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Ceragenins are active against drug-resistant *Candida auris* clinical isolates in planktonic and biofilm forms

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Background: *Candida auris* has emerged as a serious threat to human health. Of particular concern are the resistance profiles of many clinical isolates, with some being resistant to multiple classes of antifungals.

Objectives: Measure susceptibilities of *C. auris* isolates, in planktonic and biofilm forms, to ceragenins (CSAs). Determine the effectiveness of selected ceragenins in gel and cream formulations in eradicating fungal infections in tissue explants.

Materials and methods: A collection of 100 *C. auris* isolates available at CDC was screened for susceptibility to a lead ceragenin. A smaller collection was used to characterize antifungal activities of other ceragenins against organisms in planktonic and biofilm forms. Effects of ceragenins on fungal cells and biofilms were observed via microscopy. An *ex vivo* model of mucosal fungal infection was used to evaluate formulated forms of lead ceragenins.

Results: Lead ceragenins displayed activities comparable to those of known antifungal agents against *C. auris* isolates with MICs of 0.5–8 mg/L and minimum fungicidal concentrations (MFCs) of 2–64 mg/L. No cross-resistance with other antifungals was observed. Fungal cell morphology was altered in response to ceragenin treatment. Ceragenins exhibited activity against sessile organisms in biofilms. Gel and cream formulations including 2% CSA-44 or CSA-131 resulted in reductions of over 4 logs against established fungal infections in *ex vivo* mucosal tissues.

Conclusions: Ceragenins demonstrated activity against *C. auris*, suggesting that these compounds warrant further study to determine whether they can be used for topical applications to skin and mucosal tissues for treatment of infections with *C. auris* and other fungi.

Introduction

Candida auris has emerged as a global threat; since the initial report from Japan in 2009, it has spread and been isolated on five continents.[1–6] *C. auris* can be a substantial cause of nosocomial infections in some settings and is associated with high levels of mortality.[7,8] Of particular concern is the drug resistance found among many isolates. Resistance to azole antifungal agents with clinical isolates is common and resistance of some strains of *C. auris* to all three classes of commonly used antifungals (azoles, polyenes and echinocandins) has been observed.[1,9] The emergence of this pathogen has led to efforts to better track and characterize infections[1,6] and it provides a strong impetus for the development of novel antifungal agents active against drug-resistant organisms.[10]

Higher organisms have faced threats from fungal pathogens for hundreds of millions of years and evolutionary pressures have yielded antifungal innate immune functions effective against organisms in both planktonic and biofilm forms. Antimicrobial peptides (AMPs) constitute a key component of these innate immune defences and AMPs from a variety of sources have shown potent antifungal activities.[11–14] The prevalence of AMPs, in organisms ranging from mammals to amphibians to insects to plants, suggests that AMPs retain antimicrobial activities over extended periods without generation of widespread resistance. Recognizing the antifungal activities of AMPs and their anticipated retention of potency with extensive use, substantial efforts have been made to develop antifungal AMPs for clinical use.[13] Impediments to clinical use include the relatively high cost of large-scale preparation of

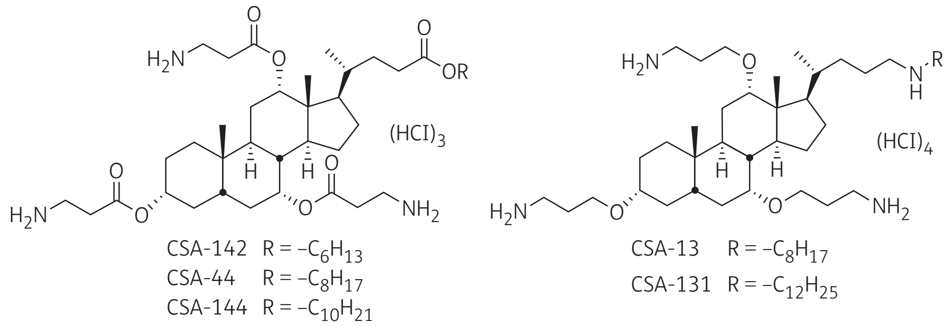

Figure 1. Structures of selected ceragenins.

peptide therapeutic agents compared with that of small molecules and the instability of linear peptides in the presence of ubiquitous proteases.

In an effort to overcome the challenges associated with these peptide therapeutic agents, while retaining the same mechanisms of antimicrobial action, we have developed a class of non-peptide molecules, based on a common bile acid, that mimic the amphiphilic morphology common to AMPs.[15,16] Molecules of this class, termed ceragenins (CSAs) (examples shown in Figure 1), can be prepared on a large scale and, because they are not peptide-based, ceragenins are not substrates for proteases. Lead ceragenins are broad-spectrum antimicrobials with activity against drug-resistant bacteria, including colistin-resistant Gram-negative bacteria.[17,18] Ceragenins also mimic ‘secondary’ activities of AMPs associated with wound healing.[19]

Recently, antifungal activities of selected ceragenins were reported,[20] with MICs of ceragenins comparable to or below those measured with amphotericin B, fluconazole and AMPs, omiganan and LL-37, for multiple strains of *Candida albicans*. Fungi in the genera of *Paecilomyces*, *Cryptococcus*, *Aspergillus*, *Scedosporium*, *Rhizopus*, *Blastomyces* and *Apophysomyces* also proved susceptible to the ceragenins tested.[20] Atomic force microscopy images of *C. albicans* untreated and treated with ceragenin CSA-13 showed changes in surface morphology of cells treated with the ceragenin, which were interpreted as showing membrane activity.[20] Studies of mechanisms of antifungal activities of AMPs have concluded that membrane perturbation plays a key role[21–23] and increases in damage by reactive oxygen species (ROS) and attenuation of mitochondrial functions, leading to apoptosis, have been observed.[24–27] In a recent study, magnetic nanoparticles adorned with either the AMP LL-37 or CSA-13 resulted in generation of ROS in *Candida* spp.[28]

