehicle groups (Fig. 6A–C). Contrary to the effect observed *in vitro*, MCT alone did not show any antibacterial activity in the *ex vivo* studies and the bioluminescence curve for MCT-treated ocular tissues remained similar to that of the negative control, possibly due to poor tissue absorption and low intracellular MCT concentrations after topical application.

A 0.5–1 $\log_{10}$ increase in bioluminescence was evident in all corneal samples treated with AZM test formulations with no significant difference between them. In contrast to the observations made *in vitro*, the antibacterial efficacy of SFA-AZM and the positive control was similar to that of the commercial formulations with their bioluminescence AUC not differing significantly in corneal tissues (Fig. 5C). Benzalkonium chloride has previously been shown to increase corneal epithelial damage [8] and elevate inflammatory and apoptotic effects in the cornea and conjunctiva [52,53], thus reducing the barrier properties to drug penetration [54] and therefore potentially enhancing drug efficacy. Nevertheless, the presence of benzalkonium chloride in the AzaSite formulation did not seem to provide any superior antibacterial effect to it. Similarly, no improvement in antibacterial effect was observed using the positive control prepared in DMSO, despite the vehicle's reported penetration enhancing effect.

Previous *in vivo* studies evaluating ocular drug pharmacokinetics after instillation of a single dose of Azyter and AzaSite have shown a corneal C_{max} of 9.3 and 40.4 µg/g, respectively [55,56]. Since the *ex vivo* model used in this study did not have any clearance mechanisms to simulate tear flow, it is reasonable to expect that corneal concentrations achieved would at least be equivalent to the MIC of 25 µg/ml. Nevertheless, the bioluminescence curves observed in corneas treated with AZM test formulations were similar to those previously observed on exposure of *S. aureus* cultures to sub-MIC AZM concentrations [42]. These observations may be related to the tendency of hydrophobic drugs to remain localised in the corneal epithelium (Fig. 2) leading to sub-MIC AZM concentrations in the inoculated stroma.

The intensity and area of bioluminescence appeared to increase more rapidly in the conjunctiva (Fig. 7) than in the cornea (Fig. 6). Rapid bacterial growth with colonisation of the entire tissue was observed in negative control tissues receiving MH medium only (Fig. 7A). Similar to the observations made in the cornea, but contrary to the *in vitro* observations, MCT did not appear to have any antibacterial effect in the *ex vivo* conjunctival tissue (Fig. 7C). Overall, the antibacterial effect of all AZM test formulations in the conjunctiva (Fig. 7D–G) was distinctly more pronounced than in the corneal tissue, possibly due to the significantly greater conjunctival absorption and permeability of drug in line with observations made in Fig. 2. Nevertheless, antibacterial efficacy of the tested AZM test formulations differed considerably. Conjunctivas treated with SFA-AZM and AzaSite (Fig. 7E and G) showed a substantial reduction in the area and intensity of bacterial

