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Adjuvant Antibiotic Activity of Acidic Sophorolipids with Potential for Facilitating Wound Healing

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ABSTRACT The sophorolipid class of biosurfactants is finding increasing use in personal care as well as pharmaceutical products and has the potential to disrupt biofilm formation and inhibit the growth of a variety of clinically relevant organisms. In order to investigate potential biomedical applications of sophorolipids derived from nonpathogenic organisms, we fractionated and purified glycolipid biosurfactant sophorolipids produced by the yeast *Starmerella bombicola*, which yielded both AQ:A acetylated and nonacetylated acidic $C_{18:1}$ congeners that were essentially free from other contaminants (>95% purity). These acidic sophorolipids have antimicrobial activities against the nosocomial infective agents *Enterococcus faecalis* and *Pseudomonas aeruginosa*, with significant reductions in CFU at concentrations of as low as 5 mg ml^{-1}. In addition, the sophorolipid showed similar effects against the same two bacterial strains when combined with kanamycin or cefotaxime. As a potential use of these sophorolipids is as a component of topically applied creams for the treatment of wound infections, it is clear that they must have no demonstrable adverse effect on wound healing. To assess this, we evaluated mammalian cell toxicity *in vitro* using viability tests, which revealed no adverse effect on either endothelial or keratinocyte-derived cell lines with sophorolipid concentrations of < 0.5 mg ml^{-1}. In addition, *in vivo* experiments using a mouse skin wounding assay revealed that the time course of healing wounds was unaffected by the application of sophorolipid-containing creams, and histological examination of regenerated skin tissue confirmed that the healing process was similar to that observed for control animals, with no evidence of inflammation. These results are consistent with the suggestion that acidic sophorolipids can be used as a component of antimicrobial creams to reduce the risk of wound infection during healing.

KEYWORDS acidic sophorolipids, microbial pathogens, growth inhibition, endothelium, keratinocytes, *in vivo*, wound healing, *Enterococcus*, *Pseudomonas aeruginosa*, adjuvants, antimicrobial activity

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AQ: B **M**icrobial contamination is a major obstacle to tissue healing, with cutaneous wounds in particular representing one of the major routes of exposure to pathogenic bacterial strains. A systematic review (1) recently identified *Pseudomonas aeruginosa* as the most commonly reported burn-wound-infective pathogen. Multiple strains of multidrug-resistant *P. aeruginosa* have been isolated from burn patients in India (2), and enterococcal in addition to pseudomonad species have been identified in cases of diabetic foot ulcer infections (3). Many medical and surgical procedures carry a significant risk of microbial infection (4, 5), with a significant proportion of these cases

demonstrating bacterial drug resistance, making the search for alternative approaches to treatment a clinical imperative.

Surfactants are a diverse group of amphiphilic compounds commonly used in detergents and products for human consumption or application (such as the food industry or in cosmetic or medical creams, for example), although their production is heavily reliant on the petrochemical industry and associated raw materials. In contrast, biosurfactants are a diverse group of surfactants produced by certain species of yeast or bacteria, which represent a more sustainable and perceived environmentally friendly alternative to traditional surfactants. There is increasing evidence that microbial bio-surfactants, as well as possessing industrially valuable properties of detergency, emul-sification, and foaming, also have significant bioactivities, including inhibitory or anti-bacterial adjuvant activities against various microorganisms (6–18) and specific anticancer activity (19).

AQ: C Sophorolipids (SLs) are a diverse group of glycolipid biosurfactants, characterized by a sophorose molecule attached to a variable-length fatty acid chain, that can be produced in significant quantities by the yeast *Starmerella bombicola*. The sophorose molecule may be acetylated in two positions, the carbon chain between 16 and 18 atoms in length, and contain no, one, or more double bonds. The arrangement of the fatty acid chain accounts for the two major SL subtypes: acidic SLs terminate in a carboxylic acid group (open chain), while the chain in the lactonic type is reattached to the sophorose molecule, forming a closed-ring structure. The antimicrobial effects of SLs are dependent on the SL structure and class of bacteria examined, yet SLs are predominantly produced and used as mixtures, with very few studies examining the effects of SL samples containing well-defined and purified single congeners. In addi-tion, sophorolipids have virucidal and antibiotic adjuvant characteristics (6, 7) and may have potential applications in infection, wound healing, or anti-inflammatory contexts (8, 19). However, the widely variable methods of production, extraction, and purifica-tion, coupled with the diversity of possible SL structures and a lack of sufficient characterization of preparations, make comparison between data from *in vitro* and *in vivo* studies difficult to interpret.

The purpose of our studies was to use a highly purified preparation of acidic sophorolipid that contained only the C_{18} congener (predominantly nonacetylated) and ascertain if it could act as an antimicrobial agent or antibiotic adjuvant against two common nosocomial infection-causing bacteria: *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Furthermore, we also explored whether these acidic sophorolipids inhibited the growth of mammalian cells (endothelium, fibroblasts, and keratinocytes) that are relevant to the wound healing process *in vitro* and are compatible with the healing of uncomplicated wounds *in vivo*.

