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Preclinical development of sodium fusidate antibiotic cutaneous spray based on water-free lipid formulation system

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ABSTRACT

Topical antibiotics are a key component in the management of mild to moderate skin and soft tissue infections. There are, however, concerns about the emerging bacterial resistance against topical antibacterial agents such as fusidic acid, due to the prolonged treatment period of its marketed dosage forms. Improving the efficacy of topical formulations could potentially shorten the treatment period and avoid the resistance growth. To provide a more effective drug delivery, a water-free lipid-based formulation system (AKVANO®) which can be applied by spraying, has been developed. In the current paper, different formulations containing sodium fusidate were evaluated for their *in vitro* skin permeability using artificial skin mimicking membranes and antibacterial properties using *ex vivo* and *in vivo* skin wound infection models. The novel formulations containing sodium fusidate showed a much higher skin permeation (up to 60% of nominal amount) than the commercially available Fucidin® cream (3%). These formulations also gave a significantly stronger antibacterial effect than Fucidin cream showing a clear dose-response relationship for the sodium fusidate content. A spray product based on the described formulation technology would therefore require a shorter treatment time and thereby lower the risk for the development of bacterial resistance. Spray administration of these formulations provides an even layer on the skin surface from which the solvent quickly evaporates and thereby facilitates a non-touch application where no rubbing is required.

1. Introduction

Topical antibiotic therapy has recently emerged as a fast-growing therapeutic field in the treatment of bacterial infections of skin and mucous membranes. Topical antibacterial agents carry some additional advantages over the systemic agents that includes high and sustained local concentrations at the infection site, better compliance, moisturizing properties, low cost of production, and less adverse effects (Mayba and Gooderham, 2017). Several classes of topical antibacterial agents, both antibiotics and antiseptics have been identified, which have shown beneficial effects on the overall management of skin diseases (Long, 2008). Fusidic acid is one such topical antibiotic which, despite having a narrow antibacterial spectrum against Gram-positive bacteria, has a high activity against *Staphylococcus aureus* (*S. aureus*), a commonly known skin pathogen (Bishop and Howden, 2007). Fusidic acid treatment has earned significant interest in the last decade due to its potential role in combat against superficial skin and soft tissue infections (Schöfer and Simonsen, 2010). There are different topical formulations available for fusidic acid and one such is Fucidin® cream which is widely marketed and used. For the treatment of primary skin infections, fusidic acid cream has been reported to be as effective as other topical and oral antibiotics (Hamann and Thorn, 1991). It is indicated against the sensitive strains of *S. aureus, Streptococcus* species and *Corynebacterium minutissimum* in the treatment of primary and secondary skin infections

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Table 1
Compositions used in the experiments.

Formulation	Sodium fusidate (% w/w)	Soy-PC (% w/w)	Isopropyl myristate (% w/w)	Medium chain monoglycerides (% w/w)	EtOH (% w/w)	Cyclomethicone D5 (% w/w)	Additional components (% w/w)
AKVF01a	2.07	20.2	–	–	77.7	–	–
AKVF01b	2.24	10.1	5.4	6.0	76.2	–	–
AKVF01c	1.94	5.0	10.4	10.5	72.1	–	–
AKVF02a Placebo	–	20.3	–	–	20.3	59.4	–
AKVF02a	1.93	20.5	–	–	77.6	–	–
AKVF02c	1.94	5.0	10.4	10.5	72.1	–	–
AKVF03a	2.07	19.9	–	–	20.2	57.9	–
AKVF03c	1.99	5.2	10.5	9.9	19.5	52.9	–
AKVF04a Placebo	–	19.8	–	–	20.1	60.1	–
AKVF04a 0.5%	0.50	19.7	–	–	20.0	59.8	–
AKVF04a 1%	1.00	19.6	–	–	19.9	59.5	–
AKVF04a 2%	1.94	19.4	–	–	19.8	58.9	–
AKVF04b 1%	0.99	9.8	5.5	5.3	19.4	59.1	–
AKVF04c Placebo	–	5.0	10.1	10.0	19.8	55.3	–
AKVF04c 0.5%	0.49	4.9	10.0	9.9	19.7	55.0	–
AKVF04c 1%	1.00	4.9	10.0	9.9	19.6	54.7	–
AKVF04c 2%	1.99	4.8	9.9	9.8	19.4	54.2	–
Na fusidate 1% in EtOH	1.00	–	–	–	99.0	–	–
AKVF05 Placebo	–	5.1	10.2	10.2	19.9	54.6	–
AKVF05	1.95	5.0	10.0	10.0	19.5	53.5	–
AKVF06 Placebo	–	9.7	5.4	10.0	20.0	49.7	Lactic acid (90%) 5.1
AKVF06	2.04	9.5	5.3	9.8	19.6	48.7	Lactic acid (90%) 5.0
AKVF07 Placebo	–	10.2	4.9	5.0	45.5	29.8	Urea 2.0 Lactic acid (90%) 2.7
AKVF07	2.07	10.0	4.8	4.9	44.5	29.2	Urea 2.0 Lactic acid (90%) 2.6
AKVF08 Placebo	–	5.2	10.0	10.1	20.0	54.8	–
AKVF08	1.99	5.1	10.0	9.9	20.2	52.7	–
AKVF09 Placebo	–	10.2	5.0	10.0	19.9	49.8	Lactic acid (90%) 5.0
AKVF09	1.99	10.0	5.0	10.0	20.2	47.8	Lactic acid (90%) 5.1

(Long, 2008). The active ingredient in Fucidin cream and other fusidic acid preparations is either the free acid or the sodium salt. In an environment with water activity such as the skin, the two forms will be in equilibrium. pKa of fusidic acid (5.35) (Compound Summary for Fusidic acid, 2022) is within the range of a typical pH of the skin, and therefore both the protonated and deprotonated forms will be present.