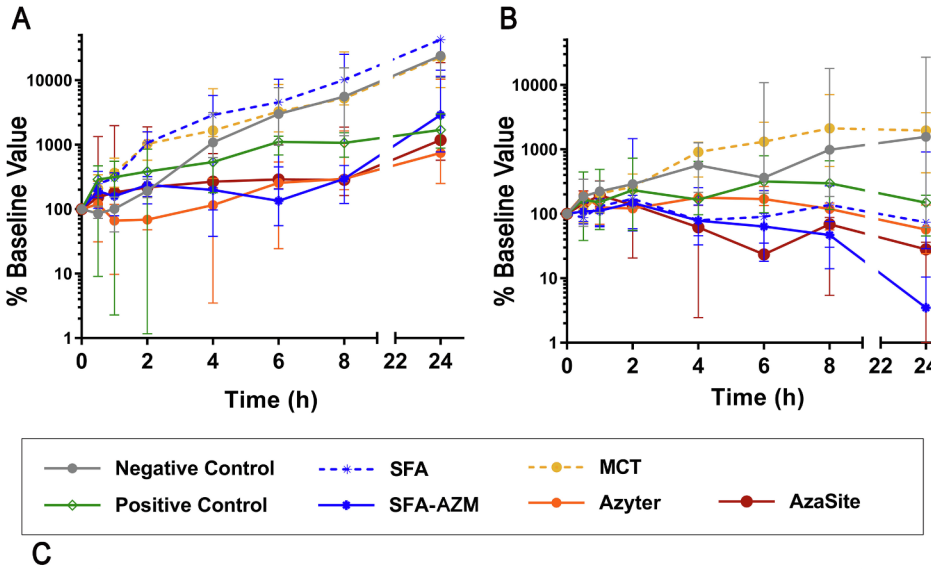


Fig. 5. Bacterial bioluminescence observed after exposure to the test formulations for 24 h *ex vivo*. The bioluminescence after application of the test formulations to colonised A. corneas or B. conjunctivas was calculated as a percentage of the baseline (time 0) value (measured as photons/s) at each time point and plotted against time (n = 3; median ± range). C. The AUC of SFA-AZM was statistically compared to the positive control (1.5% AZM solution in DMSO), Azyter and AzaSite. ns: no significant difference; *b*: antibacterial activity of SFA-AZM is significantly greater.

C

Activity of SFA-AZM *vs.*	cornea	conjunctiva
Positive Control	ns	* *b*
Azyter	ns	* *b*
AzaSite	ns	ns

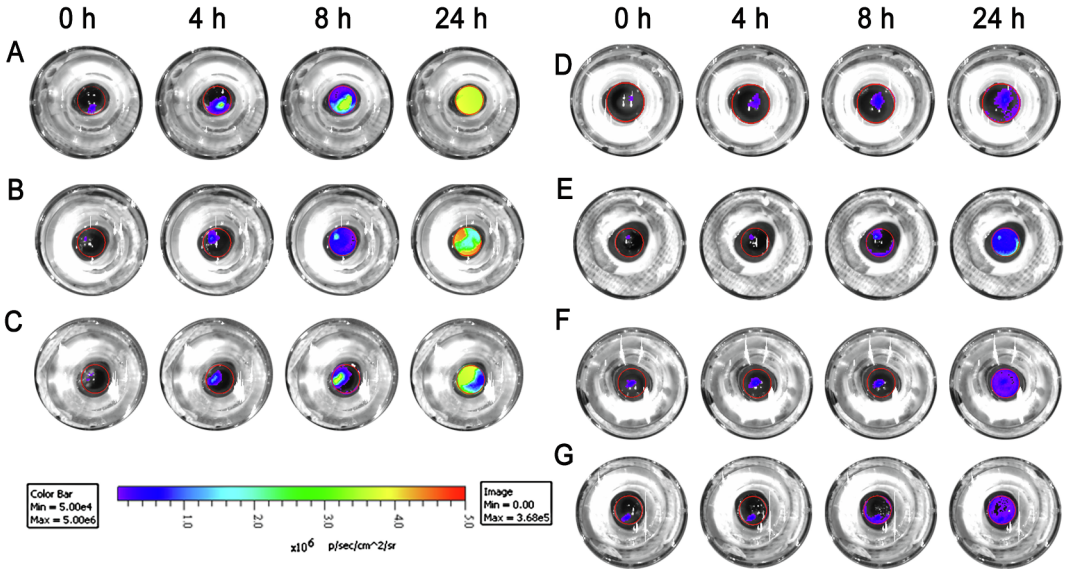


Fig. 6. Bacterial bioluminescence in corneas treated with the test formulations. Representative images showing bacterial bioluminescence over 24 h in the ROI (red circle) of corneas treated with the A. Negative Control, B. SFA, C. MCT, D. Positive Control, E. SFA-AZM, F. Azyter and G. AzaSite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

colonisation. The bioluminescence curves obtained for SFA-AZM and AzaSite treated conjunctivas were almost identical with the AUC not differing significantly (Fig. 5C). On the other hand, the intensity and area of bioluminescence in conjunctivas treated with the positive control and Azyter remained unchanged over the test period (Fig. 7D and F) with their AUC being significantly larger than that obtained with SFA-AZM or AzaSite application (a larger AUC here is indicative of more bacteria and, therefore, lower drug efficacy). The reduction in conjunctival bioluminescence after 24 h was greatest for SFA-AZM treated conjunctivas, which showed more than a $\log_{10}$ (90%) reduction in bioluminescence from baseline. It is likely that intracellular AZM concentrations in conjunctivas treated with SFA-AZM were equivalent to, or higher than, the MBC, possibly due to adhesion and enhanced absorption of AZM particles into the conjunctival tissue using the SFA vehicle. Moreover, due to the excellent spreadability of SFAs on the ocular surface [57], the SFA-AZM formulation is likely to have accessed and covered the conjunctival tissue more intimately, thus reaching the bacteria in the conjunctival folds more efficiently.