RESULTS

Analysis of bacterial growth with purified nonacetylated acidic sophorolipid treatment. Following treatment with doses of acidic sophorolipids of ≥5 mg ml^{-1}, a clear inhibitory effect on the growth of *Enterococcus faecalis* was observed: colony

AQ: D formation was zero with 20 mg ml^{-1} $C_{18:1}$ NASL in two out of three experiments (Fig.

F1 1A). Inhibition of growth of *Pseudomonas aeruginosa* was also evident at concentrations of ≥5 mg ml^{-1} acidic sophorolipid; following treatment with 20 mg ml^{-1} $C_{18:1}$ NASL, colony formation was zero in one out of three experiments (Fig. 1B).

Antibiotic adjuvant activity of sophorolipid. In culture experiments, solutions of 10 and 20 mg ml^{-1} of $C_{18:1}$ NASL successfully inhibited the growth of both bacterial species (as determined by optical density at 600 nm [OD_{600}] measurements); therefore, for the adjuvant assay, we tested subinhibitory concentrations of 2 and 4 mg ml^{-1}, respectively.

The addition of purified $C_{18:1}$ NASL to bacterial cultures reduced the MIC of kanamycin and cefotaxime in the majority of replicates; however, the value of the MIC

AQ:E/T1 itself was difficult to determine due to interexperimental variation (Table 1). The highest concentration of $C_{18:1}$ NASL was clearly the most effective at reducing antibiotic MICs
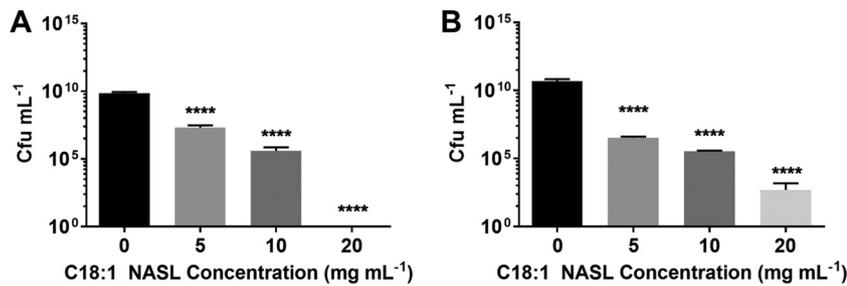
FIG 1 Growth (CFU per milliliter) of *Enterococcus faecalis* (A) and *Pseudomonas aeruginosa* (B) was significantly reduced by exposure to 0.5 to 2% purified acidic sophorolipid ($C_{18:1}$ NASL). Representative data from one of three experiments are presented (means ± standard deviations; $n = 4$ technical replicates; ****, $P < 0.0001$).

for both strains and both drugs. In fact, 4 mg ml^{-1} $C_{18:1}$ NASL alone effectively reduced the growth of *Pseudomonas aeruginosa*. The adjuvant effect was most obvious for strains treated with 4 mg ml^{-1} $C_{18:1}$ NASL together with cefotaxime. The effect of the addition of $C_{18:1}$ NASL on the kanamycin MIC was generally less marked than that on cefotaxime. Interestingly, the MIC of kanamycin against *Pseudomonas aeruginosa* was increased with the addition of 2 mg ml^{-1} $C_{18:1}$ NASL.

In vitro cell viability assay. The addition of acidic $C_{18:1}$ NASL to culture media in doses ranging from 0.01 to 500 μg ml^{-1} did not affect the cell viability of human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells (HDMVECs), or HaCaT cells (Fig. 2), as measured by an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium] bromide assay.

In vivo wound healing assay. All wounds were completely closed, with no remaining residual coagulum covering, by day 16 of the study. Treatment with cream containing $C_{18:1}$ acidic sophorolipid did not significantly affect wound size on the monitored days in comparison to the control group (Fig. 3; see also the supplemental material). The wound size for the vehicle group was initially larger than that for controls on day 4 ($P < 0.05$) but was smaller than that for controls by day 8 of the study ($P < 0.001$) (see the supplemental material).

Histology of healing wounds. Qualitative assessment of light microscopic images from samples taken 21 days after injury revealed typical morphological features of murine skin samples, including a thin cornified epithelial layer, a dermal layer with numerous obliquely sectioned hair follicles, and a deep 3- to 5-cell-layer-thick *panniculus carnosus* (skeletal muscle) layer (see Fig. S2 in the supplemental material). Morphological features of tissue structure were consistent across all treatment groups, with no evidence of fibrosis or lymphocytic infiltration being noted.

