Optimization of Ceragenins for Prevention of Bacterial Colonization of Hydrogel Contact Lenses

Xiaobo Gu, Jacob D. Jennings, Jason Snarr, Vinod Chaudhary, Jacob E. Pollard, and Paul B. Savage

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah

Correspondence: Paul B. Savage, Reed M. Izatt Professor of Chemistry and Biochemistry, Department of Chemistry and Biochemistry, Brigham Young University, C100 BNSN, Provo, UT 84602; paul_savage@byu.edu.

Submitted: June 24, 2013
Accepted: August 14, 2013

Citation: Gu X, Jennings JD, Snarr J, Chaudhary V, Pollard JE, Savage PB. Optimization of ceragenins for prevention of bacterial colonization of hydrogel contact lenses. *Invest Ophthalmol Vis Sci*. 2013;54:6217–6223. DOI:10.1167/iovs.13-12664

Purpose. We provided contact lens hydrogels with an antibacterial innate immune function using nonpeptide mimics of endogenous antimicrobial peptides.

Methods. Antimicrobial peptide mimics, ceragenins, were prepared for either covalent attachment to hydrogels or for controlled elution from lenses. The lipophilicity of the ceragenins was varied incrementally to provide differing levels of association with hydrophobic domains in lenses. Ceragenin-containing lenses were challenged repeatedly with *Staphylococcus aureus* or *Pseudomonas aeruginosa* in nutrient media. Bacterial growth and biofilm formation on lenses were quantified.

Results. A ceragenin covalently fixed in lenses effectively inhibited *S. aureus* biofilm formation on lenses in 10% tryptic soy broth (approximately 3-log reduction), but did not reduce biofilm formation in 100% tryptic soy broth. Ceragenins designed to elute from lenses were incorporated at 1% relative to the dry weight of the lenses. The ceragenin with the optimal lipid content, CSA-138, prevented bacterial colonization of lenses for 15 days with *P. aeruginosa* and for 30 days with *S. aureus* (daily exchange of growth media and reinoculation with 10^6 CFU). Measurement of CSA-138 elution showed that concentrations of the ceragenin never exceeded 5 μg/mL in a 24-hour period and that after 4 days of elution, concentrations dropped to <0.5 μg/mL, while maintaining antibacterial activity.

Conclusions. Ceragenin CSA-138 appears well suited for providing an innate immune-like function to abiotic hydrogel contact lenses for extended periods of time. Elution of even low concentrations of CSA-138 (<0.5 μg) is sufficient to eliminate inocula of 10^6 CFU of *S. aureus* and *P. aeruginosa*.

Keywords: antimicrobial peptide mimic, bacterial infection, bacterial biofilm

The ocular surface is challenged regularly with bacteria from multiple sources: airborne organisms, bacteria associated with foreign objects that come in contact with the eye, and with micro-flora growing in surrounding tissue. Innate immune defenses against these challenges include the antibacterial activities of multiple endogenous antimicrobial peptides (AMPs); for example, defensins and cathelicidins.[1] While these defenses generally are effective in limiting bacterial growth in the eye, introduction of foreign objects lacking innate immune function, such as contact lenses, provides a nidus for bacterial colonization.[2] Bacteria readily form biofilms on abiotic surfaces, including contact lenses, and in biofilm form, they are inherently resistant to most antibiotics. As biofilms mature, bacteria are released into surrounding tissues providing a continuous source of infection. With the use of contact lenses these infections can range in severity from contact lens–associated acute red eye to microbial keratitis.[3,4]

An attractive approach to prevention of bacterial colonization of contact lenses would involve providing an innate immune-like function to lenses. Multiple types of drugs, including antimicrobial agents, have been incorporated into lenses.[5] However, as noted by Cole et al.,[6] concerns over generation of drug resistance[7] and lack of sustained antimicrobial activity have hampered development of contact lenses with an innate antimicrobial function. As noted above, AMPs have a central role in innate defenses against bacterial growth on the eye. AMPs are found in organisms, ranging from insects to amphibians to mammals, and there is growing interest in clinical uses of AMPs.[8,9] Much of this interest stems from the idea that, as active bactericidal agents, AMPs do not readily engender resistance by bacteria. Noting the potential of AMPs to inhibit bacterial colonization of contact lenses, Willcox and colleagues[6,10,11] infused lenses with a designed AMP and covalently attached an AMP to lens hydrogels, and demonstrated that the AMP inhibited bacterial colonization in vitro and in vivo with a single inoculation of bacteria.

