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RESEARCH ARTICLE

Cationic amphiphiles against *Gardnerella vaginalis* resistant strains and bacterial vaginosis-associated pathogens

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One sentence summary: Cationic amphiphiles control *Gardnerella vaginalis*.

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ABSTRACT

Antibiotic resistance and infection recurrence are critical issues in treating bacterial vaginosis, the most common vaginal disorder in women of reproductive age. Novel alternatives to traditional antibiotics, such as peptidomimetics, have the potential to address this challenge. Previously, two series of cationic amphiphiles (CAms) were developed with both hydrophilic head groups and non-polar domains, giving them the ability to self-assemble into supramolecular nanostructures with membrane-lytic properties. Those CAms were shown to be effective against biofilms of *Gardnerella vaginalis* while preserving the commensal microbiota. Two new series of CAms were designed with varying levels of flexibility between the hydrophilic head groups and the hydrophobic domains. Activities against the vaginal pathogen *G. vaginalis* ranged from 1.3 to 18.5 μM, while the tested vaginal lactobacilli were significantly more tolerant of CAms, with minimal inhibitory concentration values as high as 208 μM. Minimal biofilm bactericidal concentrations of the tested CAms ranged from 21.47 to <388.3 μM, and were lowest against resistant forms of *G. vaginalis*, while *Lactobacillus* biofilms were tolerant of concentrations ≥687 μM. Safety aspects of the CAms were also investigated, and they were found to be safe for use against vaginal ectocervical tissue.

Keywords: AMP mimics; antimicrobials; *Gardnerella vaginalis*; biofilm; bacterial vaginosis

INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal disorder found in women of reproductive age and is characterized by a shift in the vaginal microbiota from a *Lactobacillus*-dominated environment to one rich in a variety of other microorganisms, primarily opportunistic anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella bivia*, *Peptostreptococcus anaerobius* and *Mobiluncus curtisii* (Bagnall and Rizzolo 2017). BV is non-inflammatory in nature; symptoms include a malodorous and homogeneous gray/white discharge that adheres to

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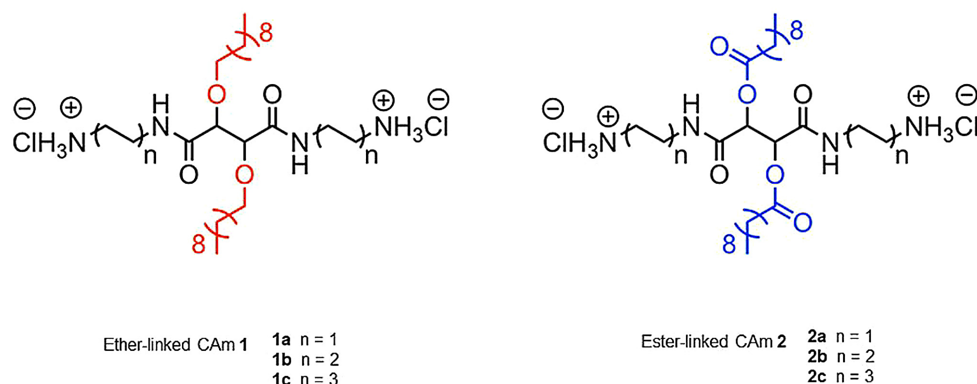


Figure 1. Chemical compositions of CAms.

the vaginal lumen, with a pH > 4.5, accompanied by an itching/burning sensation (Javed, Parvaiz and Manzoor 2019). BV is frequently asymptomatic and the composition of the microbiota differs across ethnic and cultural groups, in both healthy and BV-diagnosed patients (Ma *et al.* 2012). What is certain, however, is that BV is a polymicrobial infection involving a variety of BV-associated microbes, where *G. vaginalis* plays a central role (Muzny, Schwebke and Josey 2014; Cerca and Machado 2015).

Gardnerella vaginalis is the most comprehensively studied and commonly isolated bacterial species in BV, appearing in over 98% of BV diagnoses (Aroutcheva *et al.* 2001). It is capable of forming a robust biofilm and produces toxins such as vaginolysin as effective virulence factors, thereby increasing bacterial resistance to conventional antibiotics. *Gardnerella vaginalis* has also been demonstrated to have symbiotic relationships with other anaerobes, such as *Prevotella* ssp. (Gilbert *et al.* 2019; Randis and Ratner 2019). In BV, *G. vaginalis* is a key species in the formation and persistence of a complex, highly structured polymicrobial biofilm (Castro, Machado and Cerca 2019).