A major problem associated with the inappropriate and extreme use of antibiotics is the emergence of bacterial resistance (Pulingam et al., 2022). This is of growing concern with fusidic acid since its bactericidal effect is comparatively low as compared to the others in the class. Therefore, the treatment period with fusidic acid needs to be prolonged ($\geq$ 7 days), which creates a high risk for the development of bacterial resistance (Heng et al., 2013). Reports of fusidic acid resistance have been known for a long time in dermatology departments and other closed hospital environments having a high risk of cross-infection (Reed et al., 1999; Shanson, 1990). Looking at the global concerns regarding the antibiotic resistance and relatively fewer therapeutic options against some widespread skin pathogens, such as *S. aureus*, the appropriate development of an effective topical formulation system is critical. A mitigation of the bacterial resistance could be to improve the drug delivery characteristics of the topical formulations. Recent development of lipid-based formulations has allowed a precise release of medications at the site of infection (Holmbäck et al., 2022) and can play an important role in overcoming the problem of bacterial resistance.

The AKVANO® technology is based on water-free lipid formulations (Holmbäck et al., 2022). The active ingredient is dissolved in a volatile solvent system along with selective lipids, which provides different characteristics to the formulation. The resulting formulation is a sprayable liquid, suitable for topical administration. After evaporation of the solvent, a thin lipid layer is formed on the skin surface for effective deposition of the active ingredient. Direct deposition of lipids and active ingredients leads to an efficient physicochemical interaction with the skin. The AKVANO solvent is typically a short-chain alcohol such as ethanol. Alcohols have an inherent antiseptic property which makes them an essential component of the AKVANO system. In contrast to water, ethanol can dissolve the lipids, forming an unorganized (isotropic) solution in which the lipid molecules are apparently molecularly dispersed. The absence of organization, *i.e.*, no significant presence of ordered self-assembled aggregates, makes the solution low-viscous, with a viscosity close to that of the neat solvent. Alcohols are not only good solvents for lipids, but also for many active ingredients, which serves as an additional advantage in their usage in the AKVANO formulation. AKVANO formulations can also contain other excipients depending on the required properties. Keratolytic agents such as urea and lactic acid are especially useful due to their emollient and moisturizing effects, thereby improving the cosmetic qualities of the product.

In this study, different AKVANO formulations containing sodium fusidate were evaluated for their *in vitro* skin permeability using artificial skin membranes and antibacterial properties using *ex vivo* and *in vivo* wound infection models. Two initial screening experiments were performed in an *ex vivo* pig skin wound infection model, as described before (Boge et al., 2018; Håkansson et al., 2019; Schmidtchen et al., 2009). The best candidates were then evaluated in an *in vivo* mouse model of superficial skin wound infections (Håkansson et al., 2021).

2. Materials and methods

2.1. Composition and preparation of sodium fusidate in AKVANO

A detailed description of the AKVANO composition has been published recently (Holmbäck et al., 2022). In brief, along with active pharmaceutical ingredient (sodium fusidate) main pharmaceutical excipients are lipids (phospholipids from soybean, single chain lipids such as monoglycerides and isopropyl myristate), alcohol (anhydrous ethanol), and optionally keratolytic agents (such as lactic acid and urea) and volatile silicone oil (cyclomethicones or dimethicones). Preparation of the AKVANO formulations follows a general procedure. The aim is to

obtain a clear cutaneous solution by dissolving phospholipids with additional lipids and part of the ethanol. In a separate vial, sodium fusidate is weighed and dissolved in the remaining ethanol. The mixture is stirred using a magnetic stirrer until a clear solution is obtained, and finally the active solution and cyclomethicone (D5) are added. The preparation procedure can be further adjusted depending on the requirements of the specific formulation, and especially when the batch size is scaled up.

The composition of different AKVANO formulations without (placebo) and with sodium fusidate studied in this paper are listed in Table 1. Phospholipid used was soybean phosphatidylcholine (Soy-PC) obtained from Lipoid AG (Steinhausen, Switzerland). Monoglycerides used were medium chain monoglycerides (MCM) from Abitec Corp. (Columbus, OH, USA). Isopropyl myristate (IPM), urea and lactic acid were obtained from Sigma-Aldrich (Darmstadt, Germany). Sodium fusidate was obtained from Ercros S.A (Barcelona, Spain). Absolute ethanol (EtOH) used was from VWR (Stockholm, Sweden). Cyclomethicone was supplied by DuPont de Nemours, Inc, USA. Fucidin cream 2% (containing fusidic acid 20 mg/g) was from Leo Pharma (Denmark). HPLC solvents were procured from Rathburn Chemicals (Scotland, UK). *S. aureus* was obtained from CCUG (Gothenburg, Sweden). Brain heart infusion (BHI) medium was procured from Service Substrat, Sahlgrenska University Hospital (Gothenburg, Sweden). The pig skins used were obtained from pigs of a mixed breed of Yorkshire, Hampshire and Swedish Pigham. The pigs were used for other research studies; however, the skin was harvested after euthanization and used in the current study. Therefore, no additional ethical approval was needed for using the skin in these studies.

The AKVANO formulations used in these experiments were of three different types: (a) containing Soy-PC as the only lipid, (b) containing approximately equal amounts of Soy-PC and single chain lipids (medium chain monoglycerides and isopropyl myristate) and (c) containing a high concentration of single chain lipids. The nominal concentrations of sodium fusidate in the AKVANO formulations were 0.5%, 1% or 2% (w/w), corresponding to 4.8, 9.6 and 19.2 mg/g of fusidic acid.

2.2. HPLC method for analysis of sodium fusidate in AKVANO

The concentration of sodium fusidate in samples from *in vitro* permeation studies was analysed by RP-HPLC (Agilent Technologies Inc., Santa Clara, CA, USA) with UV detection at 240 nm. The separation for sodium fusidate was carried out on a Symmetry C8 column (150×3.9 mm, particle size 5 μm, HPLC-5) from Waters (USA) at ambient temperature. Sodium fusidate was eluted at 9.6 min using a flow rate of 1–2 ml/min with 60% A to 100% B in 18 min, where A was methanol/water (40:60) + 0.16% triethylamine + 0.16% acetic acid and B was methanol + 0.16% triethylamine + 0.16% acetic acid and a total run time of 25 min with injection volume of 5 μl. Samples containing 2% (w/w) sodium fusidate were diluted with ethanol (1 + 9) prior to injection. The detection and quantification limits of fusidic acid for the permeation study were 0.2 and 0.6 μg/ml when the signal to noise ratio were 3 and 10 respectively. The linear range was 4–40 μg/ml ($R^2 = 0.9999$). The linearity of 4–40 mg/g ($R^2 = 0.9996$) was determined for the assay of the formulation. The same method with slight modifications was applied for the better analysis of in-house stability samples. The flow rate of 0.9–1.0 ml/min was used with 100% A to 40% B in 19 mins, where A was methanol/water (64:36) + 0.16% triethylamine + 0.16% acetic acid and B was methanol + 0.16% triethylamine + 0.16% acetic acid with injection volume of 2 μl.

