cell migration into wound bends, neovascularization and acceleration of bone regrowth [31]. These properties, coupled with their broad antimicrobial activities, make ceragenins attractive targets for clinical development in replacing deficiencies of endogenous AMPs or augmenting their activities through additive or synergistic effects.

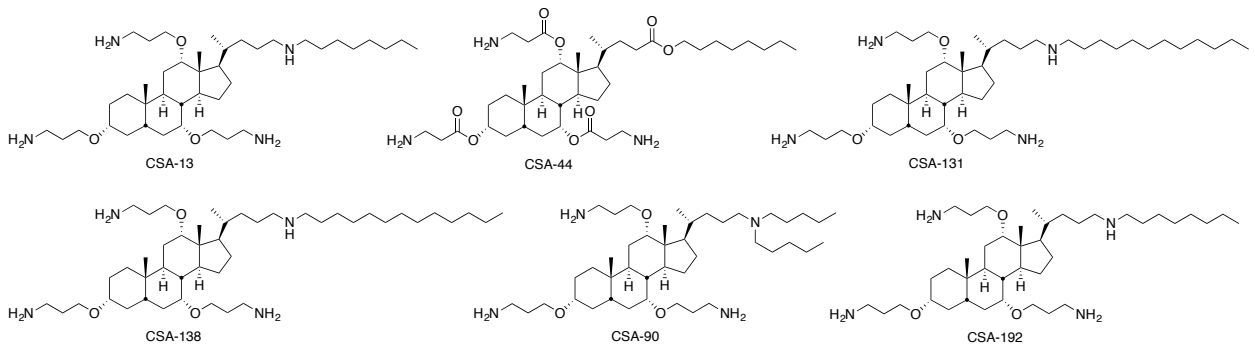

Figure 1: Structures of ceragenins CSA-13, CSA-44, CSA-90, CSA-131, CSA-138 and CSA-192.

Mechanism of action

As mimics of AMPs, ceragenins selectively target bacterial membranes [32,33], and association of ceragenins with the cytoplasmic membranes of bacteria is sufficient to cause cell death. Epand et al. [34] used the ML-35p strain of *Escherichia coli* to verify that ceragenins are able to traverse the outer membranes of Gram-negative bacteria, and observation of cytoplasmic membrane depolarization correlated well with bactericidal activity, suggesting that formation of only minor membrane defects can lead to bacterial death. Similarly, many AMPs cause transient ion fluxes through bacterial membranes, and these have been correlated to antibacterial activities [35-37]. Multiple models, including the "carpet" model, have been used to explain the origins of these ion fluxes. This model involves AMP association, *via* ionic interactions, with bacterial membranes, and when sufficient local concentrations of the antimicrobial are reached, patches of membrane are disrupted, allowing ion flux [18,38-41]. Morphological changes in Gram-negative bacteria in response to AMPs include blebbing of the outer membranes, [42-44] and the same type of blebbing has occurs upon treatment of Gram-negative bacteria with ceragenins [33,45] Bacterial endotoxins are constituents of bacterial membranes, [46] and the affinity of ceragenins for bacterial membranes translates into the ability of ceragenins to sequester bacterial endotoxins and inhibit innate immune recognition. Bucki et al. [47] demonstrated that a lead ceragenin, CSA-13, binds to lipopolysaccharide (LPS) and inhibits its ability to activate TLR-4, preventing NF-kB translocation to the nucleus; NF-kB translocation leads to inflammatory cytokine production and release. Isogai et al. [48] showed that CSA-13 sequesters LPS from multiple types of bacteria commonly found in the oral cavity at concentrations comparable to AMPs. In addition, they showed that CSA-13 also binds to lipoteichoic acid, an endotoxin found in Gram-negative and positive bacteria.

The understanding of how AMPs and ceragenins interact with viral lipid envelopes is less well understood. The activities of AMPs against lipid-enveloped viruses has been well established, but a full description of their antiviral activities has not been realized [49,50]. Howell et al. [51] studied the activities of ceragenins against vaccinia virus. They showed that CSA-13, at a concentration comparable to the human AMP, LL-37, caused viral disruption. Transmission electron micrographs showed substantial changes in the lipid envelop after treatment with CSA-13. Use of a fluorophore-labeled ceragenin allowed observation of selective binding of the ceragenin to the envelop of vaccinia virus over human keratinocytes. Topical administration of CSA-13 on vaccinia-inoculated mice resulted in a significant decrease in the number of satellite lesions, and pre-incubation of the virus with CSA-13 inhibited viral replication. Notably, in these studies, Howell et al. [51] observed that treatment of human keratinocytes with CSA-13 induced LL-37 and beta-defensin 3 production.