The etiology of BV still remains unclear, as it is difficult to determine whether BV is initiated by the loss of protective lactobacilli due to environmental changes, contact with an infectious agent (e.g. *G. vaginalis* or structured polymicrobial *Gardnerella* biofilms) or a combination of both factors (Swidsinski *et al.* 2014; Cerca and Machado 2015; Randis and Ratner 2019). In BV, a decrease in the commensal *Lactobacillus* population causes an increase in pH due to reduced lactic acid production. This decreased *Lactobacillus* population coupled with increased fatty acid production by anaerobic bacteria makes the vaginal environment more suitable for the growth of opportunistic pathogens and unfavorable for lactobacilli (Kumar *et al.* 2011). In addition to decreased production of lactic acid, bacteriocins and other antimicrobial molecules are also reduced in concentration, further shifting the environment toward BV (Bradshaw and Sobel 2016).

BV is associated with numerous negative health outcomes including, but not limited to pre-term birth, increased transmission and acquisition of various sexually transmitted infections (HIV, pelvic inflammatory disease, chlamydia), endometriosis and infertility (Unemo *et al.* 2017; Francis *et al.* 2018; Shimaoka *et al.* 2019). The prevalence of BV among different groups is 23–29%, with an estimated annual cost of ∼4.6 billion USD globally (Peebles *et al.* 2019).

Current treatments for BV are often insufficient with high rates of reoccurrence across all current treatment regimens (Bradshaw and Sobel 2016) likely due to the protection offered by the polymicrobial biofilm, and potentially linked to increasing resistance to the most commonly prescribed antibiotic treatments (clindamycin and metronidazole) (Eschenbach 2007; Nagaraja 2008; Bostwick *et al.* 2016). High rates of recidivism coupled with increasing resistance to traditional antimicrobials underscore the need for novel alternative treatments. One such potential alternative is the use of antimicrobial peptides (AMPs) (Turovskiy *et al.* 2012; Algburi *et al.* 2017). AMPs are produced by a variety of eukaryotic and prokaryotic hosts, and are involved in both host defense (Sun *et al.* 2018) and the regulation of numerous processes within microbial communities (Chikindas *et al.* 2018; Mukherjee and Bassler 2019). AMPs are short peptide molecules that are generally cationic and demonstrate a wide range of antimicrobial activity against many microbial species (Bahar and Ren 2013). AMP-mimicking cationic amphiphilic compounds (CAms) target the lipopolysaccharide layer of the cell membrane in Gram-negative microorganisms, forming pores in the cell membrane or disabling the proton motive force (Chikindas *et al.* 2018). In eukaryotes, the cell membrane has a high concentration of cholesterol and a low anionic charge, effectively protecting the cells from the membrane-lytic properties of AMPs (Wimley and Hristova 2011; Bahar and Ren 2013). AMPs interact with the negatively charged membranes of bacteria and biofilm surfaces electrostatically, where they may exhibit bacteriostatic or bactericidal activity in a concentration-dependent manner. Hydrophobic domains may also interact with and potentially disrupt the hydrophobic cell membrane, resulting in cell death (Laverty, Gorman and Gilmore 2011; Zhang *et al.* 2017).

Previously, a series of CAms was designed with hydrophilic head groups and non-polar domains. These CAms self-assemble into supramolecular nanostructures with membrane-lytic properties, as demonstrated against representative Gram-negative and Gram-positive species (Zhang *et al.* 2017). More recently, the activity of CAms against *G. vaginalis* ATCC 14018, vaginal lactobacilli and other BV-associated pathogens was demonstrated (Algburi *et al.* 2017). Based upon the previously published report (Moretti *et al.* 2019), this study explores the activity of a novel series of CAms (see Fig. 1 for structures) against both planktonic and biofilm-associated cells of *G. vaginalis* ATC 14018, vaginal lactobacilli, other BV-associated pathogens and *G. vaginalis* strains resistant to both metronidazole and clindamycin. The safety of the CAms was also investigated for potential mutagenicity and activity against a vaginal ectocervical tissue model.