TABLE 1 MICs and MECs of kanamycin or cefotaxime against *Enterococcus faecalis* or *Pseudomonas aeruginosa*, alone or in combination with purified $C_{18:1}$ NASL

| Treatment	MIC (mg/liter)	MEC (mg/liter)
Enterococcus faecalis		
Kanamycin	4–16+	2–4
+2 mg ml^{-1} $C_{18:1}$ NASL	2	2–4
+4 mg ml^{-1} $C_{18:1}$ NASL	2–4	Not determined
Cefotaxime	0.5–2	0.5–2
+2 mg ml^{-1} $C_{18:1}$ NASL	0.5–1	0.25–1
+4 mg ml^{-1} $C_{18:1}$ NASL	<0.016–1	<0.062–1
Pseudomonas aeruginosa		
Kanamycin	8	8
+2 mg ml^{-1} $C_{18:1}$ NASL	8–16	8
+4 mg ml^{-1} $C_{18:1}$ NASL	<0.25	<0.25
Cefotaxime	0.5–4	0.5–4
+2 mg ml^{-1} $C_{18:1}$ NASL	0.5	<0.62
+4 mg ml^{-1} $C_{18:1}$ NASL	<0.062	<0.062

Margin notes: F2, AQ: F, F3
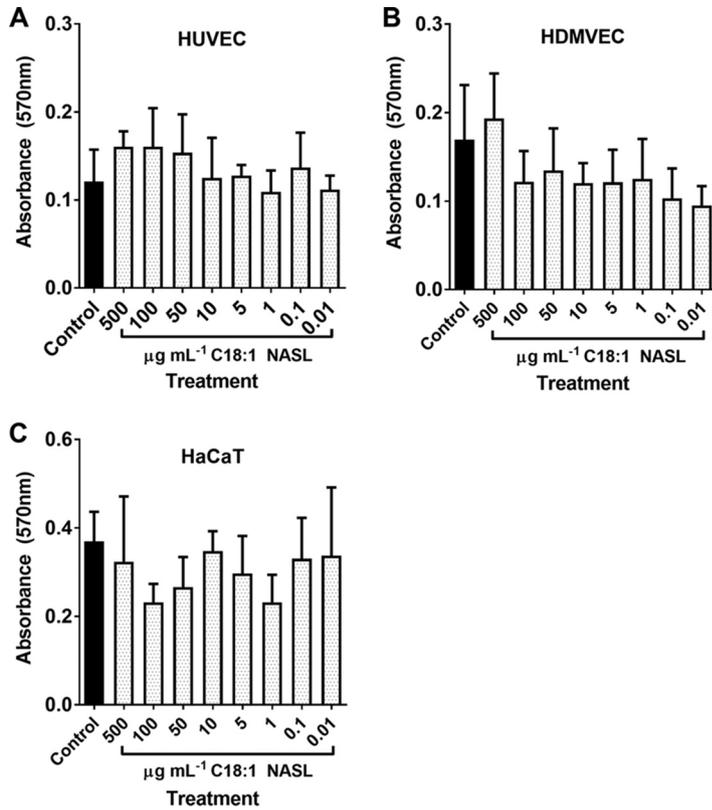
FIG 2 Reduction of formazide salt (absorbance at 570 nm) (means $\pm$ standard deviations; $n = 6$) to formazan crystals in HUVECs (A), HDMVECs (B), and HaCaT cells (C) *in vitro* is not affected by the addition of acidic $C_{18:1}$ SL. A low serum concentration (2%) served as the control conditions for endothelial cells (A and B), and a high serum concentration (10%) served as the control conditions for HaCaT cells (C). Representative data from two repeat assays are shown.

DISCUSSION

To our knowledge, the work presented here details the first investigation of the application of purified sophorolipids in the context of healing wounds. In addition to our observations of consistent antimicrobial and antibiotic adjuvant activities of this
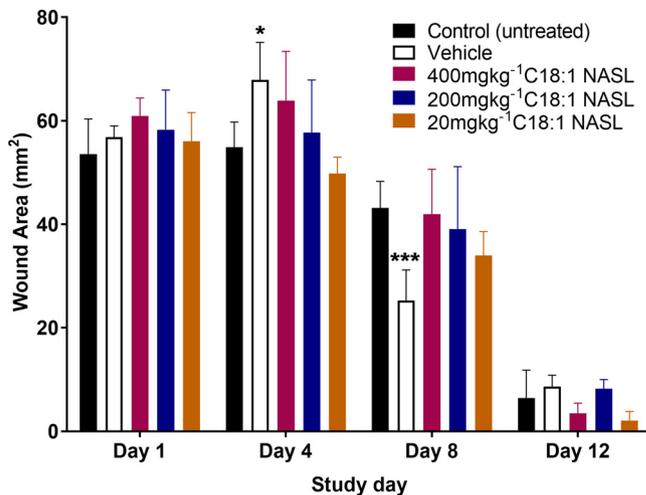