Antimicrobial spectrum ceragenin activity

Antibacterial activity

As mimics of AMPs, ceragenins display broad-spectrum activity against Gram-negative and positive bacteria, including activity against drug-resistant organisms [33,52-54]. Studies of bacteria susceptibility to ceragenins has focused on clinical isolates and bacteria, primarily clinical isolates, endemic to specific areas of the human body. For example, Isogai et al. [48] surveyed the susceptibility of a collection of Gram-negative and positive bacteria from the oral cavity to CSA-13 and found minimum inhibitory concentrations (MICs) of 1 to 16 μg/ml; Bucki et al. [55] determined the susceptibility of bacteria associated with oral and upper respiratory tract infections to ceragenins and LL-37. Ceragenin MICs against bacteria included measurements with *S. aureus* and *S. epidermidis*, Streptococcus strains (*S. salivarius*, *S. sanguinis*, *S. mutanss*, *S. pneumoniae*, *S. pyogenes*) and other pathogens including *Neisseria meningitis* B and C, *Moraxella catarrhalis* and *Tannerella forsythensis*. MICs ranged from 0.7 to 46.8 μg/ml with ceragenins, and those with LL-37 ranged from 14 to 448 μg/ml. Notably, the highest MICs for the ceragenins were with *Lactobacillus casei* (44.8 to 46.8 μg/ml), and the MIC was also elevated with LL-37 (224 μg/ml). This species of bacteria is considered a probiotic, and it is likely that it has adapted to the presence of AMPs and is similarly resistant to ceragenins. Negatively charged biopolymers in the oral cavity and respiratory tract have the potential to bind to AMPs and ceragenins, thereby deactivating them, and these compounds may increase during infection and biofilm formation. Bucki et al. [40,46] showed that DNA, F-actin, and salivary mucins can partially inhibit LL-37 antibacterial activity, but CSA-13 remains bactericidal activity in their presence [47,56]. A focal point in

determining the antibacterial efficacy of ceragenins has been with Gram-negative bacteria, including drug resistant strains. Treatment options for infections caused by Gram-negative bacteria are limited, as compared to Gram-positive bacteria, [57] due to the permeability barrier provided by the outer membranes of Gram-negative bacteria and the efflux pumps therein, and ceragenins retain activity against drug-resistant Gram-negative bacteria [58].

Susceptibility of 40 drug-resistant strains of *Pseudomonas aeruginosa*, isolated from cystic fibrosis patients to ceragenins CSA-13 and CSA-131 was significantly higher than to LL-37 [59]. MICs of the ceragenins were 0.6-16 μg/ml, while those with LL-37 were 32-256 μg/ml respectively. MICs of these strains with tobramycin were 4-256 μg/ml. In a related study, Bozkurt-Guzel et al. [60,61] showed that CSA-13 retains activity against tobramycin-resistant *P. aeruginosa* isolates from cystic fibrosis patients and carbapenem-resistant *Acinetobacter baumannii* isolates from bacteremia patients. Of increasing concern is the emergence of colistin resistance among Gram-negative bacteria. Colistin is often considered an antibiotic of "last resort," and colistin-resistance is typically accompanied by high levels of resistance to other antibiotics [62,63] because colistin is peptide based and cationic, similar to AMPs, it is possible that colistin resistance translates into resistance to AMPs and ceragenins.

Vila et al. [64] compared the susceptibility of colistin-resistant and susceptible isolates of *P. aeruginosa* and *A. baumannii* to ceragenins CSA-13, CSA-44, CSA-131 and CSA-138. MICs were identical or comparable with both the colistin-resistant and susceptible isolates, with MIC90s ranging from 2-8 μg/ml. Hashemi et al. [22] determined susceptibility of colistin-resistant isolates of *Klebsiella pneumoniae* to selected ceragenins and AMPs (Table 1). For most ceragenins, MICs were relatively low, independent of MICs with colistin. Two of the AMPs tested (LL-37 and magainin I) were relatively weakly active against all of the strains, while cecropin gave much lower MICs.