MATERIALS AND METHODS

Bacterial growth and cultivation

Gardnerella vaginalis ATCC 14018 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Metronidazole-resistant *G. vaginalis* 14019_metR was obtained from Dr Hilbert (Schuyler *et al.* 2015). All *G. vaginalis* strains were grown in Brain Heart Infusion (Difco, Sparks, MD, USA) supplemented with 3% horse serum (sBHI) (JRH Biosciences, Lenexa, KS, USA). For resistant strains, sBHI supplemented with either metronidazole (Acros Organics, Morris Plains, NJ, USA) or clindamycin (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) was used (300 and 50 μg/ml, respectively). For biofilm experiments, BHI supplemented with 1% glucose was utilized. All experiments with *G. vaginalis* were conducted in an anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI, USA) using an anaerobic gas mix (10% hydrogen, 5% carbon dioxide and 85% nitrogen).

Four species of vaginal lactobacilli were used in this study: *Lactobacillus plantarum* ATCC 39268, *Lactobacillus rhamnosus* 160 (provided by Dr Aroutcheva, Rush University Medical Center, Chicago, IL, USA), *Lactobacillus crispatus* ATCC 33197 and *Lactobacillus gasseri* ATCC 33323; all were grown for 18–24 h in DeMan, Rogosa and Sharpe broth (MRS Difco BD, Franklin Lakes, NJ, USA) under aerobic conditions at 37°C. For lactobacilli biofilm formation, MRS broth supplemented with 1% glucose and 2% sucrose (Fisher Scientific, Waltham, MA, USA) was utilized.

Peptostreptococcus anaerobius ATCC 27337, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303 were purchased from the ATCC (Manassas, VA, USA) and grown anaerobically in sBHI.

Developing resistant strains

A clindamycin-resistant strain, designated as *G. vaginalis* 14018c, was developed via exposure to clindamycin at sub-MICs (minimal inhibitory concentrations), followed by a step-wise increase in concentration to a final concentration of 50 μg/ml. Resistance was confirmed by growing the resistant strain on BHI agar plates in the absence of clindamycin for 48 h; individual colonies were then selected and subcultured again in the absence of clindamycin for an additional 48 h. Colonies were then selected and propagated in the presence of 50 μg/ml clindamycin. The resulting colonies were considered resistant, and were collected for draft genome sequencing and storage at −80°C.

Genome sequencing

The genomic DNA was extracted from bacterial cultures using Invitrogen PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The library was prepared using NEBNext® Fast DNA Fragmentation and Library Prep Set for Ion Torrent (New England Biolabs, Ipswich, MA, USA). A mixture of DNA fragments with ligated adaptors was run on 2% E-gel (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) to isolate fragments of ∼495 bp in size. The library was amplified according to the manufacturer's protocol using 10 PCR cycles, followed by analysis using the Agilent BioAnalyser and High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA) for precise estimation of DNA fragment sizes and concentrations. The library was diluted to 25 pM concentrations for template preparation using an OT2 system and Ion PGM Hi-Q View OT2 Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). After enrichment, the ion sphere particles were annealed with the sequencing primer, incubated with DNA polymerase and loaded onto chip 316v2 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). The sequencing was run using Ion PGM Hi-Q View Sequencing Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) with 850 flows. The sequences were assembled using IonTorrent SPAdes *de novo* assembly plugin, version 5.0.0.0.0. CLC Genomics Workbench suite version 6.5 (QIAGEN, Redwood City, CA, USA) was used for read mapping to the reference genomes. Sigma 70 promoter search was conducted using an online tool at http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb.

Synthesis of CAm compounds

Ether- (series 1) and ester- (series 2) linked cationic gemini-like amphiphiles (CAms) with varying cationic linker lengths (*n*C where *n* is the total carbon linker length) were synthesized following the procedure described by Moretti *et al.* (2019).