2.3. In vitro permeation experiments of sodium fusidate in AKVANO

The diffusion cell system (Holmbäck et al., 2022; Iadaresta et al., 2018; Lodén et al., 2004) consisted of a buffer reservoir containing PBS buffer (pH 7.4), an 8-channel peristaltic pump, eight flow-through diffusion cells (cross section area of 0.5 cm^2) placed on a stainless-steel platform which was kept at 37 °C, and an 8-channel fraction collector. The buffer was transported in the system through Teflon tubes (0.5 mm ID). A Strat-M® membrane (Uchida et al., 2015) of appropriate size was placed between the donor chamber and the receiving chamber. The flow rate was adjusted to around 1.5 ml/h. Approximately 5 mg of formulation was applied on top of the membranes. The top of the donor chamber was left open to allow evaporation of the volatile solvent. Fractions of the receptor fluid were collected during 0–2, 2–4, 4–6, 6–10, 10–14, 14–18 and 18–24 h. The concentration of sodium fusidate in the receptor fluid was analyzed by RP-HPLC with UV detection (as described above). The remaining amount of active substance in the membranes was analyzed after extraction with 1 ml of methanol overnight using the same method.

2.4. Antimicrobial tests of sodium fusidate in AKVANO

2.4.1. Ex vivo pig skin wound infection model

The aim was to evaluate the antibacterial effect of sodium fusidate in different formulations in a pig skin *ex vivo* wound infection model.

Bacteria preparation. A few colonies of *S. aureus* (ATCC 29213) were transferred with a sterile loop to 10 ml BHI medium in a 50 ml sterile falcon tube and incubated overnight at 250 rpm at 37 °C. 1 ml from the overnight culture was transferred to a new falcon tube with 10 ml BHI medium and incubated for 2 h at 250 rpm at 37 °C, to reach log growth phase. The tube with bacterial suspension was centrifuged for 10 min at 1500 x g. The supernatant was removed, and the pellet was re-suspended in 1 ml BHI medium diluted 100 times in sterile H_2O (BHI-100) to a master bacterial suspension. Optical density (OD) at 600 nm was used to measure the bacterial content of the suspension (Epoch plate reader, Agilent Technologies Inc., USA). The bacteria suspension was diluted to a final density of 1×10^7 CFU/ml.

Ex vivo skin wound infection. Skin from the back of the pig was shaved, packed in plastic foil and frozen at −20 °C for storage. At the time of the experiment, the skin was thawed, and the subcutaneous fat was removed with a scalpel. The skin was cleaned with 70% ethanol, put in a petri-dish with Kleenex paper tissues in the bottom and moistened with sterile H_2O. Wounds were made with punch biopsies, approximately 0.5–1 mm in thickness and 3 mm in diameter. The top cylinder of a cut 1.5 ml micro-centrifuge tube (diameter approximately 9 mm) was glued around each punch wound with ethyl cyanoacrylate glue (Loctite super glue gel). The area inside the cylinder was washed twice with sterile H_2O. Bacteria (cultured as described above) were diluted to a concentration of 1×10^7 CFU/ml and 100 μl of the bacteria suspension was applied to each wound area. The lid was applied to the petri-dish to create a moist chamber and the skin was incubated for two hours at 37 °C. Remaining fluid was carefully removed with a pipette. To obtain equal amounts of sodium fusidate in the test areas, the exact sodium fusidate concentration of each active sample and the density of the formulation was considered when calculating the volume to be applied. Thus 83–100 μl of the samples and 100 μl BHI-100 (blank) was added to each wound area in six replicates and the skin was incubated for another 4 h at 37 °C. The bacteria were harvested by adding 500 μl of phosphate buffer (0.1% Triton X-100 in 0.075 M phosphate, pH 7.9) followed by rubbing the wound area inside the cylinder gently with a plastic loop. The suspension was transferred to a 5 ml tube prefilled with 1 ml 2x diluted phosphate buffer, the procedure was repeated once and the two fractions of liquid from the infected area were pooled. The suspensions were diluted in five ten-fold steps in 2x diluted phosphate buffer and 50 μl from each dilution was seeded on horse blood agar plates. The plates were incubated at 37 °C overnight and the number of CFUs were counted.