There are obstacles to clinical uses of AMPs that commonly are acknowledged[8,9]: Peptide therapeutics typically are more expensive to prepare on a large scale (compared to small-molecule therapeutics), and most AMPs are linear peptides and, consequently, they are substrates for ubiquitous proteases. As a means of evading host defenses, some bacteria release highly active proteases to degrade AMPs before they exert their antibacterial effects.[12]

We have developed nonpeptide, small-molecule mimics of AMPs, termed ceragenins, that reproduce the amphiphilic morphology (Fig. 1) of AMPs and their antibacterial activities.[13] The ceragenins are based on a common bile acid and are relatively inexpensive to prepare at a large scale. Furthermore, because ceragenins are not peptide-based, they are not substrates for proteases. A compelling application of the

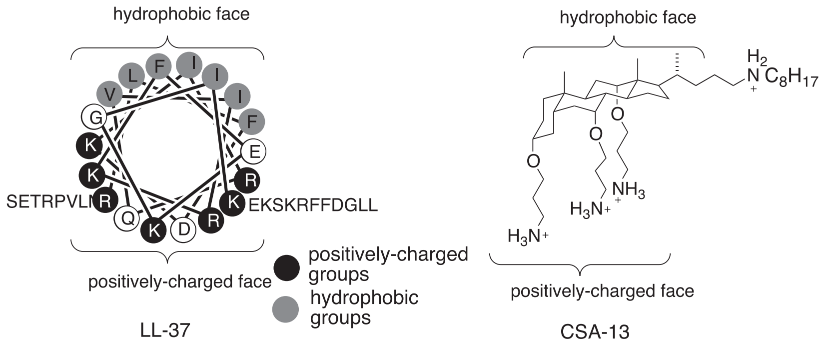


Figure 1. Helix-wheel representation of the human AMP LL-37 and CSA-13 showing the facially amphiphilic morphology common to both molecules.

ceragenins, as mimics of AMPs, is incorporation into contact lenses to provide an innate immune-like antibacterial function.

The development of antimicrobial surfaces generally involves either covalent attachment of the active on a surface, or controlled elution of the active from a device or coating. An advantage of covalent attachment is that the antimicrobial remains associated with the surface and interaction with surrounding tissue is limited.[11] However, in the presence of large inocula of bacteria or high concentrations of protein and over extended periods, surfaces can become fouled with bacterial detritus and adhered protein. Thus, the immobilized antimicrobial agent becomes isolated from the surrounding environment and antimicrobial activity is lost. Devices or coatings from which an antimicrobial elutes are less likely to be fouled, but elution of the active has to be controlled carefully to avoid toxicity and to prolong antimicrobial activity. In the context of contact lenses, a variety of methods have been used to control elution,[5] including use of loaded nanospheres[14] and fibrin coatings.[15]

As synthetic molecules, the structures of ceragenins can be modified readily to accommodate attachment to surfaces in a site-specific manner. Herein, we describe the preparation of a ceragenin containing an acrylamide group that can be incorporated directly and covalently into hydrogel polymers that make up contact lenses. The ceragenin incorporated into the hydrogel retains its antibacterial activity. However, concerns regarding fouling of the attached ceragenins led to investigation of elution of ceragenins from contact lens hydrogels.

Materials making up contact lens hydrogels typically include hydrophilic and lipophilic domains. To control the release of ceragenins from hydrogels, we prepared a series of ceragenins in which the lipophilic character of the antimicrobials was varied incrementally to provide differing affinities for the lipophilic domains of lenses. It was anticipated that increasing the associative interaction between the ceragenin and the lipophilic portion of the lenses would slow elution of the antimicrobial from the lenses. We anticipated that an optimized structure would allow controlled and sustained release of a ceragenin from contact lenses in a sufficient concentration to prevent biofilm formation on lenses over an extended period.

Materials and Methods

Preparation of Ceragenins

Ceragenin variants for optimization of elution from lenses were prepared using methods published for the synthesis of a lead ceragenin (CSA-13, Fig. 1). The variants were synthesized by substituting the appropriate primary amine for octyl amine in synthesis of CSA-13.[16] The ceragenin used in covalent attachment to the lens hydrogel was based on CSA-13, with a short oligoethylene glycol linking an acrylamide group (CSA-120, Fig. 2).