Preparation of stock solutions

Stock solutions of each CAm compound were prepared in ddH_2O and then incubated at 37°C with shaking at 250 rpm for ∼10 min. Once fully dissolved, stock solutions were sterilized under UV light for 20–25 min, and stored at 4°C for no more than 30 days. Clindamycin phosphate and metronidazole were prepared as 10 mg/ml solutions in ddH_2O, and then filter-sterilized and stored for no more than 21 days at 4°C.

MIC determination

The MIC for a given substance is defined as the lowest concentration of that substance that results in no visible growth, i.e. complete inhibition, after 24 h exposure (Clinical and Laboratory Standards Institute, CLSI, 2017). MIC values were determined using a standard broth microdilution assay as described in Algburi *et al.* (2017): A 24 h culture of a given species/strain was diluted with the appropriate culture media to a concentration of ∼10^6 CFU/ml as confirmed by spot-plating. CAm compounds were serially diluted 2-fold with sBHI in a non-tissue culture-treated 96-well microplate (Falcon, Corning Incorporated, Corning, NY, USA) to a final volume of 100 μl in each well. The 10^6 CFU/ml suspension (100 μl) was added to each test well of the microplate, and 200 μl of 10^5 CFU/ml in sBHI was used as a positive control, while 200 μl of sBHI was used as a negative control. Seventy-five μl of mineral oil (Sigma-Aldrich, St Louis, MO, USA) was added to each well to prevent evaporation. The microplate was then transferred to a plate reader (AccuSkan FC, ThermoFisher Scientific, Rochester, NY, USA) and incubated anaerobically at 37°C for 24 h. The kinetic reading was statistically analyzed, and the MIC and sub-MIC concentrations for each CAm compound were determined. In some instances, MIC concentrations were determined by visual confirmation and end-point readings after 24 h treatment, per CLSI guidelines.

Minimal biofilm inhibitory concentration (MIC-B) determination

The minimal biofilm inhibitory concentration is defined as the concentration of an antimicrobial that inhibits either 50% (MBC_{50}) or 90% (MBC_{90}) of biofilm growth compared to an untreated control (Chapot-Chartier and Kulakauskas 2014). MIC-B concentrations were determined as described in Algburi *et al.* (2017). In brief, *G. vaginalis* cells were grown overnight in sBHI

anaerobically at 37°C. Overnight growth was then diluted 1:100 (v/v) to achieve a concentration of $\sim10^6$ CFU/ml. The CAm solutions were serially diluted 2-fold into a 96-well tissue culture microplate (Falcon, Corning Incorporated, Corning, NY, USA) with a final volume of 100 µl in each well. The diluted cell suspension (100 µl) was added to each test well, and 100 µl was added to sterile sBHI as a positive control, while 200 µl of sterile sBHI served as the negative control. Sterile sealing tape (Thermo Fisher Scientific, Rochester, NY) was used to prevent evaporation. The 96-well plate was then incubated anaerobically at 37°C for 48 h. After incubation, the biofilm was stained using 0.1% crystal violet as described by Algburi *et al.* (2017).

Minimal biofilm bactericidal concentration (MBC-B)

The minimal biofilm bactericidal concentration (MBC-B) is defined as the minimal concentration of an antimicrobial compound able to reduce the number of viable cells within a biofilm by ≥3 log as compared to an untreated control (Sandoe *et al.* 2006). The assay was performed as described in Algburi *et al.* (2017) with some modifications. In brief, a 24 h culture of *G. vaginalis* was diluted to $\sim10^7$ CFU/ml in sterile BHIG. The suspension (200 µl) was then added to a 96-well tissue culture microplate and the plate was sealed using sterile sealing tape (Thermo Fisher Scientific, Rochester, NY, USA) and incubated anaerobically for 24–36 h at 37°C. After incubation, the biofilm was washed twice with fresh BHIG to remove planktonic cells, and then treated with 200 µl of the CAm compounds at predetermined concentrations. The plate was then sealed again, and incubated for 24 h at 37°C under anaerobic conditions. After incubation, the biofilm was washed twice with fresh BHIG to remove any residual compound, and the biofilm was then assayed for cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In the case of *Lactobacillus* biofilms, the MTT assay could not be utilized due to interference caused by lactic acid production. As such, biofilm-associated cells were quantified using the spot plate method as described in Algburi *et al.* (2017).