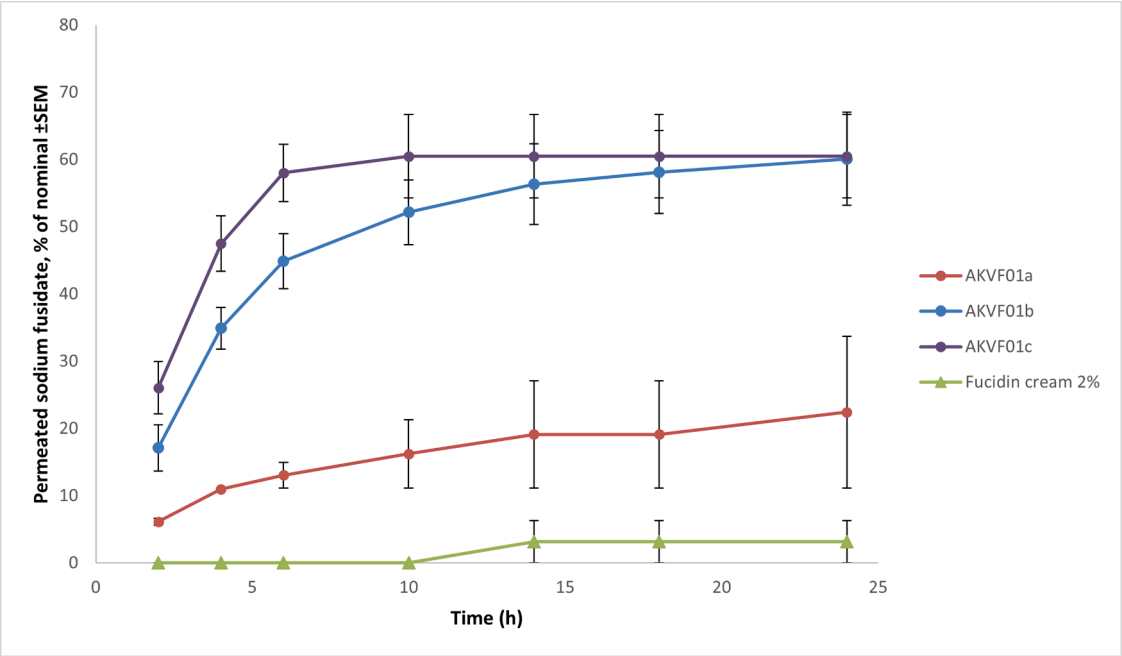

Fig. 1. Cumulative permeation of sodium fusidate in AKVANO through Strat-M membranes.

2.4.2. Mouse in vivo superficial skin wound infection model

Bacteria preparation. A few colonies of *S. aureus* (ATCC 29213) were transferred with a sterile loop to 10 ml BHI medium in a 50 ml sterile falcon tube and incubated overnight at 250 rpm at 37 ºC. The final bacterial concentration was set to 1 × 10^7 CFU/ml by diluting in BHI-100 using optical density (OD) at 600 nm.

In vivo superficial skin wound infection. The animal experiments were performed after prior approval from the local ethics committees in Gothenburg, Sweden. Sixty-six Female Balb C mice (Taconic Biosciences, Denmark), approximately nine weeks old were used. Buprenorfin (48 µg/kg, Temgesic, Indivior, Ireland) was given preoperatively by intraperitoneal injection for post-surgical pain relief. The back of the mice was shaved with a clipper and the remaining fur was removed by hair-removing lotion (Veet, Reckitt Benckiser, Denmark). The mice were anesthetized with isoflurane inhalation and an area of approximately 2 × 2 cm was tape-stripped 10 times with pieces of elastic surgical adhesive bandage (Elastoplast, Beiersdorf, Germany). The area was further injured by scalpel blade by 10 horizontal and 10 vertical light cuts on the skin in a way that no visible bleeding occurred. 10 µl of the inoculum containing ~10^5 CFU *S. aureus* was applied on the injured skin by droplets and spread with a pipette tip. Two hours after infection, 30 µl of treatment formulation (approximately 7.5 mg/cm^2) was added to the wound and four hours after treatment the mice were euthanized for tissue processing and analysis.

In vivo skin wound infection model with application by spraying. In a slightly modified version of the *in vivo* superficial wound infection model, the AKVANO formulations were applied to the wounds by spraying and the amount of formulation was reduced to approximately 3 mg/cm^2. The study was performed as described above with the difference that 1 and 4 h exposure time after treatment was used before the mice were euthanized and the tissue processed. Thirty-two and thirty-three Female Balb C mice (Taconic Biosciences, Denmark), approximately nine weeks old were used for 1 and 4 h exposure study, respectively. To ensure equal dosing of Fucidin cream and AKVANO formulations used for application on the test areas on the mice, a verification test was performed with the spray device. Six 2 × 2 cm paper squares were cut with a pair of scissors. As a distance guide, a self-lock cable was tied around the spray bottle (containing AKVANO calibration formulation). One spraying was performed on each pre-weighed paper piece and let dry for six hours, and then weighed again. The experiment was repeated twice. The volume of Fucidin cream to be used, corresponding to one spraying of AKVANO formulation was calculated as:

$V_{Fuc} = \rho_a * A / \rho_{Fuc}$, where ρ_{Fuc} is the density of Fucidin cream (0.933 g/ml)

AKVANO formulation aerial density (ρ_a, mg/cm^2) = w_{mean} / (c*A), where w_{mean} is the average weight of formulation on the paper squares after evaporation, c is the relative content of non-volatile components in the formulation (0.2) and A is the area of the paper square (4 cm^2).

Tissue processing. The infected skin was excised aseptically and homogenized in two ml ice cold BHI-100. The homogenate was centrifuged at 2000 x g for ten minutes at 4 °C. The supernatant was then discarded, and the pellet was resuspended in two ml phosphate buffer (0.05% Triton X-100 in 0.0375 M phosphate buffer). The suspension was serially ten-fold diluted five times in phosphate buffer (0.05% Triton X-100 in 0.0375 M phosphate buffer). 50 µl of each dilution was plated on horse blood plates and incubated for 16 h at 37 °C. To determine the remaining bacteria in the wound, the colonies were counted. Table 1 lists the formulation groups included in the study. In addition, one negative control (no treatment) and one positive control (Fucidin cream 2%) was used.

2.5. Statistical analysis and graphs

Comparisons between formulations were done with Mann-Whitney *U test*, and dose-response correlation was evaluated using Spearman's rank correlation test. Calculations were done in R statistical package. Adjustments for multiple comparisons were done using Hommel procedure (Chen et al., 2017). Data are graphically presented in box plots, showing median, quartiles, min and max values on a logarithmic scale. In the *ex vivo* pig skin experiments some agar plates had zero colonies (CFU) even at the first dilution, which makes it impossible to plot on a logarithmic scale. A measured CFU/plate of 1 at first dilution corresponds to 40 CFU/wound, due to initial dilutions. Therefore, zero readings were changed to 20, which is half of the lowest possible count.

Table 2
Antimicrobial effects of AKVANO formulations with and without addition of sodium fusidate 2% in a pig skin *ex vivo* wound infection model (4 h post-treatment). *N* = skin samples/group; N.S. = not significant.

Formulation	N	Median bacterial survival (%)	Adjusted P-value vs Untreated control	vs Fucidin cream 2%
Negative control	6	100	–	0.006
Fucidin cream 2%	6	42.1	0.006	–
AKVF02a Placebo	6	56.3	N.S.	N.S.
AKVF02a	6	0	0.006	0.006
AKVF02c	6	0	0.006	0.006
AKVF03a	6	0	0.006	0.006
AKVF03c	6	0	0.006	0.006

In case of several test areas with 0 CFU for a formulation, they were considered to be evenly distributed in the interval 0–40.