Contact Lenses

Contact lenses were prepared from lotrafilcon B silicone-acrylate prepolymers (Ciba Vision, Johns Creek, GA) using standard lens forms. Lens formation was initiated by addition of a photosensitizer and irradiation under UV light for 1 minute. Newly formed lenses were removed from forms by soaking in an isopropanol water mixture. Dry weights of lenses were 18.8 ± 0.21 mg. To incorporate CSA-120 covalently into lenses, the ceragenin was dissolved in the prepolymer solution at 1.25 or 2.5 weight percent (dry lens weight) before irradiation. To ensure that only covalently attached CSA-120 remained in newly-formed lenses, they were soaked in isopropanol, which swelled the lenses and solubilized unattached ceragenin. CSA-120 was incorporated into lenses at 2.50% and 1.25% relative to the dry mass of the lenses. Lenses containing 2.50% CSA-120 experienced a phase separation of the polymeric materials, which made the lenses partially opaque. Lenses with 1.25% CSA-120 retained their clarity, and this loading was used for further experiments in which the ceragenin was attached covalently to the lenses. To verify that antibacterial activity was due to covalently attached CSA-120 and not residual unattached ceragenin, lenses were soaked in 10% tryptic soy broth (TSB) in PBS for 24 hours at 37°C. The lenses were removed from solution, and the resulting solutions were inoculated with *Staphylococcus aureus* (10^6 CFU). Control growth medium was prepared with 10% TSB in PBS, and was inoculated with the same number of bacteria. Samples were incubated for 24 hours, then aliquots were serially diluted and plated on TSB agar. After 24 hours, colonies were counted to determine the number of CFUs present in the test and control samples. To incorporate ceragenins noncovalently into lenses, the ceragenins were dissolved in the prepolymer solution at 1 weight percent (dry lens weight) before irradiation. After removal from lens forms, lenses were stored in PBS (0.5 mL). Elution of CSAs from lenses during removal from lens forms and storage

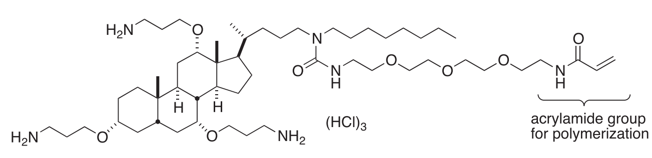


Figure 2. Structure of ceragenin CSA-120 containing an acrylamide group for copolymerization in acrylate-containing hydrogels.

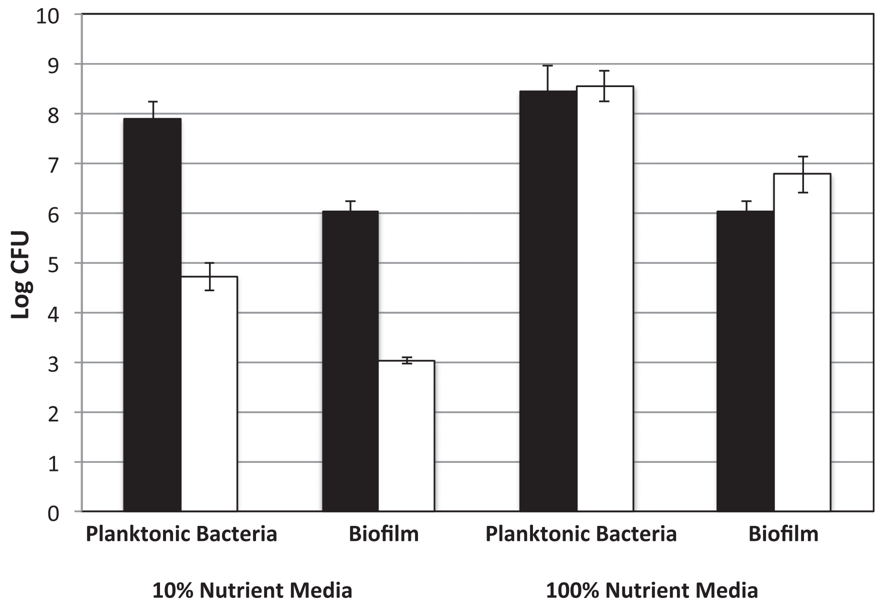


FIGURE 3. Quantification of planktonic bacteria (*S. aureus*) and bacteria in biofilm form on contact lenses after 24 hours of incubation at 37°C (initial inoculation with 10^6 CFU). Planktonic bacteria are the organisms present in the nutrient media in which the lenses were suspended. *Black bars*: control lenses. *White bars*: lenses containing 1.25% CSA-120. Growth media were 10% TSB in PBS or 100% TSB.

was measured by liquid chromatograph–mass spectrometry (LC/MS), and was estimated as less than 20%.