Poor *in vitro-in vivo* correlation with respect to antibacterial activity of antibiotic agents has frequently been reported in the literature [32,33] and several factors may be responsible for this. AZM is generally bacteriostatic against *S. aureus*, both *in vitro* and *in vivo,* with less

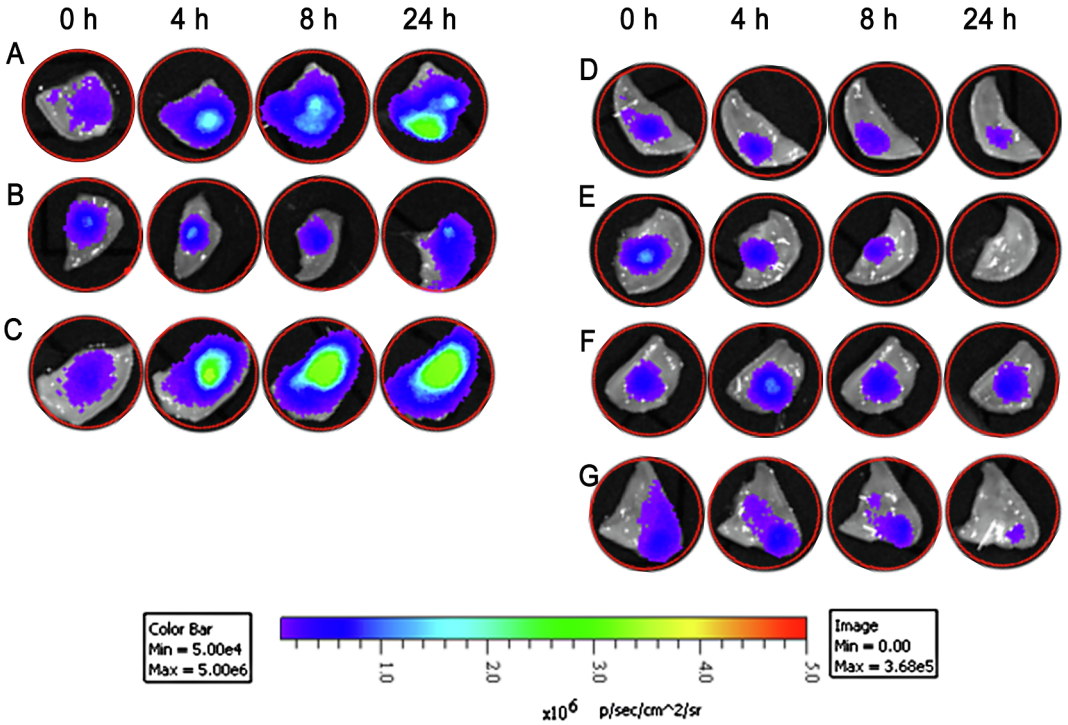


Fig. 7. Bacterial bioluminescence in conjunctivas treated with the test formulations. Representative images showing bacterial bioluminescence over 24 h in the ROI in eyelids treated with A. Negative Control, B. SFA, C. MCT, D. Positive Control, E. SFA-AZM, F. Azyter and G. AzaSite.

Table 2
Antibacterial activity of the test formulations observed *in vitro* and *ex vivo*.

	In Vitro		*Ex Vivo*	
Test Formulations	Inoculum = 10^6 CFU/ml	Inoculum = 10^7 CFU/ml	Cornea	Conjunctiva
Negative Control	Normal Growth	Normal Growth	Normal Growth	Normal Growth
SFA	Normal Growth	Normal Growth	Normal Growth	Bacteriostatic
MCT	Bactericidal	Bacteriostatic	Normal Growth	Normal Growth
Positive Control	Bactericidal	Bactericidal	Reduced Growth‡	Bacteriostatic
SFA-AZM	Bactericidal	Bactericidal	Reduced Growth‡	Bactericidal¥
Azyter	Bactericidal	Bactericidal	Reduced Growth‡	Bacteriostatic
AzaSite	Bactericidal	Bactericidal	Reduced Growth‡	Bacteriostatic