MTT cell viability assay

An MTT assay for cell viability was used to determine MBC-B concentrations, defined here as the concentration at which no formazan compound is produced (as compared to the negative control) after MTT treatment. Conditions for the MTT assay were determined experimentally after consulting Grela, Kozłowska and Grabowiecka (2018) and the manufacturer's instructions provided with an MTT assay kit (Vybrant MTT Cell Proliferation Assay Kit, Molecular Probes, Eugene, OR, USA). Briefly, washed biofilms were exposed to 0.45 mg/ml MTT (Molecular Probes, Eugene, OR, USA) in 100 µl of media for 30 min at 37°C. Plates were then rinsed with sterile phosphate buffered saline (PBS), and 100 µl dimethyl sulfoxide (DMSO, Thermo Fisher Scientific, Rochester, NY) was added to each well. The plates were incubated at 37°C for 10 min, and then 50 µl was removed from each well after thorough mixing and the absorbance was read at 540 nm. The MBC-B concentration was defined as the concentration at which the absorbance was less than or equal to the negative control, consisting of 100 µl culture media treated with MTT.

Checkerboard assay for antimicrobial combinations

The potential effectiveness of a combination treatment against *G. vaginalis* ATCC 14018 was investigated using a checkerboard assay as described by Algburi *et al.* (2017). Each strain was treated with a combination of one CAm compound coupled with either metronidazole or clindamycin. For planktonic cells, a 24 h culture of *G. vaginalis* was diluted to $\sim10^6$ CFU/ml. Each antimicrobial was diluted twofold with sBHI broth into two separate 96-well non-tissue culture microplates. From each dilution of antimicrobial B, 50 μl was added horizontally over 50 μl of antimicrobial A. Then, 100 μl of the bacterial suspension (10^6 CFU/ml) was separately added to each well. The MIC of each combination was determined after 24 h incubation anaerobically at 37°C.

Checkerboard assay, data analysis

The fractional inhibitory concentration (FIC) is defined as the concentration that kills when used in combination with another agent divided by the concentration that has the same effect when used alone (Hall, Middleton and Westmacott 1983; Krogstad and Moellering 1986). The FIC index (FICI) for the combination of antimicrobial A and antimicrobial B is the sum of their individual FIC values. By convention, the FIC values of the most effective combination are used in calculating the FICI. The FICI is not a standardized measurement and in this manuscript is defined according to EUCAST (2000): a synergistic effect (Syn_E) is observed when FICI value $\leq$ 0.5; an additive effect (Add_E) when 0.5 $<$ FICI value $\leq$1; an indifferent effect (Ind_E) when 1 $<$ FICI value $<$ 2 and an antagonistic effect (Ant_E) when FICI value $\geq$ 2.

Mutagenicity testing

The AMEs fluctuation test is a modified version of the traditional AMEs test (Ames *et al.* 1975), and was carried out as described by Hubbard *et al.* (1984) using *Salmonella enterica* serovar Typhimurium TA100. The AMEs-MOD ISO kit and TA100 reference strain were purchased from Environmental Bio-Detection Products, Inc. (EBPI, Mississauga, ON, Canada). CAm solutions were prepared at MIC concentrations for *G. vaginalis* 14018. An overnight culture of TA100 was diluted to $OD_{600} = 0.05$ and 200 µl of the suspension was added to each well in a 24-well plate, along with 50 µl of the CAm solutions and 1550 µl of ddH_2O. Negative and positive control wells were prepared with 50 µl of ddH_2O and standard mutagen (2 µg NaN_3, EBPI, Mississauga, ON, Canada) respectively. Additional sterility control wells were also included, with the bacterial suspension replaced by ddH_2O. Exposure solution (200 µl) was then added to each well, and the plate was incubated at 37°C for 100 min. Each well was then mixed with reversion mix, and 200 µl of the resulting mixture was added to 48 wells of a 96-well plate. The plates were then incubated for 2–3 days at 37°C. On the third day, the plates were scored visually as follows: Yellow and partial yellow wells are counted as positive, while purple wells are negative. The statistical significance of the results was quantified using a provided EBPI statistical table.