Strains	Colistin	CSA-13	CSA-44	CSA-131	CSA-138	CSA-142	LL-37	Cecropin A	Magainin 1
K. pneumoniae (ARLG-1127)	32	2	1	1	2	2	64	2	64
K. pneumoniae (ARLG-1340)	100	2	1	1	3	4	100	nm	nm
K. pneumoniae (ARLG-1349)	16	2	1	3	3	8	64	4	64
K. pneumoniae (ARLG-1360)	64	2	1	2	6	6	100	4	150
K. pneumoniae (ARLG-1389)	200	6	2	3	8	8	100	4	200
K. pneumoniae (ARLG-1406)	64	3	1	3	6	16	64	4	100
K. pneumoniae (ATCC-13883)	2	2	1	1	3	3	32	2	64
A. baumannii (ATCC-19606)	1	3	2	2	3	6	16	4	32
P. aeruginosa (ATCC-27853)	1	2	2	2	2	5	32	4	64

Table 1: MICs (μg/ml) of colistin, selected ceragenins and AMPs against Gram-negative bacteria including *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*.

Isolation of drug-resistant forms of *Helicobacter pylori* have led to concerns about the inability to eradicate chronic infections leading to gastric adenocarcinoma [65].

An *in vitro* study of CSA-13 susceptibility of seven strains of *H. pylori* (including clarithromycin and/or metronidazole-resistant strains) indicated MBCs of 0.275-8.9 μg/ml with CSA-13, while MBC values with LL-37 and WLBU2 (an engineered antimicrobial peptide) were 100-800 and 17.8-142 μg/ml, respectively [66].

In a further study of the susceptibility of *H. pylori* to ceragenins, McGee et al. [67] showed that membrane incorporation of cholesterol decreased substantially the susceptibility of these bacteria to ceragenins. This effect is likely due to changes in the membrane fluidity caused by cholesterol content.

Sporicidal activity

Sporulation processes in bacteria, including those from the genera *Bacillus* and *Clostridium*, enhance survival in extreme environments and decrease susceptibility to antibiotics and disinfectants. Considering the broad spectrum of antimicrobial activity of ceragenins, Bucki et al. [68] evaluated the sporicidal activity of CSA-13 against *Bacillus subtilis*. CSA-13 association with *B. subtilis* spores caused permeabilization of the spore membrane, and significantly lowered levels of calcium dipicolinic acid in the core. Since, dipicolinic acid release proceeds spore transformation into vegetative cells, it is likely that the ability of CSA-13 to eradicate dormant forms is associated with the ability to induce their germination.

Figure 2 shows TEM images of CSA-13-treated and untreated spores. Notably, assessment of surface electrical properties of spores and vegetative cells through zeta potential illustrated that CSA-13 has a higher affinity for spores than for vegetative cells, indicating that the spores present a higher level of negative charges on their surfaces as compared to vegetative cells. This activity of CSA-13 suggests that it may find use in eradicating not only vegetative forms of bacteria but spore forms as well.

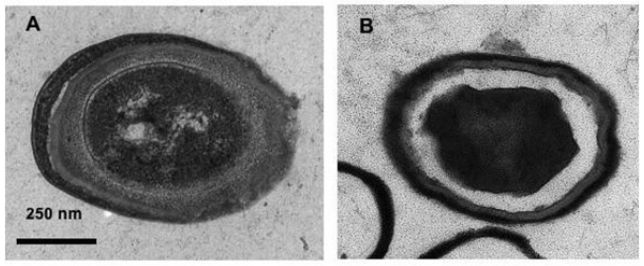

Figure 2: TEM micrographs of a *Bacillus subtilis* spore before (panel A) and after treatment with CSA-13 (50 μM at 70°C) (panel B).

Anti-fungal activity

Due to emerging resistance of fungi to commonly used antibiotics, the need for development of novel effective antifungal agents has become increasingly recognized [69-71]. Antifungal activities of AMPs have been reported, [49,72] and as mimics of AMPs, ceragenins were anticipated to have antifungal activities. Bucki et al. [24] determined the antifungal activities of ceragenins CSA-13, CSA-131 and CSA-192 and compared these to those of LL-37, a synthetic, anti-fungal AMP (omiganan) and commonly-used antifungal agents amphotericin B and fluconazole. MICs and minimum fungicidal concentrations (MFCs) for clinical isolates of *C. albicans* are given in Table 2. Ceragenins CSA-13 and CSA-192 displayed comparable activity to amphotericin B, and MICs and MFCs were much lower with ceragenins than with LL-37, omiganan and fluconazole. Against a broad array of fungal strains, including those from the genera *Candida, Cryptococcus, Aspergillus, Scedosporium, Rhizopus* and *Blastomyces*, CSA-131 proved to be the most active of compounds tested, with MICs ranging from 1 to 4 μg/ml. As with bacteria, treatment of *C. albicans* caused perturbations in the morphology of cells, suggesting that ceragenins impact cell membranes (Figure 3). The activities of ceragenins against both bacteria and fungi suggest that they have potential as antimicrobial agents against mixed infections.