Considering the need for novel antifungal agents in the face of the growing threat of *C. auris* infection, our objectives in the studies described herein were to determine the antifungal activity of selected ceragenins against isolates of *C. auris* collected by CDC and to compare these activities with those of representatives of the three major classes of antifungal agents. Activities measured included both MICs and minimum fungicidal concentrations (MFCs) on planktonic organisms. Because fungi may grow in sessile form in biofilms, we also measured the activities of selected ceragenins against established biofilm forms of *C. auris*. To better understand the antifungal activities of ceragenins, scanning electron microscopy (SEM) images of *C. auris*, with and without ceragenin treatment, were acquired. Although ceragenins are well-tolerated in a variety of routes of administration,[29–32] topical application to skin or mucosal tissues to treat fungal infections represents an attractive use of the antimicrobials. To determine the potential for use in this venue, different formulations of ceragenins CSA-44 and CSA-131 were evaluated in an *ex vivo* porcine vaginal mucosal tissue model infected with *C. albicans* or *C. auris* and compared with nystatin or a commercial cream containing miconazole.

Materials and methods

Materials and fungal strains

CDC (Atlanta, GA, USA) has catalogued over 100 clinical isolates of *C. auris* for use in determining susceptibility patterns and these were used in initial studies with ceragenin CSA-131. These isolates represent all four of the known clades of *C. auris* and originated from various countries. These isolates were characterized as susceptible or resistant to fluconazole and echinocandins based on the MICs published by CDC (https://www.cdc.gov/fungal/diseases/candidiasis/recommendations.html), although breakpoints specific for *C. auris* have not yet been established. Ten additional isolates of *C. auris* and one strain of *C. albicans* (ATCC 90028, Manassas, VA, USA) were used to further characterize susceptibility to other ceragenins. Ceragenins CSA-44, CSA-131, CSA-142 and CSA-144 were prepared as reported previously.[33,34] Three antifungal drugs belonging to the three main classes of commonly used antifungal compounds were used in comparison: amphotericin B (Fisher Scientific, Pittsburgh, PA, USA), fluconazole and caspofungin (Sigma–Aldrich, St Louis, MO, USA). The culture media used were Sabouraud dextrose broth (SDB) or agar (SDA) and Roswell Park Memorial Institute medium 1640 (RPMI) (Sigma–Aldrich) buffered at pH 7.0 with 165 mM morpholinopropanesulfonic acid (MOPS) (Sigma–Aldrich). Published protocols for growth of *Candida* spp. have used either SDB or RPMI,[35–38] and to observe any medium-dependent changes in susceptibility, MICs and MFCs were measured in both media.

Determination of susceptibility profiles of planktonic fungi

MICs of the *C. auris* strains were measured according to the broth microdilution protocol from the CLSI M27-A3 document.[39] With the entire CDC collection, RPMI medium was used in the measurement; inocula of 0.5×10^3–2.5×10^3 cfu/mL were prepared and used to seed pre-prepared drug plates containing CSA-131. These plates were incubated at 35°C and read after 24 h. With the smaller collection (10 isolates), two media (SDB and RPMI) were used to evaluate the antifungal activity of all four classes of antifungal compounds used in this study. MFCs were determined by plating 10 μL from each well of the MIC plate (after 24 h incubation) on SDA plates and measuring the lowest concentration of the antibiotic which eliminated

99.9% (3 logs reduction) of the colonies formed on the plates after 48 h of incubation at 35°C.[40]

Determination of susceptibility profiles of fungal biofilms

To quantify the biofilm formed by clinical isolates of *C. auris* and determine the sessile susceptibility profile of ceragenins, metabolic activity within biofilms was imaged using XTT as described by Moss *et al.*[41] In this assay, metabolic activity of cells in the biofilm is measured based on their reduction of XTT. Initially, biofilms were formed in sterilized polystyrene flat-bottomed 96-well microtitre plates (Sarstedt, USA) incubated at 35°C for 48 h and washed three times with PBS (Sigma–Aldrich) to remove planktonic cells. Ceragenins were then added to the wells in a concentration range of 1–128 mg/L and incubated for 24 h. A 10 mM menadione (Sigma–Aldrich) solution was prepared in 100% acetone and added to an XTT solution (0.5 mg/mL) to achieve a final concentration of 1 μM menadione. Aliquots of the XTT/menadione solution (100 μL) were added to each well containing biofilm and negative control wells. Plates were wrapped in aluminium foil and incubated at 37°C for 2 h. Aliquots of the supernatant (70 μL) were removed from each well and the OD was measured at 490 nm using a microtitre plate reader. Corresponding OD values for each strain were subtracted from the negative control values to calculate sessile MICs (SMICs). The $SMIC_{50}$ and $SMIC_{80}$ represent the concentration of the drug where the absorbance of the biofilm decreased by 50% or by 80%, respectively, compared with the biofilm formed by the same strain in the absence of the drug. Results are from three independent experiments.