EpiVaginal ectocervical tissue model

The EpiVaginal (VEC-100) ectocervical tissue model (MatTek Corporation, Ashland, MA, USA) was used and maintained as described by Dover *et al.* (2007). The tissues were exposed to MIC and MBC-B concentrations of the Cam compounds for 4, 24 and 48 h. For time points greater than 24 h, a 12-well plate with an ALI cell culture insert was used, with each well receiving 5 ml of the assay media. ddH_2O was used as a negative control, while

Table 1. MIC values of CAms (μM) against *G. vaginalis* and other vaginal pathogens in comparison to healthy *Lactobacillus* species.

	MIC (μM)		MIC (μM)		MIC (μM)	
Microorganism	1a	2a	1b	2b	1c	2c
G. vaginalis 14018	2.5 ± 0.98[a]	18.5 ± 7.9[a]	6.7 ± 3.1	16.3 ± 4.4	3.4 ± 1.1	4.3 ± 1.2
G. vaginalis 14018c	4.5 ± 1.8	7.6 ± 3.6	4.4 ± 1.1	10.2 ± 5.1	3.8 ± 1.5	3.9 ± 1.0
G. vaginalis 14019_metR	1.3 ± 0	3.8 ± 1.8	7.3 ± 3.4	9.3 ± 0.0	3.3 ± 1.6	3.2 ± 1.5
L. rhamnosus 160	38.5 ± 17.6[a]	94.03 ± 32.1[a]	80.9 ± 28.0	155 ± 53.7	148.8 ± 51.6	143.1 ± 49.6
L. plantarum ATCC 39268	118 ± 34.1[a]	185 ± 64.4[a]	81.3 ± 27.4	155.6 ± 54.1	208 ± 136.4	204 ± 131.2
L. gasseri ATCC 33323	9.7 ± 4.3[a]	46.2 ± 16.6[a]	16.2 ± 7.0	23 ± 0.0	37.2 ± 12.9	28.6 ± 12.4
L. crispatus ATCC 33197	7 ± 0.0[a]	55.8 ± 0.0[a]	18.2 ± 13.2	8.7 ± 5.0	22.3 ± 0.0	16.1 ± 9.3
P. bivia ATCC 29303	28.4[a]	111.6[a]	29.1	40.9	37.5	29.2
M. curtisii ATCC 35241	28.4[a]	93.1[a]	21.4	38.0	50.9	29.2
P. anaerobius ATCC 27337	3.6[a]	13.9[a]	4.9	9.3	8.93	4.3

[a]For comparison, results from Algburi *et al.* (2017).

Table 2. MIC-B_{90} values for CAm compounds against *G. vaginalis* species and strains.

Microorganism	1a	2a	1b	2b	1c	2c
G. vaginalis 14018	3.6[a]	>28.8[a]	1.6	3.7	2.9	2.7
G. vaginalis 14018c	3.4	4.9	1.6	4.5	2.9	2.7
G. vaginalis 14019_metR	0.9	1.6	0.8	1.1	0.4	0.3

[a]For comparison, results from Algburi *et al.* (2017).

1.00% Triton X-100 was used as a positive control. At the end of each exposure time point, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine overall cell viability, per the manufacturer's instruction. All plates were incubated at 37°C/5% CO_2 (Model 5215, Shel Lab, Radnor, PA, USA).

MTT assay for ectocervical tissue

The MTT assay for ectocervical tissue (VEC-100) was carried as described by Dover *et al.* (2007). The viability of ectocervical cells after exposure to the CAms was determined via the conversion of the tetrazolium compound to the insoluble purple compound formazan, as this reaction can only occur in live cells (Mosmann 1983). At the end of each time point, any remaining liquid was decanted off of the surface of the tissue, and the tissues were rinsed twice with sterile PBS. The cell culture inserts were then transferred to a 24-well plate containing 300 μl of 1 mg/ml MTT solution in each well and incubated at 37°C/5% CO_2 for 3 h. Cell culture inserts were then transferred to a fresh 24-well plate and immersed in 2.0 ml of extractant solution and left overnight at room temp in the dark. The extractant solution was then assayed spectrophotometrically (Spectrophotometer UV-1600 PC, VWR, Radnor, PA, USA) to determine the level of tetrazolium degradation. Viability (%) of the treated tissue inserts was calculated according to an equation provided by the manufacturer: % viability = OD570 (treated tissue)/OD570 (negative control tissue). The ectocervical tissue assay was carried out once in duplicate for each concentration tested.