FIG 3 Sizes of wounds (square millimeters; means $\pm$ standard deviations; $n \leq 8$) 8 days after an excision was created on depilated dorsal skin of male C57BL/6J mice and treated with aqueous cream with PBS (vehicle; $n = 8$) or various doses of purified $C_{18:1}$ NASL in aqueous cream for 7 days or left untreated (control). Asterisks indicate a significant difference in wound sizes between a test group and the control group (*, $P < 0.05$; ***, $P < 0.001$).

purified sophorolipid product, we show that $C_{18:1}$ NASL does not affect cell viability in human endothelial cells (HUVECs and HDMVECs) and keratinocytes (HaCaT cells) *in vitro* or the rate of wound healing in a murine model of longitudinal wound healing *in vivo*. Taken together, these data provide evidence that purified sophorolipid application is compatible with healing wounds and could be beneficial in the context of wound contamination or infection with opportunistic bacterial pathogens.

In terms of the effects of sophorolipids on cultured cells, much of the reported data focuses on relatively impure preparations, and these studies were primarily directed at antimigratory or cytotoxic effects on phenotypically invasive cell lines in the context of anticancer therapy (9–14) rather than compatibility with and low toxicity to normal (nontransformed) cells. Our observations are consistent with the suggestion of continuing research into possible therapeutic applications. More detailed investigations of the interactions between sophorolipids and cultured human cells, such as assays of membrane integrity, biomarkers of irritancy, or inflammation, for example, will be vital to inform the progression of these agents into clinical application.

A range of sophorolipid preparations, including mixtures containing both acidic and lactonic structures, were shown to be ineffective against Gram-positive and Gram-negative bacteria (*Escherichia coli* and *Staphylococcus aureus*), with MICs above 512 μg ml^{-1}, similar to the sophorolipid concentrations used to demonstrate adjuvant activity in our study (15). However, when ethanol was employed as a vehicle, inhibition of *E. coli* and *S. aureus* was observed at concentrations of >128 μg ml^{-1}. A "natural SL" mixture (approximately 75% lactonic and 25% acidic structures) was effective against *E. coli* at 1 mg ml^{-1} and against *S. aureus* at 15 to 150 μg ml^{-1} (6), concentrations approximately 5- to 10-fold lower than the concentrations applied in our antimicrobial studies. The mixture previously reported (6) also displayed adjuvant activity at lower concentrations, <1 mg ml^{-1}, than those that we have reported here. Another study of natural SL mixtures with a variety of sugar head groups reported antimicrobial activity against a range of bacteria, which were predominantly Gram positive (16) and evident at 100- to 1,000-fold-lower concentrations than the ones that we tested. These differences in antimicrobial activities may be associated with the presence of lactonic structures in natural SL mixtures. The range of activities presented by sophorolipid structures is further highlighted by the observation that acidic SL structures are virucidal against HIV *in vitro* at doses of 3 mg ml^{-1} (7), similar to the doses that we observed to exert an adjuvant effect on bacterial pathogenic strains. The dosages employed during our *in vitro* antimicrobial studies that were effective in bacterial killing are consistent with those that were administered intravenously *in vivo* (1- to 1.2-mg dose of a natural SL mixture) and were subsequently shown to prevent lethal septic shock in two distinct rat models of peritonitis (8, 19).

While research activity in the fields of antimicrobial, antiviral, antiadhesion, or adjuvant activities of various types of biosurfactants is increasing, their applications in the context of wound healing are rare. The dirhamnolipid ("BAC-3") produced by *Pseudomonas aeruginosa* is well tolerated and promoted faster healing in studies examining burn wounds in mice and rats; these studies led to its successful application in a single clinical case of chronic decubitus ulcer (17, 18). However, differences in wound types, wound sizes, animal models, surfactant types, and biosurfactant mixtures make comparisons between these studies difficult. In order to improve our understanding of the potential benefits or risks of this diverse group of compounds in wound healing, information on the composition of biosurfactant mixtures and standardization of the experimental wound healing model that is employed will be crucial.

In comparison to the control group, only the addition of cream that contained the vehicle-only solution resulted in a significant change in wound size, with no histological differences being detected between treatment groups. We interpreted these results to indicate that topical creams containing $C_{18:1}$ NASL play a passive role in the process of healing uncomplicated, noninfected wounds. Factors affecting the percutaneous penetration of sophorolipids, such as molecular weight and lipophilicity, may be important for choosing an appropriate delivery mechanism or vehicle. Investigating the roles of

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acidic sophorolipid preparations in critical micelle concentrations and characteristics of dissociation from suitable creams or their extent of percutaneous penetration may thus be beneficial to determine their efficacy for antibiotic prophylaxis of wounded skin.