SEM of C. auris

To observe the effect of ceragenins on cell membranes, *C. auris* CDC390 was cultured to mid-log phase and washed three times with PBS. Fungi were resuspended in PBS (OD_{600} = 0.2). CSA-131 (25 or 50 mg/L) was added and the mixtures were incubated at 37°C for 1 h. A control was prepared by incubating the fungal suspension without adding CSA-131. After collection via centrifugation, cells were washed with PBS three times. Glutaraldehyde (2.5%) in PBS was added to fix the cells at 4°C overnight. Resulting material was washed five times with PBS at 5000 rpm for 10 min using a microhaematocrit centrifuge (Hettich Mikro 20, Hettich, Tuttlingen, Germany) to remove the glutaraldehyde. Osmium tetroxide (0.5 mL) was used as a second fixative reagent and samples were stored at room temperature for 2–3 h. Cells were washed with PBS five times at 14 000 rpm for 8 min. A graded ethanol series (10%–100%) was used to dehydrate the cells. Samples were collected by centrifugation and the supernatant was discarded after each cycle. Finally, the samples were exposed to air in a desiccator at room temperature for 24 h. Dried fungal specimens were sputter-coated with 5–10 nm of a gold/palladium alloy and visualized under a scanning electron microscope (FEI Helios NanoLab 600 SEM/FIB, Hillsboro, OR, USA).

Confocal laser scanning microscopy

To observe the effects of treatment of fungal biofilms with CSA-131, aliquots (300 μL) of *C. albicans* and *C. auris* CDC383 (10^6 cfu/mL) were placed into separate single wells in a Lab-Tek® Chamber Slide™ (Nunc, Inc., Naperville, IL, USA) and incubated for 48 h. The biofilms were treated with CSA-131 (50 μL of a 50 mg/L solution) and incubated for 24 h at 35°C. After incubation, the solution was carefully removed and the wells were washed three times with PBS. Biofilms were stained with BacLight Live/Dead Viability Kit (L13152, Molecular Probes, Inc) according to the manufacturer's instructions and observed at ×60 magnification using a confocal laser scanning microscope (Olympus FLUOVIEW FV1000).

Table 1. MICs of CSA-131 for fluconazole-resistant, fluconazole-susceptible and echinocandin-resistant *C. auris* isolates

		MIC (mg/L)			
C. auris isolate	*n*	range	mode	MIC_{50}	MIC_{90}
Fluconazole-susceptible	30	0.5–1.0	NA	0.5	1.0
Fluconazole-resistant	70	0.5–1.0	1.0	1.0	1.0
Echinocandin-resistant	7	0.5–1.0	1.0	1.0	1.0

NA, not applicable (bimodal with equal numbers of MICs of 0.5 and 1.0 mg/L).

Assays in ex vivo tissue

Ex vivo experiments were performed using porcine vaginal mucosal explants as previously described.[42] Normal healthy porcine vaginal tissue was excised from animals at slaughter (Theurer's Quality Meats, Lewiston, UT, USA) and transported to the laboratory on ice. Tissue was trimmed and collected in RPMI-1640 medium supplemented with penicillin (50 IU/mL, MP Biomedicals, Solon, OH, USA) and streptomycin (50 IU/mL, MP Biomedicals). Antibiotics were included to decolonize normal flora, which may interfere with biofilm formation. Explants of uniform size were obtained using a 5 mm biopsy punch. Excess muscle was trimmed away with a scalpel. After antibiotic washout (three changes of RPMI-1640 medium followed by 30 min incubation at 37°C), explants were transferred mucosal side up onto a polyethylene terephthalate 0.4 μm cell culture insert (BD Falcon, Franklin Lakes, NJ, USA) and inoculated with 2 μL (10^5 cfu/explant) of *C. auris* (CDC390) or *C. albicans* (ATCC 90028) prepared from overnight cultures in Todd Hewitt broth. Following 2 and 24 h incubation at 37°C, explants ($n = 3$) were treated with CSA-44L or CSA-131L (100 μL, 0.5% active) or CSA-44H and CSA-131H (100 μL, 2.0% active) in a hydroxyethylcellulose (HEC) gel formulation and subsequently incubated for 24 h at 37°C. For studies of cream formulations, aliquots of the test articles (100 μL) were spread evenly over explants. After treatment, tissues were suspended in a neutralizing broth [250 μL, Dey/Engley (HiMedia Laboratories, West Chester, PA, USA)], vortex-mixed (highest setting for 4 min) and then serially diluted in PBS and plated. Samples were spread on tryptic soy agar containing 5% sheep blood (BD Falcon) using a spiral plater (Microbiology International, Frederick, MD, USA) then incubated for 24 h at 37°C. The cream vehicle was assayed for antifungal activity by placing aliquots (100 μL) in a 96-well plate and adding fungal inocula (*C. albicans* ATCC 90028, 100 μL of 10^3, 10^4 or 10^5 cfu/mL cultures). Resulting samples were incubated (37°C) for 24 h. Aliquots from each sample were plated, incubated for 48 h and counted. Results were compared with controls that were not treated with the cream vehicle.

Statistical analysis

Analyses of variance (ANOVA) were performed by Dunnett's multiple comparison post-test using the GraphPad PRISM software (GraphPad Software, Inc., La Jolla, CA, USA).