Elution Profiles

Freshly prepared lenses were submerged in PBS (1.5 mL) for 24 hours and moved to fresh aliquots of PBS (1.5 mL) after each 24-hour interval. Ceragenin concentrations were determined using mass-labeled internal standards (deuterium labeling) and liquid chromatography (C_{18} column with a water-acetonitrile gradient [formate counter ion]) coupled to mass spectrometry. Detection limits were approximately 0.5 μg/ml.

In Vitro Testing

Minimum inhibitory concentration (MIC) values were determined using Clinical and Laboratory Standards Institute (CLSI) protocols (microbroth dilution). To test antibacterial activity, lenses were submerged in PBS, 10% broth (TSB) or 100% broth (2 mL), and inoculated with bacterial suspensions (10^6 CFU) in 100% broth. Strains used were *S. aureus* (IBG 031) and *Pseudomonas aeruginosa* (ATCC 27853). The former was isolated from a contact lens-induced peripheral ulceration and the latter is a standard strain used for susceptibility testing. The CSAs showed comparable activity against varied strains of these organisms, including drug-resistant forms.[13,17] Inoculated samples were incubated at 37°C for 24 hours. Lenses were removed and rinsed gently in PBS to remove nonadhered bacteria, resubmerged in broth and reinoculated. This procedure was repeated after each 24-hour interval. Bacterial counts (CFUs) were determined by plating serially-diluted samples on TSB agar, incubating for 24 hours at 37°C, and counting colonies. Biofilms on lenses were quantified by gently rinsing lenses in PBS to remove nonadhered organisms, submerging lenses in neutralizing media, sonicating (bath sonicator) for 2 minutes to break up biofilms, serially diluting the resulting media, plating serially-diluted media on TSB agar, incubating at 24 hours at 37°C, and counting colonies. All experiments were performed in triplicate.

In Vitro Toxicity

The in vitro toxicity and potential eye irritation of a lead ceragenin, CSA-13, was determined using the EpiOcular Eye Irritation assay (MatTek Co., Ashland, MD).[18] Assays were performed with PBS-buffered (pH 7.2) solution of CSA-13 (100 μg/mL).

RESULTS

The antibacterial activity of lenses containing CSA-120 were quantified using *S. aureus* (10^6 CFU) in either 10% TSB in PBS or 100% TSB and incubation for 24 hours. To verify that any unreacted (nonattached) CSA-120 had been washed from the lenses, CSA-120–containing lenses were soaked in nutrient media, then lenses were removed and the nutrient media were inoculated with *S. aureus*. Bacterial growth was control-like, indicating that insufficient CSA-120 was eluting to affect bacterial growth. The impact of the attached antimicrobial on planktonic bacteria (i.e., bacteria growing in the medium) and on bacteria adhered to lenses in biofilms was determined in both growth media (Fig. 3). Substantial decreases in the number of CFUs were observed in samples with 10% TSB in PBS: a greater than 3-log reduction in planktonic bacteria and an approximately 3-log reduction in adhered organisms. However, in 100% nutrient media, no reduction in planktonic or biofilm bacterial counts was observed.

The concern that covalently attached CSA-120 was overwhelmed by bacteria in the presence of full-strength nutrient media prompted an investigation of eluting forms of ceragenins incorporated into contact lenses. To optimize the elution rate of the ceragenins, the lipid portion of CSA-13 was varied incrementally to identify the amount of lipid character necessary to interact with the corresponding lipophilic domain in lenses providing a sustained release of the antimicrobial at the minimal rates necessary to eliminate bacterial inocula. We altered the hydrophobic chains extending from the amine group at C24 on ceragenins (Fig. 4). We have demonstrated that modifications at this position are well tolerated; the corresponding compounds maintain their antibacterial activi-