‡ The rate of bioluminescence increase was lower than for the control formulation but the test formulations were not bacteriostatic.
¥ More than a $\log_{10}$ reduction in bioluminescence.

than a $\log_{10}$ reduction in bacterial colony counts typically being observed after 24 h. However, even this bacteriostatic effect has previously been shown to be submaximal *in vivo*, irrespective of the level of intracellular drug accumulation [33,58,59]. Differences in intra-/extracellular drug concentrations and the duration of exposure to AZM, as well as other formulation excipients, may play an important role in determining the overall antibacterial efficacy [42,58]. Several other intrinsic and extrinsic factors, such as different subcellular locations of the antibiotic and the bacteria (as is believed to be the case in the corneal tissue) or impaired expression of the antibacterial activity in the intracellular environment due to drug metabolism, protein binding or pH alterations (as may have been the case with AzaSite which being slightly acidic may have reduced the pH of the bacterial medium *in vitro* but not *ex vivo*), could also be responsible for the poor *in vitro-ex vivo* correlation observed in the present study (Table 2). Additionally, altered bacterial responsiveness due to a change in bacterial metabolism or growth rate within the tissue may also influence the level of intracellular drug activity [59].

An expected limitation of the current *ex vivo* model is the absence of the lid blinking and tear turnover mechanisms, which would likely reduce the precorneal residence time of all formulations *in vivo*. It should be noted though that the precorneal residence time of SFAs has previously been evaluated *ex vivo* and was found to be significantly longer than that of typical oil-in-water emulsions [27]. It is anticipated that due to the adherence of the suspended AZM particles to the mucinous conjunctival tissue, the drug may be retained on the ocular surface for longer with gradual dissolution and absorption of the adhered particles further enhancing the antibacterial effect in the conjunctiva. *In vivo* pharmacokinetic studies with SFA-AZM in rabbits confirmed therapeutic AZM levels in anterior segment tissues up to 144 h post dosing [60], with the formulation also being well tolerated.

Overall, the observations made in this study raise significant concerns about the relevance of *in vitro* tests in predicting the efficacy of different formulations and stress the importance of using suitable *ex vivo* and *in vivo* models to measure the actual intracellular activity instead of making predictions based on extracellular pharmacokinetics and MICs [32,33].

4. Conclusion

A preservative free AZM suspension was developed using the SFA perfluorohexyloctane as the non-aqueous vehicle for topical application to the eye and its efficacy in the management of ocular infections was evaluated *in vitro* and *ex vivo*. Bioluminescent bacteria were used to compare the antibacterial efficacy of the different AZM formulations and their vehicles in real-time. Substantial differences between the *in vitro* and *ex vivo* antibacterial activity of the test formulations were observed. Moreover, the *ex vivo* antibacterial efficacy of the test formulations in corneal and conjunctival tissues also differed significantly. These observations raise concerns about the validity of *in vitro* studies when comparing the efficacy of ocular antibacterial formulations since numerous intrinsic and extrinsic factors can influence the antibacterial activity within tissues.

The additive antibacterial effects of formulation excipients in Azyter and AzaSite observed *in vitro* were not translated to the *ex vivo* setting. Moreover, *ex vivo* studies suggested that topically applied AZM eye drops have poor antibacterial efficacy against stromal infections, possibly due to preferential localisation of hydrophobic AZM in the corneal epithelium. Thus, no significant differences were observed between the antibacterial potential of SFA-AZM, Azyter and AzaSite in the cornea. On the other hand, a significant reduction in area and intensity of bacterial bioluminescence was observed in the conjunctiva on application of SFA-AZM and its antibacterial efficacy was similar to that observed with AzaSite, but better than that of Azyter, suggesting that the here presented *ex vivo* model may be more suitable to distinguish between the antibacterial efficacy of various formulations than currently applied *in vitro* tests.

Overall, the results suggest that SFA-based suspensions have high potential to enhance ocular drug absorption, especially in the conjunctiva, due to adhesion of the suspended drug particles to the conjunctival surface. With SFAs being a preservative-free, non-aqueous vehicle, they are likely to be well tolerated on the ocular surface and SFA-AZM may thus provide a safe and effective treatment option for ocular surface infections. However, parameters such as particle size, suspension stability, production costs and patient acceptance need to be further considered when formulating SFA-based suspensions.

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