Results and discussion

Susceptibility of planktonic fungi to ceragenins

The susceptibilities of 100 clinical isolates to a lead ceragenin, CSA-131, were determined and the distribution of CSA-131 MIC values ranged from 0.5 to 1 mg/L (Table 1). The overall mode was 1 mg/L and both the MIC_{50} and the MIC_{90} were 1 mg/L. CSA-131 showed activity against all four clades of *C. auris* with no variation in activity between the clades. There was no loss in activity for those isolates

Table 2. Comparison of the susceptibility of clinical isolates of *C. auris* to selected ceragenins and three major classes of antifungal agents in SDB and RPMI

	MIC (MFC) mg/L						
Strains	CSA-44 SDB [RPMI]	CSA-131 SDB [RPMI]	CSA-142 SDB [RPMI]	CSA-144 SDB [RPMI]	CPF SDB [RPMI]	AMB SDB [RPMI]	FLC SDB [RPMI]
Candida auris							
CDC381	0.5 (2.0)[a]	0.5 (8.0)[a]	4.0 (32)[a]	0.5 (2.0) [1.0 (8.0)]	2.0 (64)[a]	1.0 (48) [1.0 (64)]	16 (>100) [32 (>100)]
CDC382	0.5 (4.0)[a]	0.5 (8.0)[a]	4.0 (24)[a]	1.0 (8.0)[a]	NM	NM	NM
CDC383	0.5 (8.0) [0.5 (16)]	1.0 (10) [0.5 (8.0)]	2.0 (64)[a]	1.0 (8)[a]	32 (64) [16 (48)]	1.0 (32) [1.0 (64)]	64(>100) [>100 (>100)]
CDC384	0.5 (4.0)[a]	0.5 (4.0)[a]	4.0 (24)[a]	1.0 (8.0)[a]	NM	NM	NM
CDC385	0.5 (16)[a]	0.5 (4.0)[a]	8.0 (32)[a]	1.0 (8.0)[a]	NM	NM	NM
CDC386	0.5 (8.0)[a]	1.0 (8.0) [0.5 (8.0)]	4.0 (32)[a]	0.5 (8.0) [1.0 (16)]	2.0 (64)[a]	2.0 (48) [1.0 (64)]	64(>100) [>100(>100)]
CDC387	0.5 (8.0)[a]	0.5 (8.0)[a]	4.0 (32)[a]	0.5 (8)[a]	NM	NM	NM
CDC388	1.0 (8.0)[a]	0.5 (4.0)[a]	4.0 (24)[a]	2.0 (8.0)[a]	NM	NM	NM
CDC389	0.5 (8.0)[a]	0.5 (8.0)[a]	4.0 (24)[a]	1.0 (8.0)[a]	NM	NM	NM
CDC390	0.5 (8.0) [1.0 (32)]	0.5 (4.0) [0.5 (8.0)]	4.0 (16)[a]	1.0 (8.0)[a]	2.0 (100)[a]	4.0(64)[a]	64 (>100) [>100 (>100)]
Candida albicans							
ATCC 90028	0.5 (8.0) [0.5 (16)]	0.5 (4.0) [0.5 (8.0)]	2.0 (8.0)[a]	2.0 (8.0)[a]	2.0 (32) [1.0 (8.0)]	2.0 (100) [2.0 (64)]	24 (>100) [32 (>100)]

CAS, caspofungin; AMB, amphotericin B; FLC, fluconazole; NM, not measured.
[a]Same result in both media.

that were previously determined to be resistant to fluconazole and/or an echinocandin.

To verify that other lead ceragenins also demonstrated activity against *C. auris*, studies were performed with a smaller collection of isolates and activities were compared with those of a strain of *C. albicans*. Both MICs and MFCs were measured in SDB or RPMI of CSA-44, CSA-131, CSA-142, CSA-144 and representatives of the three major classes of antifungal agents. As observed with the larger collection of *C. auris* isolates, MICs of CSA-131 were either 0.5 or 1.0 mg/L and MICs of CSA-44 and CSA-144 were similar (Table 2). MICs of CSA-142 were relatively higher. The strains tested were associated with relatively high MICs of fluconazole and one of the strains (CDC383) showed resistance to caspofungin. Compared with caspofungin, amphotericin B and fluconazole, the ceragenins gave lower MFCs, suggesting greater fungicidal activity compared with these other antifungal agents. Among the representative antifungal agents, caspofungin is the most closely related to ceragenins; it is a cationic molecule appended with a lipid chain. Similarly, ceragenins are cationic and a lipid chain is required for activity. Nevertheless, echinocandin-resistant *C. auris* strains (CDC383 for example) were fully susceptible to ceragenins (Table 1). In general, the medium in which MICs and MFCs were determined did not play a substantial role; MICs of the majority of the antifungal agents were identical in both media, as were MFCs.

SEM of ceragenin-treated fungi

As described above, membrane disruption has been identified as a major mechanism of antifungal activity of AMPs,[21–23] among other possible mechanisms. Changes in cell morphology upon treatment with AMPs have been attributed to membrane interactions; these include loss of smooth cell surfaces with marked invagination at high AMP concentrations. Similar morphological changes in *C. albicans* were observed with the human AMP, LL-37, and the ceragenin, CSA-13.[20] The impacts of CSA-131 on the morphology of *C. auris* are shown in Figure 2. As with *C. albicans* treated with LL-37 or CSA-13, CSA-131 alters cell shape; at 50 mg/L, CSA-131 causes cells to buckle in upon themselves and some cells appear to have merged. These changes in cell morphology were observed at concentrations well above MFCs and antifungal activity may be due to more subtle changes in cell permeability at lower concentrations of the ceragenin. At MFCs, little or no morphological changes in fungal cells were apparent.