While the majority of studies report that the lactonic sophorolipids are more effective in terms of antimicrobial effects or adjuvant activity, the $C_{18:1}$ NASL sample tested was selected for evaluation in cultured cells *in vitro* and *in vivo* because of its purity (verified at the source by nuclear magnetic resonance [NMR]) and lyophilized state. The relative solubility of our NASL stock solutions varied according to the vehicle used in each assay. In this study, we used the highest final concentrations possible in both microbiological assays (4 mg/ml in phosphate-buffered saline [PBS]) and mammalian cell culture assays (0.5 mg/ml [500 mg/ml] in culture medium). We were unable to achieve higher doses in either of these cases, as they led to precipitation in the growth medium and thus an inability to calculate accurate final concentrations. This is not ideal, as there is no overlap in dosages between the antimicrobial effect that we observed (maximal at 4 mg/ml) and the highest dose used for cell toxicity testing (0.5 mg/ml). However, we believe that observations of full-thickness skin healing *in vivo* following the topical application of high dosages (200 and 400 mg/kg of body weight, well above the equivalent dose in tissue culture) are consistent with a lack of toxicity of purified NASL.

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Furthermore, the *in vitro* cytotoxic effects of purified lactonic sophorolipids observed by our group (20) extended to control cell lines as well as cancer cell lines; this was not the case for similarly tested purified acidic sophorolipid. Overall, we conclude that the topical application of purified acidic sophorolipid did not impair wound healing *in vivo*, which, together with evidence of antibacterial, antibiofilm, and antibiotic adjuvant activity, supports the future evaluation of sophorolipids as novel wound healing agents, particularly in the contexts of acutely or chronically infected wounds.

MATERIALS AND METHODS

Sophorolipids. A purified acidic sophorolipid sample was produced from the yeast *Starmerella bombicola* by using the culture and purification methods outlined previously by Van Bogaert et al. (21) and comprised >90% nonacetylated $C_{18:1}$ acidic sophorolipid ($C_{18:1}$ NASL) as determined by NMR spectroscopy (at the point of purification). Other congeners present include a mixture of sophorolipid structures with chain lengths of C_{16} to C_{18} containing 0 to 2 double bonds. Sophorolipid solutions of various concentrations (by mass) were prepared in the relevant culture media for *in vitro* experiments or sterile PBS for *in vivo* studies.

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Analysis of bacterial growth with purified nonacetylated acidic sophorolipid treatment. *Enterococcus faecalis* (ATCC 29212) and *Pseudomonas aeruginosa* (PAO1) were obtained from Ulster University Microbiology Research Group stock. Nutrient broth (5 ml; Oxoid, Basingstoke, UK) was inoculated with a single colony and then transferred to a shaking incubator overnight (16 to 18 h). Each culture grown overnight was adjusted, under sterile conditions, to an OD_{600} of 0.05 (~1 × 10^8 CFU/ml) and then diluted further into broth containing sophorolipid (20 mg ml^{-1} $C_{18:1}$ NASL), such that the final concentration of the culture was 1/100 of the inoculum adjusted to an OD of 0.05. Inoculated broth (100 μl) with or without $C_{18:1}$ NASL was loaded, alongside blank controls, into 96-well microtiter plates (Nunc; Thermo Fisher, UK), which were incubated at 37°C for 12 h with no agitation. Cultures were then appropriately diluted, spread onto nutrient agar plates (Oxoid), and incubated at 37°C. Following 12 h of incubation, agar plates were photographed for colony counting by using ImageJ software (v1.50b; NIH). The above-described procedure was performed three times in total for each strain, starting with cultures of separate colonies grown overnight.

Antibiotic adjuvant activity of sophorolipid. Antibiotics (cefotaxime and kanamycin) were purchased from Sigma (Dorset, UK). Stock antibiotic solutions were prepared in sterile water and filter sterilized as required. Appropriate dilutions of antibiotic (<512 mg/liter) were prepared in sterile nutrient broth (Oxoid, Basingstoke, UK) before being loaded (100 μl) into separate columns of a 96-well plate. Each plate contained a 2-fold dilution series of antibiotic ($n = 8$ per concentration). Purified acidic SL solutions were prepared in nutrient broth. Diluted sophorolipid samples and loaded microtiter plates were stored at 4°C and used within 5 days of preparation.

Initial bacterial cultures were prepared by inoculating 5 ml nutrient broth with a single colony from an agar plate. The inoculum was placed into a shaking incubator (37°C) for 6 to 8 h and then adjusted to an optical density (at 600 nm) of 0.05. Cultures were then diluted (1/50) into nutrient broth alone (control) or into nutrient broth containing purified acidic SL (4 or 8 mg ml^{-1} $C_{18:1}$ NASL). The control inoculum was dispensed (100 μl) into one half of the plate (antibiotic plus culture only; $n = 4$ wells), and the SL-containing inoculum was dispensed (100 μl) into the other half of the plate (antibiotic plus culture plus SL at 2 or 4 mg ml^{-1}; $n = 4$ wells). Nutrient broth containing the appropriate concentration of sophorolipid or no SL was included as a control ($n = 4$ wells). The plates were sealed with Parafilm and

incubated in a shaking incubator at 37°C for 12 h, at which point the optical density (at 600 nm) was recorded and the plates were visually inspected for growth, indicated by opacity. Each experiment was performed three times.