3. Results

3.1. In vitro permeation experiments of sodium fusidate in AKVANO

The permeation of sodium fusidate in AKVANO formulations (AKVF01a, AKVF01b and AKVF01c) was compared with Fucidin cream 2% in a flow-through cell equipment as described earlier (Holmbäck et al., 2022). The results showed that all AKVANO formulations gave a faster average permeation as compared to Fucidin cream (Fig. 1). AKVF01a, containing Soy-PC as the only lipid, gave a relatively slower permeation (22% after 24 h) as compared to AKVF01b and AKVF01c (both 60%), which contained increasing amounts of single chain lipids (medium chain monoglycerides and isopropyl myristate). The average permeation of Fucidin cream was only 3% in this experiment. The observation that increasing amounts of single chain lipids in AKVANO promote the permeation rate agrees well with the results from our earlier studies on diclofenac salts and ketoprofen (Holmbäck et al., 2022).

3.2. Antimicrobial tests of sodium fusidate in AKVANO

3.2.1. Pig skin ex vivo skin wound infection model

Four different AKVANO formulations containing sodium fusidate and one AKVANO placebo formulation were tested in the first *ex vivo* experiment and compared with Fucidin cream 2% (Table 2 and Fig. 2). AKVF02a Placebo formulation showed a 44% reduced bacterial content, whereas Fucidin cream 2% showed a reduction of 58%. AKVANO formulations (AKVF02a, AKVF02c, AKVF03a, AKVF03c) gave a full elimination of bacteria in most of the wound areas, thereby showing a statistically significant stronger antibacterial effect as compared to Fucidin 2% cream. Due to almost complete eradication of bacteria for all AKVANO formulations containing sodium fusidate in this experiment, it was not possible to compare the efficiency among them.

In the second *ex vivo* experiment, AKVANO formulations of type a, b and c with different concentrations of sodium fusidate were tested and compared with two AKVANO placebo formulations, Fucidin cream 2% and sodium fusidate solution in ethanol (see Table 3 and Fig. 3). In this experiment, the median reduction for Fucidin cream 2% was only 22% after 4 h, whereas the two AKVANO placebo formulations gave much higher reduction, which could be attributed to the intrinsic antiseptic effect of the ethanol content. Although not fully consistent, increasing

Table 3
Antimicrobial effects of AKVANO formulations with different concentrations of sodium fusidate in a pig skin *ex vivo* wound infection model (4 h post-treatment). *N* = skin samples/group; N.S. = not significant.

Formulation	N	Median bacterial survival (%)	Adjusted *P*-value vs Untreated control	vs Fucidin cream 2%
Negative control	12	100	–	N.S.
Fucidin cream 2%	6	77.9	N.S.	–
AKVF04a Placebo	6	31.2	0.002	N.S.
AKVF04a 0.5%	6	14.1	0.008	0.02
AKVF04a 1%	6	28.6	0.01	N.S.
AKVF04a 2%	6	8.04	0.002	0.03
AKVF04b 1%	6	5.19	0.008	0.02
AKVF04c Placebo	6	0.23	0.002	0.02
AKVF04c 0.5%	6	0.026	0.008	0.02
AKVF04c 1%	6	0	0.008	0.01
AKVF04c 2%	6	0	0.008	0.01
Na Fusidate 1% in EtOH	6	0	0.008	0.02

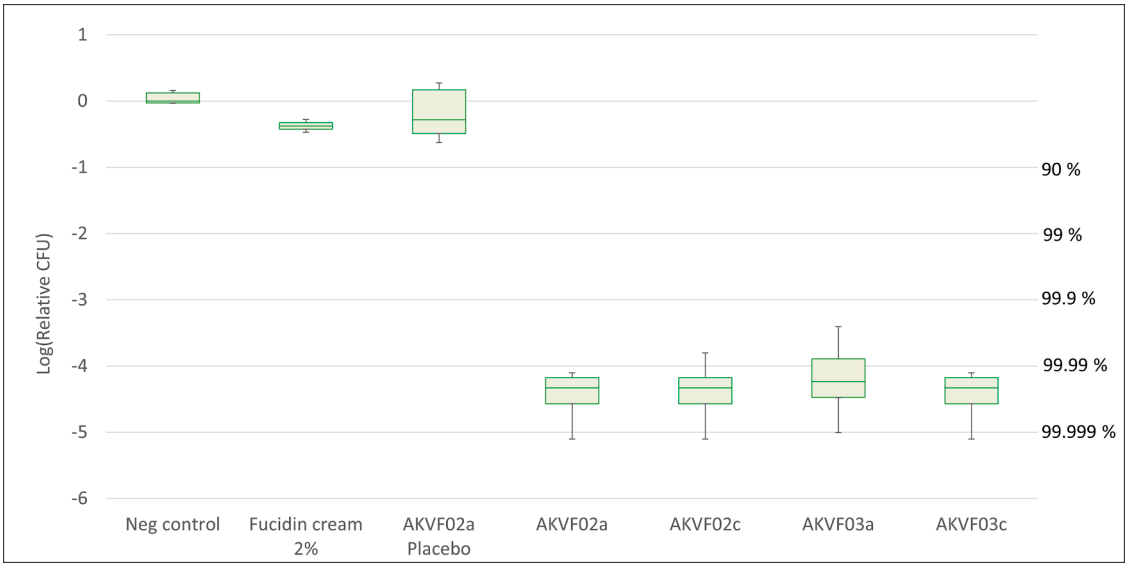

Fig. 2. Antimicrobial effect of AKVANO formulations with and without addition of sodium fusidate 2% in a pig skin *ex vivo* wound infection model (4 h post-treatment).