Susceptibility of fungal biofilms to ceragenins

The abilities of bacteria and fungi to form biofilms, in which sessile organisms reside, contribute to drug resistance and protection of persister organisms that provide sources for chronic infections. The abilities of *C. albicans* to form biofilms are well-established;[43–46] however, differing results for biofilm-forming propensities of

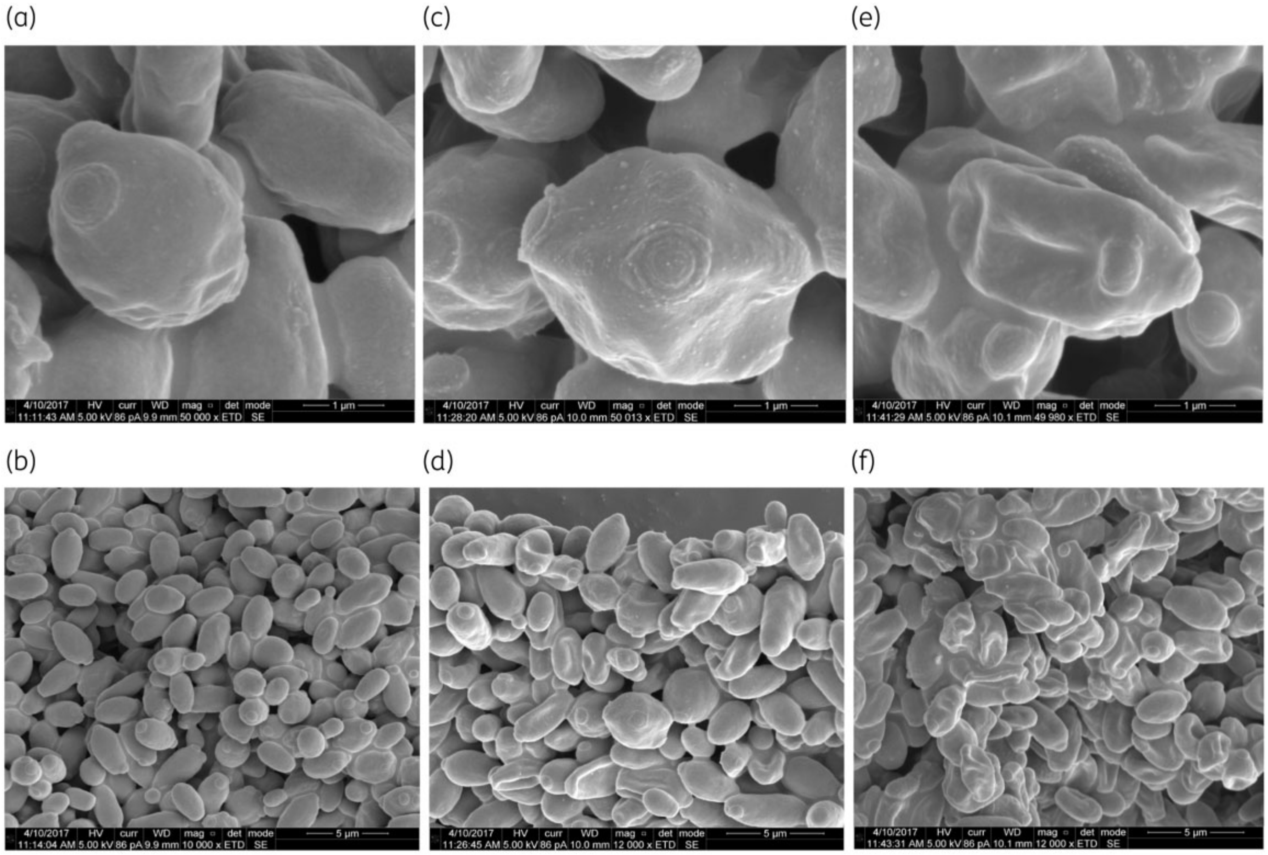

Figure 2. Scanning electron photomicrograph of untreated *C. auris* CDC390 (a and b) and treated with 25 mg/L CSA-131 (c and d) and 50 mg/L CSA-131 (e and f).

C. auris have been reported.[47,48] Using a measure of metabolic activity, we compared biofilm-forming properties of a *C. albicans* reference strain with those of four isolates of *C. auris* (CDC381, CDC383, CDC386 and CDC390). Biofilms were grown under identical conditions and, as reported,[47] *C. auris* strains were less efficient than the *C. albicans* strain at biofilm formation. Using the XTT-based assay, metabolic activity of the *C. auris* biofilms was measured as approximately 50% of that of *C. albicans* (data not shown).

Having established the biofilm-forming characteristics of *C. auris* isolates, we used metabolic activity of biofilms to determine the activities of representative ceragenins and antifungals against sessile organisms making up these biofilms. Concentrations necessary to reduce biofilms by 50% and 80% are shown in Table 3. The ceragenins, amphotericin B and caspofungin demonstrated strong activity against the biofilms, while fluconazole was much less active. Because fluconazole was weakly active against these organisms in planktonic form, it is not surprising that biofilms were less susceptible to this antifungal compared with others included in the study. Observing $SMIC_{80}$ results, it is apparent that the sessile form of *C. auris* CDC390 becomes particularly resistant to all of the antifungals.