Strains	CSA-13	CSA-131	CSA-192	LL-37	Omiganan	Amphotericin B	Fluconazole
C. albicans 1407	0.5 [1]	8 [16]	2 [4]	64 [128]	64 [128]	1 [1]	128 [128]
C. albicans 1408	1 [1]	8 [8]	1 [2]	>256 [>256]	64 [128]	1 [1]	16 [32]
C. albicans 1409	4 [4]	32 [32]	2 [4]	>256 [>256]	64 [128]	0.5 [1]	16 [32]
Candida species	0.5 [1]	8 [16]	1 [2]	64 [128]	128 [128]	1 [2]	>256 [>256]

Table 2: MICs [MFCs] (μg/ml) of selected ceragenins, LL-37, omiganan, amphotricin B and fluconazole against Candida strains.

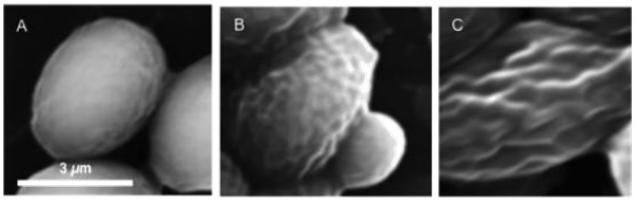

Figure 3: Scanning electron micrographs of untreated *C. albicans* cells (panel A) and cells subjected to CSA-13 at 10 μM (Panel B) and 50 μM (panel C).

Antibiofilm activity

Biofilms are microbial communities with altered phenotypes, relative to planktonic or free-growing microorganisms, that agglomerate through a extracellular polymeric matrix [73,74]. The impact of biofilms, both bacterial and fungal, are increasingly recognized as threats to public health due to the fact that biofilm formation is involved in more than 60% of bacterial infections, as well as being recognized as a causative agent in most medical device-related infections [75,76]. In their biofilm forms, bacteria and fungi are resistant to most antimicrobials, due to decreased metabolism and the barrier provided by the extracellular matrix [77-79]. The relatively small size of ceragenins allows them to permeate this matrix, and because their antimicrobial activity does not require that organisms be actively growing, they eradicate biofilms at relatively low concentrations [80,81].

Pollard et al. [82] compared the antibiofilm activity of CSA-13 to ciprofloxacin with established bacterial biofilms in a bioreactor developed at the Centers for Disease Control. CSA-13 proved to be better than ciprofloxacin against biofilms formed from methicillin-resistant *S. aureus*. Nagant et al. [83] conducted further studies using confocal laser scanning microscopy of biofilms of *P. aeruginosa* and their responses to CSA-13. Images revealed that the ceragenin effectively penetrated an established biofilm within 30 min and caused cell death without substantial changes to the extracellular matrix. In a later study, Nagant et al. [80] investigated the relationship between zeta potential of different strains of *P. aeruginosa* and the ability of CSA-13 to prevent biofilm formation. At a relatively low concentration (1 μg/ml) CSA-13 prevented biofilm formation, and the strains most strongly affected by the ceragenin were those with a zeta potential lower than -50 mV. This observation is consistent with the model of ceragenins interacting with bacterial membranes through ionic interactions.

Recently, the effects of CSA-13, CSA-131 and LL-37 on biofilms formed by drug-resistant *P. aeruginosa* were evaluated, including the Liverpool epidemic strain, a dominant strain of *P. aeruginosa* found in the airways of cystic fibrosis patients [59]. Not only did ceragenins prevent biofilm formation better than LL-37, but ceragenins retained antibiofilm activity even in presence of polyelectrolytes such as Pf1, F-actin, and DNA, which can induce biofilm formation.

Comparative antibiofilm activities of ceragenins CSA-13 and CSA-131 and LL-37 on biofilms formed from *Candida albicans* in

presence of DNA, which stimulates fungal biofilm formation, demonstrated that each of the compounds inhibited the DNA-dependent biofilm growth, and that ceragenins exhibited a stronger inhibitory effect on development of biofilm and maintained that effect for a much longer period of time than LL-37 [20]. Additionally, the combination of DNase I with both LL-37 and ceragenins improved their inhibitory effect on biofilm formation by breaking down complexes of DNA with either LL-37 or ceragenins, which are then more able to penetrate biofilm matrices.