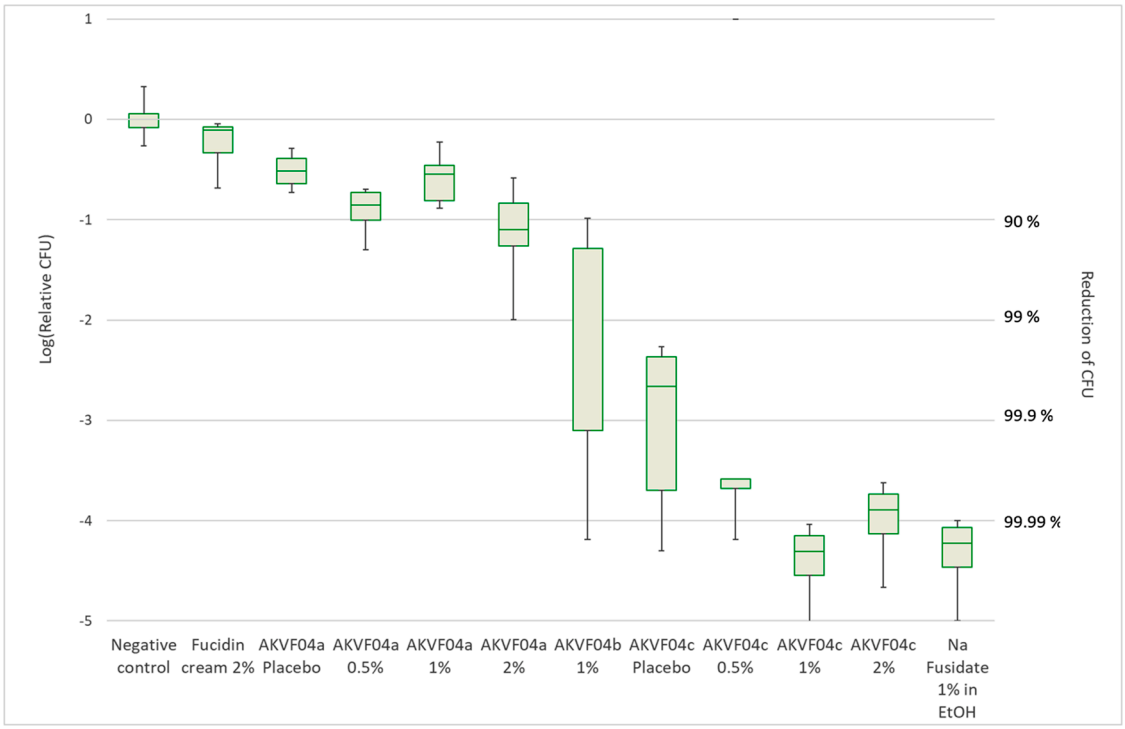

Fig. 3. Antimicrobial effects of AKVANO formulations with different concentrations of sodium fusidate in a pig skin *ex vivo* wound infection model (4 h post-treatment).

Table 4
Antimicrobial effect of AKVANO liquid formulations with and without addition of sodium fusidate 2% in an *in vivo* mouse model of superficial skin wound infection (4 h post-treatment). *N* = mice/group; N.S. = not significant.

Formulation	N	Median bacterial survival		Adjusted *P*-value	
		CFU	%	vs Untreated control	vs Fucidin cream 2%
Untreated control	8	6.88×10^6	100	–	0.004
Fucidin cream 2%	8	2.34×10^4	0.34	0.004	–
AKVF05 Placebo	10	2.79×10^6	40.6	0.003	0.003
AKVF06 Placebo	8	1.89×10^5	2.75	0.001	0.005
AKVF07 Placebo	8	1.69×10^6	24.6	0.002	0.004
AKVF05	8	2.61×10^4	0.38	0.001	N.S.
AKVF06	8	4.86×10^3	0.071	0.001	N.S.
AKVF07	8	1.60×10^4	0.23	0.001	N.S.

concentration of sodium fusidate gave statistically significant stronger reduction (tested by Spearman's rank correlation), both for AKVF04a ($P = 0.03$) and AKVF04c ($P = 0.03$). The strongest reduction was observed for AKVF04c 1% and AKVF04c 2%, in which the test items killed all bacteria. Considering that AKVF04c contains a high amount of single chain lipids and that these showed permeation enhancing effects in the diffusion cell experiments, it could be hypothesized that deeper permeation is beneficial for the bactericidal effect of sodium fusidate.

3.2.2. *Mouse in vivo superficial skin wound infection model (AKVANO liquid)*

In the first experiment using the *in vivo* superficial skin wound infection model, three different AKVANO formulations with sodium fusidate (AKVF05–07) were tested along with their respective vehicles. AKVF05 was equivalent to AKVF04c, the most efficient formulation screened in the *ex vivo* experiments. AKVF06 contained more Soy-PC but less isopropyl myristate whereas AKVF07 had equal amounts of Soy-PC and single chain lipids (type b formulation). This experiment had an additional aim to studying the effect of added keratolytic agents in the composition. Thus, AKVF06 contained lactic acid while AKV07 had both lactic acid and urea. All AKVANO vehicle formulations (AKVF05–07 Placebo) showed a significant antimicrobial effect as compared with the untreated control; AKVF05 Placebo with 59%, AKVF06 Placebo with 97% and AKVF07 Placebo with 75% bacterial reduction. Like for the *ex vivo* experiments, this effect could be explained by the antiseptic effect of ethanol. The addition of sodium fusidate to the AKVANO formulations further increased the antibacterial effect as compared to their respective placebo formulations (see Table 4 and Fig. 4). AKVF06 and AKVF07 showed stronger median reduction in bacterial count than Fucidin cream 2%, but the difference was not statistically significant.

It should be noted that due to the low viscosity of the AKVANO formulations, some of the liquid flowed down from the infected area onto the sides of the mouse after application. Fucidin cream 2% was more viscous and therefore could stay on the superficial wound after the application. Therefore, it can be assumed that the effect of AKVANO formulations was underestimated as compared to a situation when the entire formulation could have stayed on the wound.