Table 3. Susceptibility profiles (mg/L) of sessile fungi (biofilm) to CSA-44, CSA-131 and three antifungal compounds

	$SMIC_{50}$ ($SMIC_{80}$)				
Antimicrobial	*C. albicans* ATCC 90028	*C. auris* CDC381	*C. auris* CDC383	*C. auris* CDC386	*C. auris* CDC390
CSA-44	2.0 (8.0)	4.0 (16)	2.0 (8.0)	2.0 (4.0)	4.0 (64)
CSA-131	2.0 (4.0)	2.0 (32)	2.0 (4.0)	2.0 (4.0)	4.0 (64)
Amphotericin B	2.0 (8.0)	4.0 (16)	2.0 (4.0)	<1.0 (1.0)	8.0 (>64)
Caspofungin	2.0 (8.0)	4.0 (32)	8.0 (32)	8.0 (64)	8.0 (100)
Fluconazole	64 (200)	64 (>200)	64 (>200)	32 (200)	100 (>200)

Confocal laser scanning microscopy of fungal biofilms

To observe the antibiofilm properties of a lead ceragenin, biofilms of *C. albicans* and *C. auris* were treated with CSA-131, stained and imaged via confocal microscopy (Figure 3). Untreated biofilms exhibited expected aggregates of live cells, while treated biofilms showed comparable aggregates of dead cells. As with Gram-negative bacteria,[49] the ceragenin was able to penetrate the extracellular matrix and exert antifungal activity without significantly compromising the biofilm morphology. The antibiofilm property of the ceragenins complements their ability to inhibit biofilm formation as reported in a recent study in which CSA-131, incorporated into a hydrogel, prevents biofilm formation on a medical device for extended periods.[50]

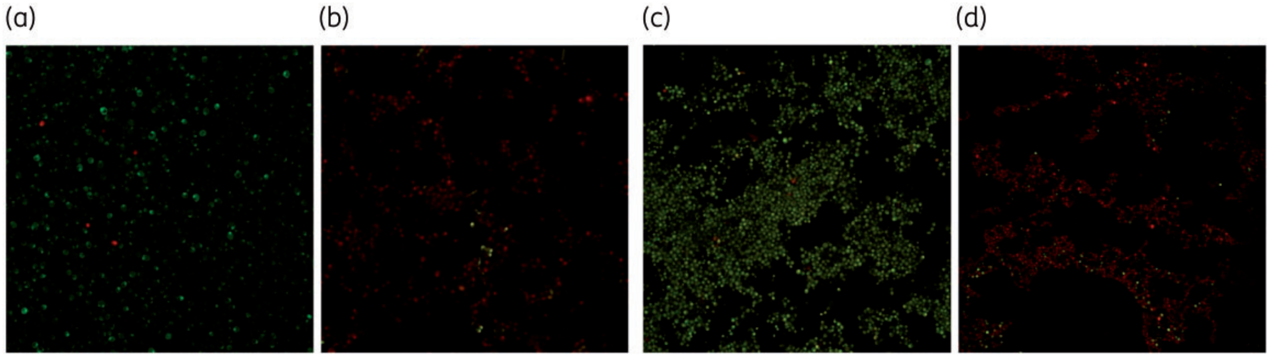

Figure 3. Confocal laser scanning micrographs (×60 magnification) of stained fungal biofilms. Green: live cells; red: dead cells. (a) *C. albicans*, untreated. (b) *C. albicans*, treated with CSA-131 (50 mg/L). (c) *C. auris*, untreated. (d) *C. auris*, treated with CSA-131 (50 mg/L).