Data and statistical analysis. Statistical analyses were performed by using GraphPad Prism (v6.01). Data sets were analyzed by comparing the mean endpoint for each test group to that for the relevant control group by one-way analysis of variance (ANOVA) with Holm-Sidak's multiple-comparison tests. Any value differing by more than 0.2 OD units from at least 2 other values in the group were omitted from analysis for all assays. This step was informed by previous experience whereby air bubbles, excessive precipitation, or contamination was linked to intragroup variation of this magnitude, up to the detection limits of the equipment (approximately 1 OD unit). This approach was not applied where mean values were above 1 OD unit, which was classed as "positive."

The highest concentration of purified acidic sophorolipid applied in the adjuvant assay (4 mg ml^{-1}) invariably increased the optical density of broth (indicated visually and by Mann-Whitney comparisons of OD measurements); therefore, raw OD data were adjusted by taking into account the background from the relevant SL-positive or SL-negative control. To determine the adjuvant efficacy of purified $C_{18:1}$ NASL, the MIC of the antibiotic was defined as the lowest concentration at which the OD_{600} was not significantly different between culture and antibiotic conditions and the relevant blank control, and the minimal effective concentration (MEC) was defined as the lowest concentration of antibiotic at which the OD_{600} was significantly lower than that of the relevant culture control.

Tissue culture. HDMVECs and HUVECs (Caltag Medsystems, Cambridgeshire, UK) were cultured in M200 phenol red-free (PRF) culture medium supplemented with low-serum growth supplement (LSGS) (giving a final serum concentration of 2%; Gibco, UK). Human keratinocytes (HaCaT cells) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (4.5 g liter^{-1} glucose) supplemented with 10% fetal bovine serum (FBS; Gibco, UK).

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In vitro **cell viability assay.** Cells were trypsinized as normal, counted by using trypan blue, and dispensed at various concentrations into 96-well plates. HaCaT cells were washed with PBS after adhering to plates and incubated overnight (16 to 18 h) in serum-free medium, whereas endothelial cells remained in LSGS-supplemented medium. The medium was then removed, and the wells were washed once more in PBS before the application of fresh complete medium containing purified acidic sophorolipid (0.01 to 500 μg ml^{-1}) or etoposide (5 μM) or of medium with a low (2%) or high (10%) fetal calf serum content. Plates were subsequently incubated for 22 h, after which MTT was added (under low-light conditions) to a final concentration of 0.5 mg ml^{-1}, and the plates were incubated for a further 4 h. Media were then removed, and dimethyl sulfoxide (DMSO) (200 μl) was dispensed into each test well with gentle mixing before the optical density at 570 nm was recorded.

Animals and surgical procedures. Adult (8- to 14-week-old) male C57BL/6J mice were obtained from Harlan UK. Animals were housed singly with freely available food and water and provided with soft adsorbent pads as bedding for at least 1 week prior to surgery, with their body weights being measured during this time. In order to reduce the risk of infection associated with contaminated bedding and to reduce stress, animals were placed into fresh cages the day before surgery, and a clean bedding pad was placed on top of the previous pad on the day of the experiment.

Mice were anesthetized by intraperitoneal injection with ketamine and xylazine (100 mg kg^{-1} and 10 mg kg^{-1}, respectively) and provided with subcutaneously administered preemptive pain relief (2 mg kg^{-1} meloxicam [Metacam]). Following appropriate anesthesia, the dorsal surface was shaved with electric clippers, and hair was removed by the application of a commercially available depilatory cream. The skin was prepared for surgery by wiping with three repeat applications of gauze soaked in warmed
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Hibiscrub followed by warmed 70% IMS. Animals were then placed onto a warming mat for the duration of the surgical procedure.

Circular dressings, 2 cm in diameter, were cut from sheets of Opsite Flexigrid transparent adhesive dressings (Smith & Nephew, Hull, UK). The mouse was placed in ventral recumbency, and the dressings were then placed onto the depilated dorsal skin, centrally across the midline and between the forelimbs, and allowed to adhere for a few moments. The mouse was then placed into flank recumbency, and the dressing was used to create a fold of the dorsal skin surface. A uniform, circular wound was created by using an 8-mm dermal biopsy punch, by applying half the blade of the punch to the folded skin (Fig. 1A) while the dressing remained in place. Animals were placed into a warming cabinet, with moistened food in the cage, until they recovered from anesthesia.