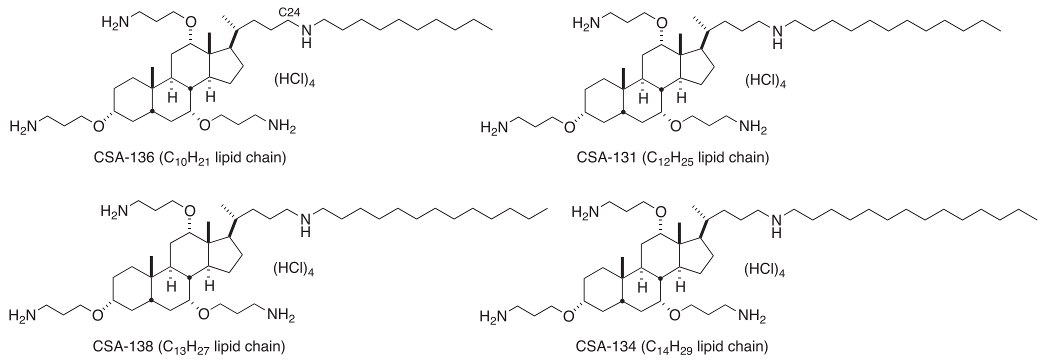


Figure 4. Structures of ceragenins used in optimizing hydrophobic character for control over elution from lenses.

ties.[16] A lead ceragenin, CSA-13 (Fig. 1), has a C_8 chain at this position, and this served as a starting point for further investigation.

In initial experiments with 1% CSA-13 in lenses (data not shown) antimicrobial activity was observed for 3 days (three 24-hour intervals), but then antibacterial activity decreased dramatically. This observation suggested the ceragenin eluted from lenses too quickly and that more hydrophobic character was required to control release of the ceragenin from lenses. Therefore, ceragenins were prepared with longer hydrophobic chains (C_{10}, C_{12}, C_{13}, and C_{14}).

Ceragenins are highly soluble in most polar organic solvents, and these compounds dissolve readily in the prepolymers used in forming the lenses. A concentration of 1% of the ceragenin (190 μg/lens) relative to the mass of the dry lenses was used. Because the ceragenins do not contain strong chromophores, addition of the ceragenins did not impact lens formation, and no physical differences were apparent in comparison with physical and optical properties of control lenses, and those containing the ceragenins.

Quantifying the duration of antibacterial activity of the ceragenins in lenses required ongoing experiments in which the growth medium was exchanged daily and reinoculated with bacteria. While this process was straightforward, assaying for bacterial colonization of lenses presented a greater challenge. Quantification of biofilm formation requires sonication of lenses, which liberates biofilm that forms on lenses. Thus, repeated sonication would clean lenses and not provide an accurate measure of bacterial colonization with repeated bacterial challenges. However, in the initial experiments with CSA-13, we observed that if bacterial growth was not supported in the surrounding medium, then no detectable bacterial biofilm formed on lenses; that is, an eight-log decrease in bacteria colonizing the lenses was achieved. Consequently, bacterial colonization of lenses was monitored by quantifying bacterial growth in the surrounding medium. Once the surrounding medium supported bacterial growth, the potential existed for biofilm formation on lenses.

Lenses containing 1% of the indicated ceragenin were placed in 2 mL of 10% TSB in PBS and challenged with *S. aureus* (10^6 CFU). Samples were incubated for 24 hours with gentle agitation. After 24 hours, lenses were removed, rinsed gently with PBS, then placed in fresh media, and reinoculated. This procedure was repeated every 24 hours until the growth medium supported bacterial growth at levels comparable to controls (approximately 10^7 CFU/mL). Controls were performed on days 6, 12, 18, 24, and 30. CSA-138 (Fig. 4) withstood colonization for 30 days with *S. aureus* under these conditions (Fig. 5).

The assay described above was performed using *P. aeruginosa*, with results shown in Figure 6. Controls were run on days 1, 2, 5, 8, 11, and 16. As observed with *S. aureus*, CSA-138 gave the longest duration of protection, which lasted 15 days. In the Table, the structures of the ceragenins and the duration during which they eliminated bacterial inocula are listed.