Antiviral activity

AMPs have been characterized as antiviral agents with activity against lipid-enveloped viruses [84]. Pox viruses are lipid enveloped, and LL-37 and its murine equivalent have been shown to play a central role in inhibition of propagation of vaccinia virus (cow pox). Deficiencies in LL-37 may allow propagation of vaccinia virus (the live-virus vaccine for small pox) in humans. Howell et al. [51] studied the antiviral activity of CSA-13 against vaccinia virus and showed that it inactivates 60%, 91% and 96% of the virus at 5, 10, and 25 μM, respectively. Microscopy of vaccinia virus with CSA-13 and LL-37 showed similar morphological changes to the virus with apparent damage to the envelope and internal structure. Topical administration of CSA-13 to mice infected with vaccinia virus showed that satellite lesions, monitored for 10 days, were substantially suppressed. In a companion study, fluorophore-labeled CSA-13 was shown to penetrated the outer layers of the skin and thereby gain access to the virus during infection.

Anti-parasite activity

Investigations of the anti-parasite activities of ceragenins have included studies with *Trypanosoma cruzi*, the causative agent of Chagas disease, *Leishmania major*, *Acanthamoeba castellanii* and *Trichomonas vaginalis*, one of the most prevalent protozoan infections in humans. Lara et al. [85] reported ceragenin activity against trypanosomatids. In an *in vitro* study of ceragenins CSA-8, CSA-13, and CSA-54 with *T. cruzi* trypomastigotes and *L. major* promastigotes, CSA-13 gave an LD_{50} of ca. 9 and 5 μ M, respectively. Polat et al. [86] observed activity of CSA-13 against *A. castellanii* at concentrations ranging from 25 to 100 μg/ml. This protozoan is the causative agent of acanthamoeba keratitis, which is a rare but devastating infection of the cornea often associated with wearing of contact lenses. *T. vaginalis* is a highly contagious, sexually-transmitted protozoan parasite, hosted only in humans. Activities of ceragenins CSA-13, CSA-44, CSA-131 and CSA-138 were evaluated against metronidazole-susceptible and resistant organisms. With the exception of CSA-44, these ceragenins, at a concentration of 50 μM, eliminated a four-log inoculum of either organism within approximately 24 hours [87].

Synergistic effects of ceragenins with other antibiotics

Association of ceragenins with the outer membranes of Gram-negative bacteria increases the permeability of the barrier provided by this membrane, [33,34] and this allows improved access of antimicrobials to cytoplasmic targets. In a study designed to assess *in vitro* synergistic effects of ceragenins, combinations of CSA-13 with colistin, tobramycin, and ciprofloxacin were studied against *P. aeruginosa* strains isolated from cystic fibrosis patients [60]. CSA-13-colistin combinations proved the most synergystic (54% of tested strains), while CSA-13-tobramycin combinations showed the lowest level of synergistic interactions (25% of tested strains). Bucki et al. [88] demonstrated that a combination of CSA-13 with AMPs, including LL-37, lysozyme, lactoferrin and secretory phospholipase A, enhanced antibacterial activity of CSA-13 against bacteria causing topical infections. This observation is consistent with the hypothesis that endogenous AMPs synergize with administered antimicrobials and antibiotics [89].

Ceragenins in medical devices, coatings, bone fractures and nanoparticles

Medical devices provide abiotic surfaces on which microorganisms can adhere, form biofilms, and provide a nidus for further infection. For example, biofilms form rapidly on implanted endotracheal tubes, and ventilator-associated pneumoniae is a leading cause of nosocomial infections [90]. Administration of systemic antibiotics does not reduce biofilm burdens and progression to ventilator associated pneumoniae; [91] consequently, there is a need for methods to directly prevent microbial colonization of endotracheal tubes. Endogenous AMPs provide an antimicrobial innate immune function in tissues that controls bacterial growth and protects against biofilm formation [12]. As mimics of endogenous AMPs, ceragenins can provide a comparable, innate immune-like function with the abiotic surfaces found in medical devices.