Treatment and monitoring. Mice were assigned to a treatment group of 20, 200, or 400 mg kg^{-1} sophorolipid or a control group ($n = 8$ per group). Sophorolipid was prepared in PBS and then mixed with commercially available aqueous cream in a 1:1 (vol/wt) ratio. The control group was treated with an aqueous cream similarly mixed with an equal volume of PBS. Animals were briefly anesthetized with isoflurane to permit the administration of treatment and consecutive photographs to be made of the wounds. Treatment was applied daily to the surface of the wounds for 7 days (Fig. 1B), with photographs being taken on day 1 (day of surgery), day 2, and then every 2 days thereafter. The plunger of a 1-ml syringe was used to smooth the edges of the cream around the wound edge if necessary. The dressing, if not detached already, was removed on day 3 postsurgery. Intake of food and water was recorded periodically throughout the study. On day 21, animals were euthanized in a CO_2 atmosphere, and the skin surrounding the wound area was collected, laid flat onto moistened filter paper, and placed flat into 10% neutral buffered formalin. Following fixation at 4°C, skin samples were processed by using an autoprocessor, embedded in paraffin blocks, sectioned at 5 μm, and stained with hematoxylin and eosin according to conventional protocols.

Image and statistical analysis. Statistical analyses for all assays were performed with the aid of GraphPad Prism (v6.01). Wound size in the *in vitro* and *in vivo* assays was measured by using ImageJ software (22). *In vitro* data sets were analyzed by comparing the mean endpoint for each test group to that for the control group by a Kruskal-Wallis test with Dunn's multiple-comparison test. For *in vivo* assays, mean wound sizes on each monitoring day were compared between each treatment group and the control group as described above.

All relevant international, national, and/or institutional guidelines for the care and use of animals were followed. Specifically, approval from the local (Ulster University) ethics committee was granted, and experiments were conducted according to the guidelines provided by the UK Animals (Scientific Procedures) Act 1986 and according to appropriate national ethical approval from the UK Home Office.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02547-16.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENTS

REFERENCES

1. Azzopardi EA, Azzopardi E, Camilleri L, Villapalos J, Boyce DE, Dziewulski P, Dickson WA, Whitaker IS. 2014. Gram negative wound infection in hospitalised adult burn patients—systematic review and metanalysis [*sic*]. PLoS One 9:e95042. https://doi.org/10.1371/journal.pone.0095042.

2. Biswal I, Arora BS, Kasana D, Neetushree. 2014. Incidence of multidrug resistant Pseudomonas aeruginosa isolated from burn patients and environment of teaching institution. J Clin Diagn Res 8:DC26–DC29. https://doi.org/10.7860/JCDR/2014/7483.4383.

3. Turhan V, Mutluoglu M, Acar A, Hatipoglu M, Onem Y, Uzun G, Ay H, Oncul O, Gorenek L. 2013. Increasing incidence of Gram-negative organisms in bacterial agents isolated from diabetic foot ulcers. J Infect Dev Ctries 7:707–712. https://doi.org/10.3855/jidc.2967.

4. Sganga G, Spanu T, Bianco G, Fiori B, Nure E, Pepe G, D'Inzeo T, Lirosi MC, Frongillo F, Agnes S. 2012. Bacterial bloodstream infections in liver transplantation: etiologic agents and antimicrobial susceptibility profiles. Transplant Proc 44:1973–1976. https://doi.org/10.1016/j.transproceed.2012.06.055.

5. Uchino M, Ikeuchi H, Matsuoka H, Bando T, Ichiki K, Nakajima K, Takahashi Y, Tomita N, Takesue Y. 2014. Catheter-associated bloodstream infection after bowel surgery in patients with inflammatory bowel disease. Surg Today 44:677–684. https://doi.org/10.1007/s00595-013-0683-5.

6. Joshi-Navare K, Prabhune A. 2013. A biosurfactant-sophorolipid acts in synergy with antibiotics to enhance their efficiency. Biomed Res Int 2013:512495. https://doi.org/10.1155/2013/512495.

7. Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R. 2005. Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob Agents Chemother 49:4093–4100. https://doi.org/10.1128/AAC.49.10.4093-4100.2005.

8. Bluth MH, Kandil E, Mueller CM, Shah V, Lin YY, Zhang H, Dresner L, Lempert L, Nowakowski M, Gross R, Schulze R, Zenilman ME. 2006. Sophorolipids block lethal effects of septic shock in rats in a cecal ligation and puncture model of experimental sepsis. Crit Care Med 34:188–195.

9. Chen J, Song X, Zhang H, Qu Y. 2006. Production, structure elucidation and anticancer properties of sophorolipid from Wickerhamiella domer-

cqiae. Enzyme Microb Technol 39:501–506. https://doi.org/10.1016/j.enzmictec.2005.12.022.

10. Fu SL, Wallner SR, Bowne WB, Hagler MD, Zenilman ME, Gross R, Bluth MH. 2008. Sophorolipids and their derivatives are lethal against human pancreatic cancer cells. J Surg Res 148:77–82. https://doi.org/10.1016/j.jss.2008.03.005.