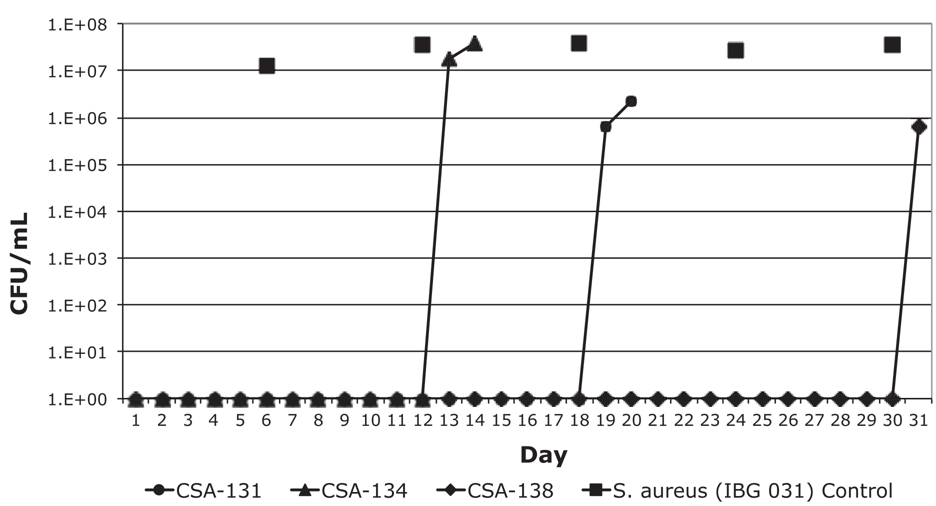


Figure 5. Bacterial populations in nutrient media (10% TSB in PBS) after initial inoculation with *S. aureus* (10^6 CFU) and incubation for 24 hours. Controls were performed with lenses formed without added ceragenin. After each 24-hour incubation, lenses were placed in fresh media and reinoculated.

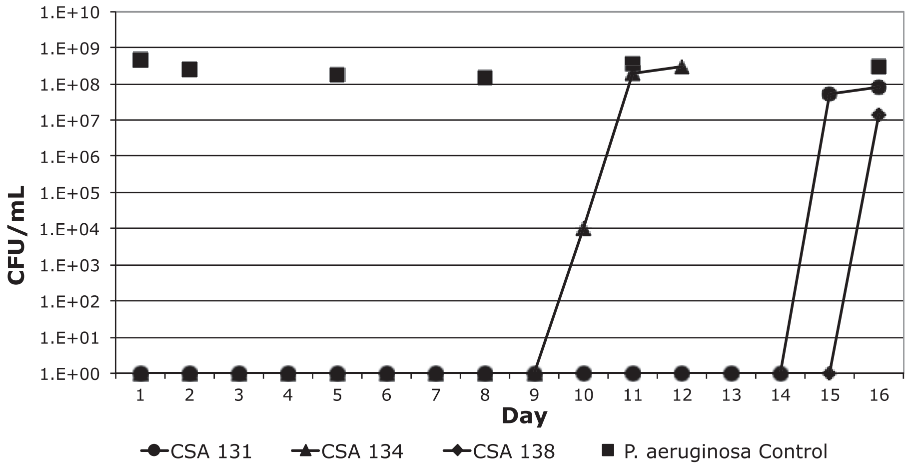
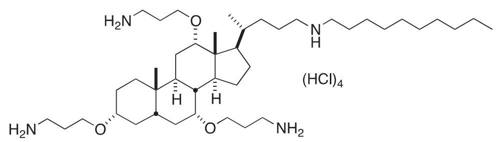
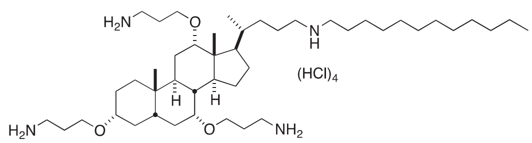
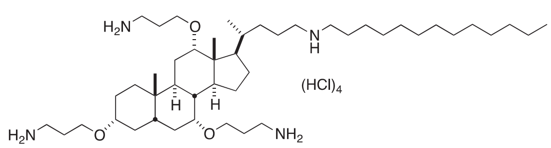
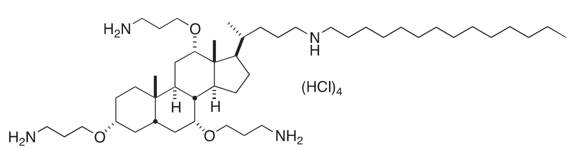


Figure 6. Bacterial populations in nutrient media (10% TSB in PBS) after initial inoculation of *P. aeruginosa* (10^6 CFU) and incubation for 24 hours. Controls were performed with lenses formed without added ceragenin. After each 24-hour incubation, lenses were placed in fresh media and reinoculated.