Incorporation of ceragenins into contact lenses

Contact lenses, as abiotic surfaces, provide bacteria a scaffold on which biofilm can form, potentially leading to microbial keratitis. AMPs are present in the conjunctival sac, and covalent attachment of AMPs to contact lenses has been shown to inhibit bacterial biofilm formation [92]. However, the costs of AMPs and their susceptibility to proteases complicates their use in contact lenses. Ceragenins offer an attractive alternative, in part due to their antimicrobial activities, relatively low cost and stability; additionally, in the specific context of contact lens manufacture, ceragenins offer these attributes: colorless nature of ceragenins, their solubility in the pre-polymers used to produce lenses, their lack of interference in polymerization processes used in lens formation and the thermal stability of ceragenins, allowing autoclaving of finished products [93,94]. Gu et al. [94] reported that adding an acrylamide group to a ceragenin, giving CSA-120 (Figure 4), allow it to participate in the radical reaction leading to lens formation, thus covalently attaching the ceragenin to the contact lens. With CSA-120 present as 1.25% of the dry mass of the lens, bacterial biofilm formation was decreased by three logs over 24 hours incubation, when tested in a 10% nutrient medium. However, this inhibitory effect was not observed in 100% growth medium, likely because bacteria and bacterial detritus covered the permanently bound ceragenin.

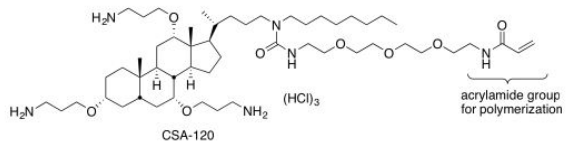

Figure 4: Structure of ceragenin CSA-120, containing an acrylamide group for polymerization.

To circumvent this problem, unbound ceragenin was added to lenses during their formation. A series of ceragenins, with varied lipid character, were added to lenses, and their duration of activity in

protecting lenses from bacterial colonization were determined. From the series tested, CSA-138 proved to provide the longest lasting protection: with the ceragenin comprising 1% of the total weight of the lens, bacterial colonization was prevented for up to 30 days with *S. aureus* and for up to 15 days with *P. aeruginosa* (Figure 5).

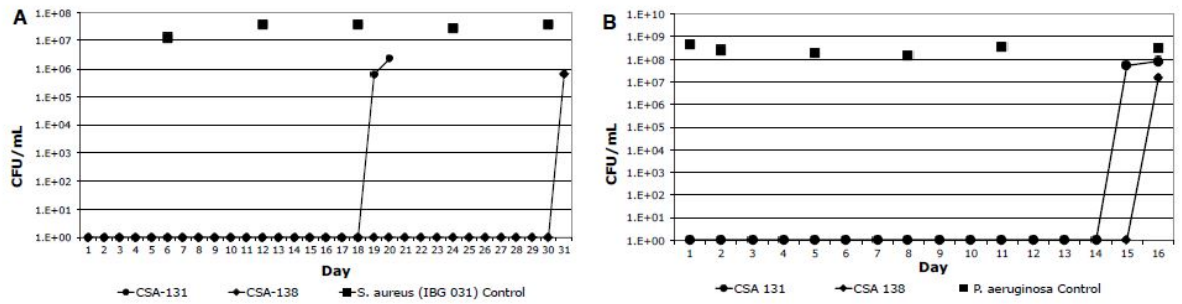

Figure 5: Bacterial populations in nutrient media (10% TSB in PBS) after initial inoculation with bacteria (10^6 CFU) and incubation for 24 hours. Controls were performed with lenses formed without added ceragenin. Lenses contained 1% of the indicated ceragenin, relative to the dry weight of the lens. After each 24 hours incubation, lenses were placed in fresh media and re-inoculated. A: *S. aureus* (IBG 031); B: *P. aeruginosa* (ATCC 27853).

Medical device coatings containing ceragenins

Implant-related biofilm infections take a toll on thousands of patients each year [95]. To address this issue, active-release antimicrobial coatings have been designed in which an antimicrobial agent is placed in a coating on an implanted device. The antimicrobial agent is released into the surrounding tissues and fluids with the expectation that it will prevent biofilm growth on the implanted device and also inhibit infection in the surrounding tissue. This approach has been met by limited success [96-98].

Active-release coatings containing ceragenins are attractive because of the bactericidal properties of ceragenins, their activity against established biofilms, their stability in the presence of proteases and other enzymes, and the ease by which they can be manipulated [4,20,83]. Williams et al. [99] characterized physical and chemical properties of a coating containing CSA-13 as a novel active release agent in a medical grade polymer coating on fracture fixation plates. Studies of polymerization of the silicone coating demonstrated that incorporation of CSA-13 (18% w/w) did not impact the physical properties of the coating. Even distribution of CSA-13 throughout the coating was observed using SEM.