11. Joshi-Navare K, Shiras A, Prabhune A. 2011. Differentiation-inducing ability of sophorolipids of oleic and linoleic acids using a glioma cell line. Biotechnol J 6:509–512. https://doi.org/10.1002/biot.201000345.

12. Ribeiro IA, Faustino CM, Guerreiro PS, Frade RF, Bronze MR, Castro MF, Ribeiro MH. 2015. Development of novel sophorolipids with improved cytotoxic activity toward MDA-MB-231 breast cancer cells. J Mol Recognit 28:155–165. https://doi.org/10.1002/jmr.2403.

13. Saini HS, Barragan-Huerta BE, Lebron-Paler A, Pemberton JE, Vazquez RR, Burns AM, Marron MT, Seliga CJ, Gunatilaka AA, Maier RM. 2008. Efficient purification of the biosurfactant viscosin from Pseudomonas libanensis strain M9-3 and its physicochemical and biological properties. J Nat Prod 71:1011–1055. https://doi.org/10.1021/np800069u.

14. Shao L, Song X, Ma X, Li H, Qu Y. 2012. Bioactivities of sophorolipid with different structures against human esophageal cancer cells. J Surg Res 173:286–291. https://doi.org/10.1016/j.jss.2010.09.013.

15. Sleiman JN, Kohlhoff SA, Roblin PM, Wallner S, Gross R, Hammerschlag MR, Zenilman ME, Bluth MH. 2009. Sophorolipids as antibacterial agents. Ann Clin Lab Sci 39:60–63.

16. Shah V, Badia D, Ratsep P. 2007. Sophorolipids having enhanced antibacterial activity. Antimicrob Agents Chemother 51:397–400. https://doi.org/10.1128/AAC.01118-06.

17. Piljac A, Stipcevic T, Piljac-Zegarac J, Piljac G. 2008. Successful treatment of chronic decubitus ulcer with 0.1% dirhamnolipid ointment. J Cutan Med Surg 12:142–146. https://doi.org/10.2310/7750.2008.07052.

18. Stipcevic T, Piljac A, Piljac G. 2006. Enhanced healing of full-thickness burn wounds using di-rhamnolipid. Burns 32:24–34. https://doi.org/10.1016/j.burns.2005.07.004.

19. Hardin R, Pierre J, Schulze R, Mueller CM, Fu SL, Wallner SR, Stanek A, Shah V, Gross RA, Weedon J, Nowakowski M, Zenilman ME, Bluth MH. 2007. Sophorolipids improve sepsis survival: effects of dosing and de-

rivatives. J Surg Res 142:314–319. https://doi.org/10.1016/j.jss.2007.04
.025.

20. Callaghan B, Lydon H, Roelants SL, Van Bogaert IN, Marchant R, Banat IM,
Mitchell CA. 2016. Lactonic sophorolipids increase tumor burden in
Apcmin+/− mice. PLoS One 11:e0156845. https://doi.org/10.1371/journal
.pone.0156845.

21. Van Bogaert INA, Holvoet K, Roelants SLKW, Li B, Lin Y-C, Van der Peer Y,
Sotaert W. 21 March 2013. The biosynthetic gene cluster for sophorolipids:
a biotechnological interesting biosurfactant produced by Starmerella bom-
bicola. Mol Microbiol https://doi.org/10.1111/mmi.12200.

22. Abramoff MD, Magalhaes PJ, Ram SJ. 2004. Image processing with
ImageJ. Biophotonics Int 11:36–42.

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AQA—"both acetylated and nonacetylated acidic $C_{18:1}$ congeners" as meant? If not, please rewrite for clarity.

AQB—To ensure sequential order, references have been renumbered in text and in References. Please check and correct renumbering, if necessary. If any references are to be deleted from the References list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQC—"The sophorose molecule may be acetylated in two positions, the carbon chain between 16 and 18 atoms in length, and contain no, one, or more double bonds" as meant? If not, please rewrite for clarity.

AQD—If NASL is an abbreviation, please define it.

AQE—Please make sure left column of Table 1 is as meant, or rewrite for clarity.

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AQF—"All wounds were completely closed, with no remaining residual coagulum covering, by day 16 of the study" as meant? If not, please rewrite for clarity.

AQG—If BAC-3 is an abbreviation, please define it.

AQH—"as there is no overlap in dosages between the antimicrobial effect that we observed (maximal at 4 mg/ml) and the highest dose used for cell toxicity testing" as meant? If not, please rewrite for clarity.

AQI—"C_{16} to C_{18}" as meant? If not, please rewrite for clarity.

AQJ—Please clarify what is meant by "normal" in "Cells were trypsinized as normal."

AQK—If IMS is an abbreviation, please define it.