Table. Structures of Ceragenins Incorporated (1%) into Hydrogel Contact Lenses, Duration of Antibacterial Activity, and MIC Values Against *S. aureus* and *P. aeruginosa*

		S. aureus		*P. aeruginosa*	
Ceragenin*	Lipid Chain	Duration, d	MIC Values, µg/ml	Duration, d	MIC Values, µg/ml
 CSA-136	C_{10}	13	1	13	1
 CSA-131	C_{12}	18	1	14	1
 CSA-138	C_{13}	30	2	15	2
 CSA-134	C_{14}	12	4	9	4

The ceragenins are ordered based on lipid chain length.

* All lenses were soaked/washed with 10% isopropanol in water and stored in 0.5 mL of PBS.

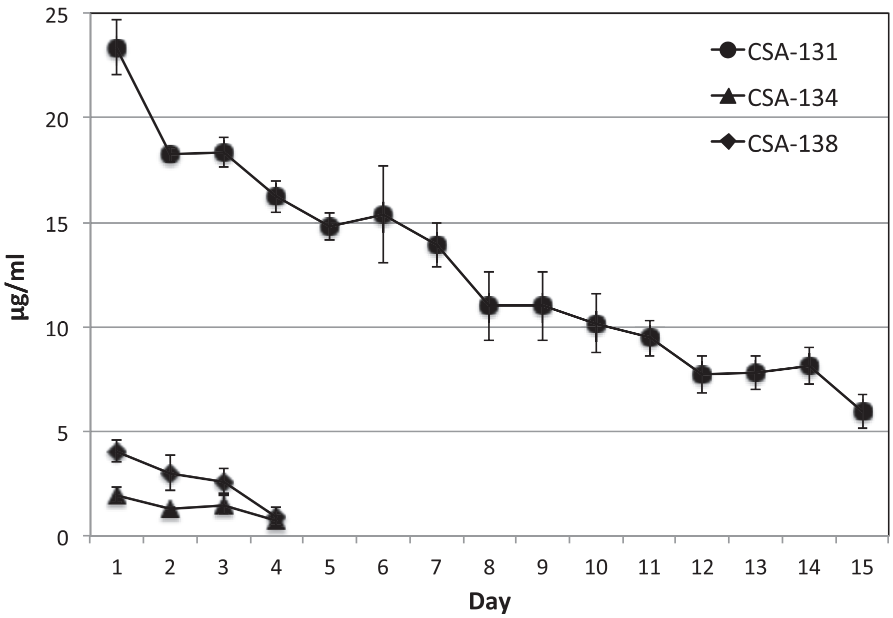


FIGURE 7. Elution of ceragenins from lenses containing 1% (wt/wt) of the indicated ceragenin. Lenses were soaked in PBS (1.5 mL) for 24 hours, and ceragenin concentrations were determined via LC/MS solutions in which lenses were soaked and were replaced with fresh PBS every 24 hours.

The MIC values of the ceragenins described in Figure 4 were determined against the test organisms using CLSI protocols. Ceragenins with longer lipid chain lengths (CSAs 134 and 138) gave elevated MIC values compared to ceragenins with shorter lipid chains. Notably, MIC values were the same with Gram-negative and -positive organisms. The MIC values of CSA-13 against these organisms are 0.5 and 2 µg/mL, respectively. To observe possible cytotoxicity of ceragenins with corneal epithelial cells, an EpiOcular Eye Irritation assay was performed with CSA-13 in saline at 100 µg/ml. No cytotoxicity was observed at this concentration.

The release of the ceragenins from lenses was quantified by soaking lenses in PBS (1.5 mL), exchanged with fresh PBS every 24 hours. Aliquots were removed at each 24-hour time point, and analyzed for ceragenin concentration using LC/MS and mass-labeled internal standards (i.e., each ceragenin was prepared with deuterium replacing two hydrogen atoms). The detection limit for quantification of these ceragenins was approximately 0.5 µg/ml. When the amount of the ceragenin fell below the detection limit, analysis was halted. As expected, the length of the lipid chain at C24 impacted the elution of the ceragenin from lenses. The ceragenin, CSA-131, with a C_{12} chain, eluted more rapidly than CSA-134 and CSA-138, with C_{14} and C_{13} lipid chain lengths, respectively (Fig. 7).