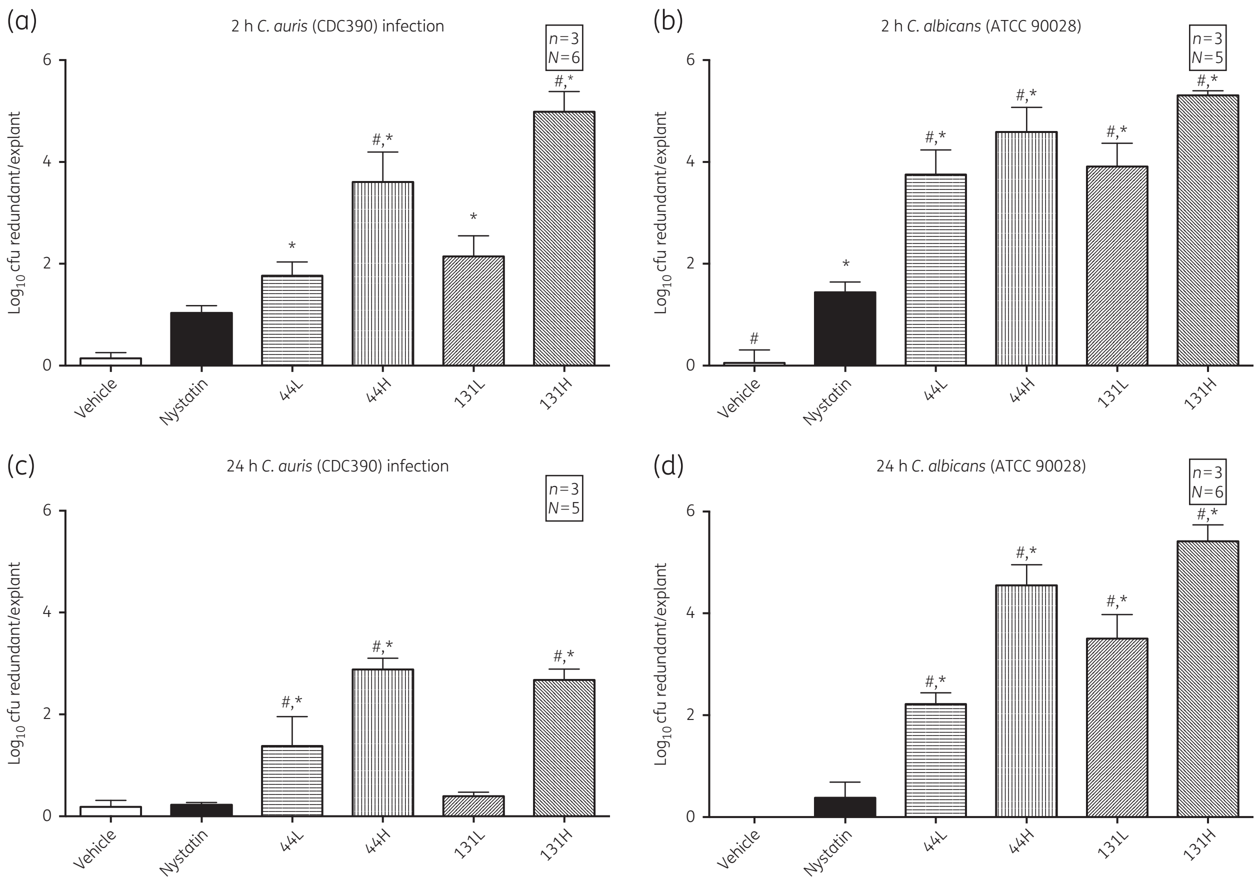

Figure 4. Antifungal activities of nystatin (100 000 USP nystatin units) compared with HEC/Pluronic® F-127 formulations of CSA-44 and CSA-131 (CSA-44L and CSA-131L contain 0.5% ceragenin and CSA-44H and CSA-131H contain 2% ceragenin) in porcine vaginal mucosal tissue explants. Log_{10} cfu reduction from untreated growth control following 2 h (a and b) and 24 h (c and d) infections of *C. auris* or *C. albicans* on PVM. Data presented are means and SEMs. Analyses of variance (ANOVA) followed by Dunnett's multiple comparison post-test were performed using the GraphPad PRISM software. *Significantly different from growth control ($P < 0.5$); #Significantly different from nystatin ($P < 0.5$).

Antifungal activity of formulated ceragenins in tissue explants

There are many potential applications of novel antifungal agents and some of the most widely useful would involve topical application to skin or mucosal tissues. For these types of applications, lead ceragenins would need to be formulated into gels or creams. HEC (typically used at 2.7% in water) can be used to form a lubricating gel into which antimicrobial compounds can be formulated. Pluronic® F-127 is a non-ionic surfactant that has shown compatibility with ceragenins with concomitant decreases in cytotoxic