3.2.3. *Mouse in vivo superficial skin wound infection model (AKVANO spray)*

Based on earlier two formulations (AKVF05 and AKVF06), two equivalent spray formulations, AKVF08 and AKVF09 along with their corresponding vehicles were developed and tested *in vivo* superficial skin wound infection mouse model (as described in Section 2.4.2.3.). Since the AKVANO formulations were applied by spraying and in a lesser amount than in the first mouse model experiment, no problem with formulation flowing off the treatment area was observed this time. Infected wounds without treatment were used as negative controls and Fucidin cream 2% was used as a positive control. One hour after

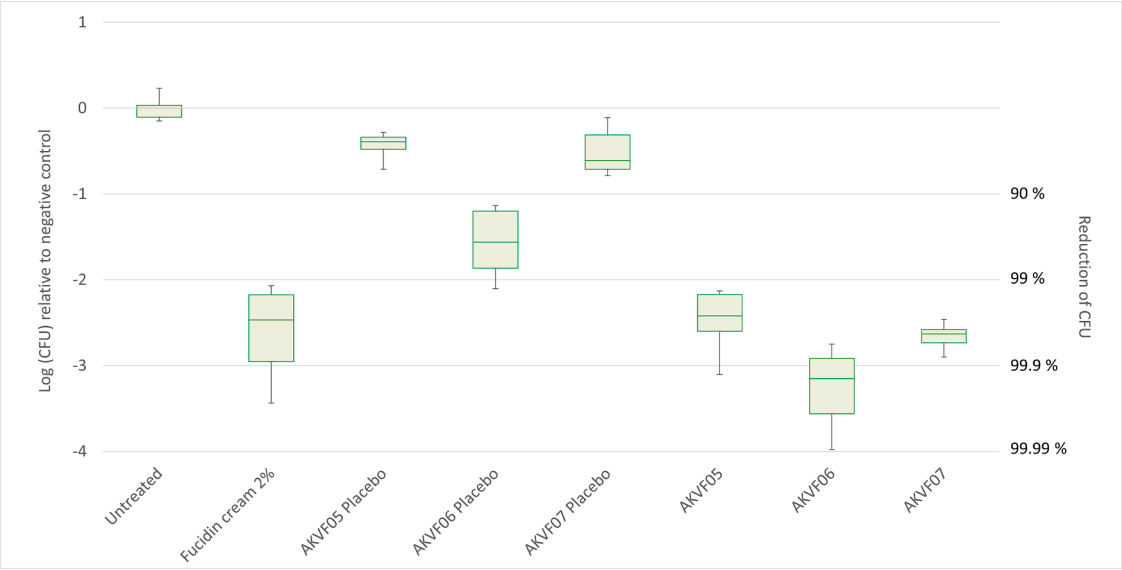

Fig. 4. Antimicrobial effect of AKVANO liquid formulations with and without addition of sodium fusidate 2% in an *in vivo* mouse model of superficial skin wound infection (4 h post-treatment). The Y axis represents the logarithm of CFU values relative to negative control (no treatment).

Table 5
Antimicrobial effect of AKVANO spray formulations with and without addition of sodium fusidate 2% in an *in vivo* mouse model of superficial skin wound infection (1 h post-treatment). *N* = mice/group; N.S. = not significant.

Formulation	N	Median bacterial survival		Adjusted *P*-value	
		CFU	%	vs Untreated control	vs Fucidin cream 2%
Untreated control	6	1.9×10^5	100	–	N.S.
Fucidin cream 2%	6	1.0×10^5	54.7	N.S.	–
AKVF08 Placebo	4	1.9×10^5	97.2	N.S.	N.S.
AKVF09 Placebo	4	2.8×10^4	14.5	N.S.	N.S.
AKVF08	6	7.5×10^4	39.5	N.S.	N.S.
AKVF09	6	8.1×10^3	4.2	0.03	0.02

Table 6
Antimicrobial effect of AKVANO spray formulations with and without addition of sodium fusidate 2% in an *in vivo* mouse model of superficial skin wound infection (4 h post-treatment). *N* = mice/group; N.S. = not significant.

Formulation	N	Median bacterial survival		Adjusted *P*-value	
		CFU	%	vs Untreated control	vs Fucidin cream 2%
Untreated control	6	9.1×10^6	100	–	0.01
Fucidin cream 2%	6	8.6×10^4	0.9	0.01	–
AKVF08 Placebo	4	3.5×10^5	3.8	0.04	N.S.
AKVF09 Placebo	5	1.1×10^5	1.2	0.02	N.S.
AKVF08	6	7.0×10^4	0.8	0.01	N.S.
AKVF09	6	9.0×10^3	0.1	0.02	0.02

treatment, all groups had a median CFU lower than the untreated group (see Table 5). However, the effect was only statistically significant for AKVF09 which showed the strongest antibacterial effect (96% reduction of the CFU). AKVF09 was also significantly more effective compared to Fucidin cream 2%, with an antibacterial effect of more than one Log_{10} order of magnitude.

Four hours after treatment, all formulations showed a statistically significant antibacterial effect (see Table 6). AKVF09 was still the most effective treatment with 99.9% killed bacteria which was one Log_{10} order of magnitude more effective than Fucidin cream 2% (statistically significant). The antibacterial effect over the course of the experiment is illustrated in Fig. 5.

The stability studies on several AKVANO formulations have been performed during the specified time. The formulation batches as used in the second *in vivo* mouse study (AKVF08 and AKVF09) were subjected for long term (12 months) stability at ambient temperature (15–25 °C) and refrigerated conditions (5 ± 3 °C), and for 6 months at accelerated conditions (40 ± 1 °C) in sealed glass vials under nitrogen. Both long and short-term stability was expressed as relative concentration (% of nominal) for sodium fusidate in AKVANO formulations using the specified analytical methods (described in Section 2.2). The stability study for formulations with and without lactic acid (AKVF09 and AKVF08 respectively) indicate that lactic acid contributed to an enhanced stability for sodium fusidate, especially at higher temperature (40 °C). The relative concentration (% of nominal) for sodium fusidate in AKVF09 was 92% at accelerated condition and in AKVF08 and AKVF09 was within the limit of 95–105% at lower temperature.

4. Discussion

Permeation experiments showed that AKVANO formulations of sodium fusidate had much higher penetration rate through artificial skin mimicking membranes than commercial Fucidin cream. It is anticipated that although transdermal delivery is not desired in case of topical antibiotics, still some permeation through epidermis and dermis is required for fusidic acid to effectively reach the site of infection. These experiments clearly showed that skin permeation can be controlled by changing the proportions of the lipid components in the formulation. An increasing amount of single chain lipids enhances the rate and extent of permeation as compared to the compositions only containing the double-chain lipid Soy-PC.