DISCUSSION

Due to the simplicity of the ceragenins, they are modified easily for covalent attachment to lenses and to optimize elution from lens materials. Covalent incorporation of CSA-120 into lenses at 1.25% (wt/wt) did not appear to interfere with the polymerization process in lens formation, and we were able to demonstrate that the ceragenin did not elute from lenses at concentrations sufficient to impact bacterial growth. Interestingly, covalently attached CSA-120 impacted bacterial growth in the surrounding medium as well as inhibited bacterial colonization of the lenses in 10% growth media (Fig. 3). Activity of immobilized CSA-120 against planktonic bacteria presumably occurs as bacteria come in contact with the surfaces of lenses. This impact was lost in full media likely due to fouling of the lens surface by protein, bacterial detritus, and bacteria. Considering the presence of optimized growth media and a high inocula of bacteria, these obviously are harsh conditions and unlikely to be duplicated in normal wear of lenses. Nevertheless, this result highlights the limitations presented by covalent attachment of this antimicrobial in lenses.

Optimization of the lipophilic character of ceragenins for controlled release from lenses required synthesis of a small series of incrementally varied compounds. As expected, increasing the length of the lipid chains in ceragenins slowed their elution from lenses (Fig. 7). After 15 days, approximately 80% of CSA-131 eluted from lenses, while less than 10% of CSAs 134 and 138 eluted over the same time period. Addition of a single CH_2 slowed elution markedly. However, while increasing lipophilicity of the ceragenins slowed elution, it resulted in decreased antibacterial activity (i.e., increased MIC values, see Table). Thus, a balance had to be struck between slowed elution and antibacterial activity.

As demonstrated by the duration of antibacterial activity of the ceragenins (Figs. 5, 6), this balance was well struck with CSA-138. It prevented bacterial growth in solution and on lenses for 30 and 15 days with *S. aureus* and *P. aeruginosa*, respectively (daily exchange of growth medium and daily inoculation). It is important to note that, while antibacterial activity extended for more than 2 weeks, the amount of CSA-138 eluting from lenses dropped below detection limits (approximately 0.5 µg/mL) after day 4. This concentration was well below the MIC values of CSA-138 with the targeted organisms. This observation suggested that the CSA-138 that remains associated with lenses retains the ability to eliminate bacteria. We observed that CSA-120, covalently attached to lenses, impacts bacterial growth, and apparently CSA-138, noncovalently attached to lenses, also exerts antibacterial activity.

Considering scenarios of covalent attachment of an antimicrobial to lenses and elution of an antimicrobial from lenses, incorporation of CSA-138 in lenses appears to have adopted aspects of both scenarios. That is, some amount of CSA-138 elutes from lenses, and is likely to be able to escape through protein and bacterial detritus deposits, while the majority of CSA-138 remains associated with the lenses. This associated ceragenin also provides antibacterial activity at the surface of the lenses.

AMPs have central roles in controlling bacterial growth on the ocular surface; however, introduction of contact lenses offers an abiotic surface on which bacteria can grow and infect the eye. As mimics of AMPs, the ceragenins appear well-suited for providing an innate immune-like function to contact lenses to prevent bacterial colonization for extended periods. The optimized structure of CSA-138 allows extended protection of lenses, while only small amounts of the antimicrobial elute from lenses. These amounts are below concentrations that are expected to cause irritation or prove to be cytotoxic, based on irritation and cytotoxicity studies with CSA-13. From a manufacturing perspective, the ceragenins are attractive antimicrobial additives for lenses: They are robust molecules (we routinely autoclave them) and they can be prepared in large quantities relatively inexpensively. While CSA-138 was optimized for use with a single lens type, it is anticipated that ceragenin elution and activity can be matched readily for other lens types.

Acknowledgments

Supported by the Technology Transfer Office at Brigham Young University.

Disclosure: **X. Gu**, None; **J.D. Jennings**, None; **J. Snarr**, None; **V. Chaudhary**, None; **J.E. Pollard**, None; **P.B. Savage**, None

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