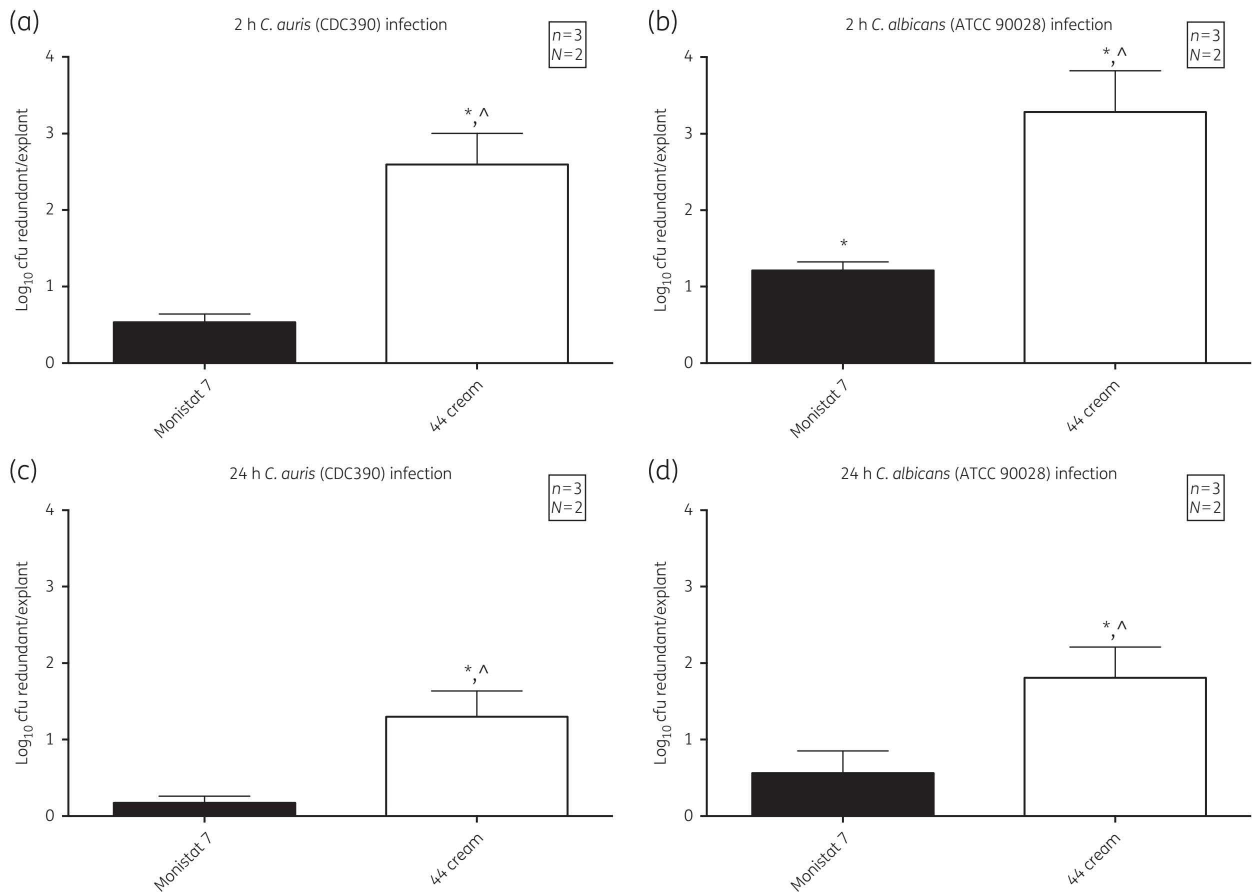

Figure 5. Antifungal activities of Monistat 7 (2% miconazole) compared with a cream formulation of CSA-44 (1%) in porcine vaginal mucosal tissue explants. Log_{10} cfu reduction from untreated growth control following 2 h (a and b) and 24 h (c and d) infections of *C. auris* or *C. albicans* on PVM. Data presented are means and SEMs. Analyses of variance (ANOVA) followed by Dunnett's multiple comparison post-test were performed using the GraphPad PRISM software. *Significantly different from growth control ($P<0.5$); ^Significantly different from Monistat 7 ($P<0.5$).

properties.[51,52] Combining HEC, Pluronic® F-127 and either CSA-44 or CSA-131 at 0.5% (CSA-44L and CSA-131L) or 2.0% (CSA-44H and CSA-131H) gave stable, lubricious gels. These percentages of ceragenins are comparable to those of antifungals incorporated into commercially available antifungal products.

Porcine vaginal mucosal explants were used to determine the antifungal activities of these gels. Nystatin (a polyene antifungal related to amphotericin B) was used as a comparator and the HEC/Pluronic® F-127 combination (vehicle) was used to observe effects of these compounds on the fungi. Fungal infection with either *C. albicans* or *C. auris* was established over 2 and 24 h of incubation, followed by treatment for 24 h with the test formulations. Over this time course, fungal populations grew to over 10^6 cfu/explant (Figure 4). In all experiments, the vehicle had no impact on fungal growth.

At 2 h, nystatin decreased fungal counts with *C. auris* and *C. albicans* by ~1 log, but only with *C. albicans* was this reduction statistically significant. In contrast, both ceragenins, at both concentrations, significantly reduced fungal counts at 2 h, with reductions as great as 5 logs (CSA-131H). At 24 h, nystatin-treated explants supported fungal counts comparable to controls with both species of fungi. At 24 h, the most potent activity of ceragenins was observed with *C. albicans*, with fungal counts significantly reduced relative to both control and nystatin-treated explants. With the highest concentrations of ceragenin (2%), fungal counts were reduced by >4 logs. Although the activity of the ceragenins at 24 h was not as dramatic with explants colonized with *C. auris*, significant reductions (~3 logs) were observed with the highest concentration of the ceragenins. Significant reduction was also observed with the lower concentration (0.5%) of CSA-44.

CSA-44 was also formulated into a cream and compared with a commercial minocycline product, Monistat 7, in the porcine vaginal mucosa (PVM) infection model. Based on efficacy of the ceragenins in the gel formulation, a concentration of 1% CSA-44 in a cream was used and compared with Monistat 7 (2% miconazole in cream formulation). No other formulations were evaluated in this assay. When tested for antifungal activity, the cream vehicle did not impact growth (i.e. growth of *C. albicans* in the presence of the cream formulation was not different from a blank control.) With *C. albicans*, Monistat 7 reduced fungal counts by a statistically significant amount at 2 h, compared with the growth control, but this effect was negligible at 24 h. With this organism, the CSA-44 cream formulation reduced counts by >3 logs at 2 h and by ~2 logs at 24 h; both of these measurements were significantly different from both the vehicle and the Monistat 7-treated explants (Figure 5). Explants colonized with *C. auris* were not significantly affected by Monostat-7 treatment at 2 and 24 h. In contrast, the CSA-44-containing cream significantly reduced *C. auris* counts at both timepoints (>2 logs and >1 log reductions, respectively).

Conclusions

The spread of *C. auris*, the high mortality rates in patients with infection and the high prevalence of drug resistance among clinical isolates underscore the need for the development of novel antifungal agents. The mechanisms common to endogenous AMPs provide direction for investigation of therapeutic agents that mimic antimicrobial activities that nature has honed over millions of years. Due to their simple structures, ceragenins are attractive candidates for development of novel antifungal agents; they can be prepared on a large scale and they are stable in the presence of ubiquitous proteases. The ceragenins display antifungal activities with *C. auris* comparable to the most active agents tested and they do not show cross-resistance with other antifungals. The ceragenins also retain activity against sessile organisms in established biofilms. In formulated forms, ceragenins are active against high inocula of fungi in mucosal tissue. The substantial antifungal activities of these ceragenin formulations compared with nystatin and Monistat 7 highlight their potential for use as novel antifungals for topical or mucosal applications.

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Transparency declarations

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