As a critical aspect of the coating, CSA-13 particles located in the pores of the polymer were released over a span of 30 days in aqueous solution. Determination of thermal stability of the coating indicated that coated materials retain their stability at elevated temperatures, suggesting that the CSA-13-silicone combination was well-suited option for coating implantable devices. To evaluate these coatings *in vivo*, [22] biofilms of methicillin-resistant *S. aureus* (MRSA) were grown on a polymer mesh to populations of over 10^9 CFU/cm^2. This mesh was implanted against the tibia of sheep and immediately on top of the mesh was placed a fracture fixation plate coated with CSA-13-silicone. All control animals developed infection, while those with CSA-13-coated plates were fully protected from infection during a 12-week study.

Histology of surrounding tissue showed that the ceragenin was well tolerated, with no evidence of cytotoxicity or interference with wound healing. It was also noted that there was evidence of an increase in bone healing in the presence of the ceragenin. The ability of this coating to prevent infection from infections of medical devices by planktonic bacteria was evaluated *in vitro* and *in vivo* by Sinclair et al. [27,29]. The coating was applied to porous titanium (Ti) plug implants infected with 5×10^8 CFU of MRSA [27].

While control mice had to be euthanized shortly after the start of the study due to effects of infection, mice with implants with the polymer coating were protected from infection throughout the 12-week study. Notably, release of CSA-13 from the coating did not damage skeletal attachment sites to the porous coated Ti plug implant compared to the control group without CSA-13 coating. Taken together, these *in vivo* studies show that ceragenins provide an effective innate immune-like function to implanted devices, which are able to eradicate bacteria in both planktonic and biofilm form.

Ceragenins in bone fractures

Among the "secondary" activities of AMPs is observation that AMPs may influence bone healing [100]. Bone morphogenetic protein-2 (BMP-2) is used clinically to accelerate bone regrowth, [93] and Schindeler et al. [24] investigated whether ceragenins would impact bone regrowth alone and in combination with BMP-2. A series of ceragenins were tested for their impacts on bone regrowth, and they found that CSA-90 displayed the most potent activity. In a rat femur-based model of bone regrowth, with and without infection, they observed that CSA-90 potentiated the activity of BMP-2 and prevented infection from an inoculum of *S. aureus*. In contrast, untreated mice showed declining health and were all euthanized per protocol within two weeks after fracture. Open fractures are frequently complicated by infection, most commonly by pathogens that dwell on the skin such as *S. aureus*, and the ability of CSA-90 to accelerate bone regrowth while preventing infection demonstrates the primary antimicrobial effects of ceragenins as well as the "secondary" activities seen with AMPs.

Ceragenins on nanoparticles

Metal-based nanoparticles provide a platform from which ceragenins can be presented in a pre-aggregated form. Furthermore,

the distribution of magnetic nanoparticles in tissues can be controlled, and silver nanoparticles display inherent antibacterial activities. Two methods have been used to attach ceragenins to nanoparticles. The first is to use a ceragenin, CSA-124, appended with a thiol group (Figure 6); the thiol coordinates with thiophilic metals, such as silver, generating a monolayer on the surface of the nanoparticles [101,102].

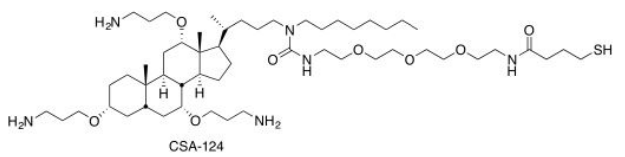

Figure 6: Structure of the thiol-containing ceragenin, CSA-124, for attachment to thiophilic nanoparticles.

The second involves generation of an aldehyde-containing surface on nanoparticles. The amines in ceragenins reversibly form Schiff bases (imines), which allow the nanoparticles to aggregate ceragenins [103]. Hayes et al. [101] prepared silver nanoparticles coated with CSA-124 and found that they were five times more bactericidal than silver alone, with MICs against *S. aureus* and *E. coli* of ca. 12 and 24 ppm respectively. Bucki et al. [103] showed that ceragenin-coated, magnetic nanoparticles retained potent bactericidal activity. In addition, sequestering ceragenins on nanoparticles enhanced biocompatibility. Even without direct conjugation, ceragenins display synergistic antibacterial activity with magnetic nanoparticles [104].