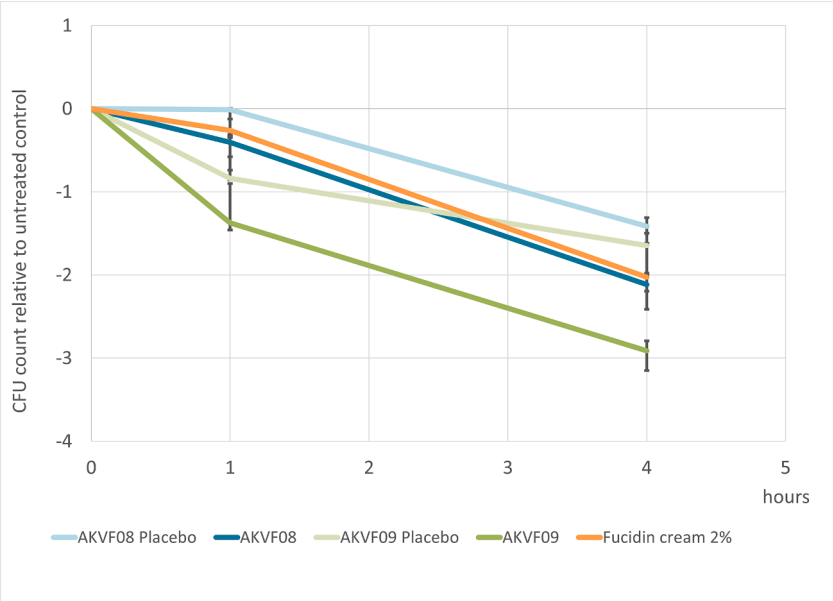

Fig. 5. Antimicrobial effect of AKVANO spray formulations, AKVF08 and AKVF09 with and without addition of sodium fusidate 2% in an *in vivo* mouse model of superficial skin wound infection (1- and 4 h post-treatment). Median bacterial survival in the different formulation examples relative to untreated control. Error bars indicate 1st and 3rd quartiles.

Since the first *ex vivo* experiment resulted in almost full bacterial elimination for all AKVANO formulations, it could not clearly guide as which level of permeation is needed for the desired antibacterial effect. However, in the second *ex vivo* experiment, a formulation of type c, which gave a higher skin permeation, was also the most efficient antibacterial formulation. The second experiment also indicated a dose-response relationship when the concentration of sodium fusidate was increased from 0.5% to 2%, where the concentration of 2% w/w was found to be most effective. The *in vivo* mouse studies further confirmed that the AKVANO formulations of sodium fusidate could increase the antibacterial activity as compared to Fucidin cream. The stronger and faster effect on bacterial reduction indicates that the treatment time could be shortened for a product based on sodium fusidate in AKVANO and thereby reduce the risk for the development of antibiotic resistance.

The skin barrier in the treated area is likely to be compromised as a result of an infection or in case of a secondary infection, due to the underlying condition. The permeation enhancing effects of single chain lipids in AKVANO formulation are thus needed to be accompanied by the barrier strengthening components. This could be provided by the double chain lipid Soy-PC which interacts with bilayer structures in the extracellular lipid matrix of the skin. These effects are planned to be reported in a separate article.

The difference in results between AKVANO formulations and Fucidin cream can be explained by the difference in dosage forms being used for the topical delivery of sodium fusidate: cutaneous solution (AKVANO) *vs.* cream of the oil-in-water emulsion type (Fucidin). AKVANO seems to be a more or less isotropic system as per our earlier physicochemical studies (Holmbäck et al., 2022). Sodium fusidate is fully soluble in AKVANO and is thus delivered in a molecularly dispersed and dissolved form to the skin. In Fucidin cream, the API is formulated with liquid paraffin and white soft paraffin, both being nonpolar oily components, together with water and emulsifier as the main excipients. The oily components are necessary for solubilising fusidic acid in small oil droplets, but because of this, they also encapsulate the active component in a nonpolar environment which leads to a reduced exposure at the site of action. Spray administration of AKVANO provides an even layer on the skin surface from which the solvent quickly evaporates, resulting in a thin lipid layer that beneficially interacts with the lipid matrix in *stratum corneum*. This facilitates a non-touch application of the formulation where no rubbing is required.

5. Conclusion

The presented set of studies can serve as an example of a preclinical program for the stepwise selection of candidates from several possible AKVANO formulations. After each experiment new candidates were chosen to proceed for the next step based on their efficacy, and after the last preclinical study the most promising candidate could be taken forward for future clinical evaluation. The AKVANO platform allows for a relatively high flexibility in the formulation components, which facilitates good possibilities to identify an optimal composition. To conclude, in the given set of experiments, AKVANO technology has led to the development of a promising candidate formulation of sodium fusidate that can potentially shorten the treatment time for superficial and soft tissue infections and thereby reduce the risk of antibiotic resistance development.

Related Patents

1. Carlsson, A.; Holmbäck, J.; Lipid Layer Forming Composition for Administration onto a Surface of a Living Organism. International patent application WO 2011/056,115 A8, 30–06–2011.

2. Herslöf, B.; Holmbäck, J.; Topical Composition and Carrier for Administration of Pharmaceutical or Cosmetic Active Ingredients. International patent application WO 2014/178,789 Al, 06–11–2014.

3. Herslöf, B.; Holmbäck, J.; Sprayable Topical Carrier and Composition Comprising Phosphatidylcholine. International patent application WO 2015/072,909 Al, 21–05–2015.

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CRediT authorship contribution statement

Jan Holmbäck: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Vibhu Rinwa:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Jenny Johansson:** Investigation,

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jan Holmbäck, Vibhu Rinwa, Puneet Rinwa, Anders Carlsson and Bengt Herslöf reports financial support was provided by Lipidor AB. Jan Holmbäck and Vibhu Rinwa reports a relationship with Lipidor AB that includes: employment and equity or stocks. Puneet Rinwa reports a relationship with Lipidor AB that includes: employment. Anders Carlsson and Bengt Herslöf reports a relationship with Lipidor AB that includes: consulting or advisory and equity or stocks. Jan Holmbäck, Anders Carlsson and Bengt Herslöf has patents published: See patents as separate list issued to Lipidor AB. Jenny Johansson and Joakim Håkansson reports: No declaration of interests.

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