Use of ceragenins in imaging infections

The primary targets of AMPs and ceragenins are bacterial membranes, and the affinity of these antimicrobials for bacteria has led to efforts to use them for imagining bacterial infections [105]. Diagnostic antimicrobial nanoparticles consisting of CSA-124 bound on a silver shell with a maghemite core were successfully synthesized and introduced as a novel multifunctional theranostic conjugate by Hayes et al. [88,101]. Confocal imaging showed that the nanoparticles displayed higher selectivity for *S. aureus* than *E. coli*. *In vitro* MRI studies demonstrated that nanoparticles were able to adhere to *S. aureus*, indicating the possible application of nanoparticles as diagnostic contrast agents in imaging of deep tissue infections. Roohi et al. [106] investigated technetium (99mTc)-labeling of CSA-13 and abilities of the complex to image infection in mice. The multiple amine groups in CSA-13 allowed stable coordination to the metal ion. Mice were prepared with a *S. aureus* infection in the thigh muscle, and the CSA-13-99mTc complex was administered. Imaging (gamma) showed that the complex accumulated at the infected site and in the kidneys. Zahoor et al. [107] prepared a ceragenin with a separate binding site for technetium; they proposed that affinity for bacterial membranes might increase with the amine groups on the ceragenin free to form ionic interactions. To date, this modified ceragenin has been complexed with technetium and shown to associate with *S. aureus*.

Conclusions

The roles that AMPs play in maintaining health in higher organisms are multifaceted and include antimicrobial activities against a broad spectrum of pathogens, abilities to sequester endotoxins, and wound healing properties. By mimicking the morphology of AMPs, ceragenins display the same combination of activities and properties. Many of these have been demonstrated through *in vitro* studies by multiple research groups, and results from a smaller number of *in vivo* studies have been reported. As the impacts of ceragenins are better understood, their full potential and their limitations in clinical applications will become more apparent. As amphiphilic compounds, concerns about the hemolytic and general cytotoxic properties of ceragenins arise, and these concerns are common to AMPs [108]. For potential systemic applications of ceragenins, hemolytic activity may present an initial concern, and while hemolytic activity with CSA-13 has been observed, it comes at a concentration 10 times higher than bactericidal concentrations [47]. Another study conducted by Garcia et al. [109] showed that CSA-13 at concentration of 10 mg/ml exhibited less than 10% hemolysis on human red blood cells and total permeabilization of the cells occurred only at a concentration of 50 mg/ml.

The amphiphilic nature of ceragenins allows them to associate well with micelles, and Nagant et al. [78] and Leszczynska et al. [88] studied the effects of pluronic F-127 on the cytotoxicity of ceragenins. They found that micelles derived from pluronic F-127 decreased cytotoxicity toward eukaryotic cells and possibly improved antimicrobial activities. Alone CSA-13 toxicity towards human keratinocytes was not significantly different than that of LL-37, and CSA-13 caused no toxicity to HaCat cells at bactericidal concentrations [55]. Bal et al. [110] administered CSA-13 intraperitoneally to mice at varied concentrations and compared the LD50 to efficacious doses of CSA-13. Their conclusion was that "the cytotoxicity of ceragenin CSA-13 is not considerable." Bucki et al. [30] used a fluorophore-labeled form of CSA-13, injected intraperitoneally, in a model of *P. aeruginosa* infection. No adverse events were observed, while bacterial counts were decreased dramatically. Similarly, studies involving medical devices [26,94] and bone regrowth [28] demonstrated no toxicity or local cytotoxicity, suggesting that in these venues ceragenins are well tolerated. Considering potential applications of ceragenins, it is likely that they will find use in localized fashion (e.g., topical treatment for infection) and in specific tissues. AMPs are found in the skin, airways, gastrointestinal tract and reproductive tracts, and ceragenins are well suited for use in these venues. Results with bone regrowth and bone-related medical devices demonstrate the effectiveness of ceragenins in these localized applications. Further studies are underway to evaluate the potential of harnessing the antimicrobial, anti-inflammatory and wound-healing properties of ceragenins *in vivo*. Nature has provided a family of compounds, AMPs, that play a central role in innate immunity, while maintaining antimicrobial activity over eons. Mimicry of the morphology of AMPs provides ceragenins with antimicrobial activities as well as "secondary" activities. And there are many potential uses of ceragenins, some of which have been studied in detail and others that are just beginning to be explored.

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