Statistical analysis

Each experiment was repeated at least three times in duplicate, unless stated otherwise. All calculations were performed in Microsoft Excel, and statistical analysis was carried out using SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA). The Student's *t*-test (with significance for P values ≤ 0.01 and P values ≤ 0.05) was also performed using SigmaPlot 11.0.

RESULTS

CAms inhibit the growth of BV-associated pathogens at concentrations where vaginal lactobacilli are tolerant

The antimicrobial activity of the CAms against planktonic *G. vaginalis* was determined and compared to their activity against vaginal lactobacilli (Table 1). Compounds from series 1 (compounds containing ether linkages) were generally more active than those of series 2 (compounds containing hydrolyzable ester linkages) against *G. vaginalis*. The MICs of CAms against *G. vaginalis* were significantly lower ($P < 0.01$) than their MICs against both *L. plantarum* ATCC 39268 and *L. rhamnosus* 160. No significant difference between the susceptibility of *G. vaginalis* and *L. gasseri* ATCC 33323 and *L. crispatus* ATCC 33197 was detected for the 1b, 1c, 2b and 2c compounds, matching previous results for similar compounds (Algburi *et al.* 2017). CAms were also evaluated against *M. curtisii* ATCC 35241, *P. anaerobius* ATCC 27337 and *P. bivia* ATCC 29303. *Peptostreptococcus anaerobius* ATCC 27337 was susceptible to similar CAm concentrations as seen with *G. vaginalis*, while *P. bivia* ATCC 29303 and *M. curtisii* ATCC 35241 were generally more tolerant to higher concentrations of the different CAms.

MIC and sub-MIC concentrations of CAms inhibit G. *vaginalis* biofilm formation

MIC-B_{90} values were calculated for each *G. vaginalis* species and strain, and the results are presented in Table 2. MIC and sub-MIC concentrations effectively inhibited biofilm formation in each species tested. The recorded MIC-B_{90} values for *G. vaginalis* ATCC 14018 and strain 14018c were the same, with the exception of compounds 2b (3.7 vs. 4.5 μM), 1a and 2a. They are

Table 3. Minimum biofilm bactericidal concentrations (MBC-B) of CAms (μM) against *G. vaginalis* strains in comparison to *Lactobacillus* species.

Microorganism	1a	2a	1b	2b	1c	2c
G. vaginalis 14018	58.8[a]	117.6[a]	>388.3	372.1	178.6	343.5
G. vaginalis 14019_metR	106.3	101.5	24.27	46.52	44.65	21.47
G. vaginalis 14018c	425.6	101.5	48.54	46.52	44.65	42.93
L. rhamnosus 160	940[a]	>890[a]	>777	>744	>714	>687
L. plantarum ATCC 39268	940[a]	>890[a]	>777	>744	>714	>687
L. gasseri ATCC 33323	940[a]	>890[a]	>777	>744	>714	>687
L. crispatus ATCC 33197	940[a]	>890[a]	777	744	714	687

[a]For comparison, results from Algburi *et al.* (2017).

Table 4. FIC values of metronidazole and clindamycin together with CAms against planktonic cells of *G. vaginalis* 14018.

Antimicrobial	1a	2a	1b	2b	1c	2c
Clindamycin	1.0	1.0	1.0	0.65	0.84	0.50
Metronidazole	0.61[a]	0.75[a]	0.60	0.74	0.71	1

[a]For comparison, results from Algburi *et al.* (2017).

Table 5. Characterization of draft genome sequences of *G. vaginalis* ATCC 14018 and 14018c.

G. vaginalis isolates	Assembly size	Number of contigs	Largest contig, nt	G + C content, %
ATCC 14018[a]	1 657 111	14	550 404	41.3
14018c[b]	1 657 819	11	372 021	41.3
ATCC 14018 ref[c]	1 667 406	1	1 667 406	41.4

Accession numbers:
[a]TBA.
[b]TBA.
[c]AP012332.1.

also not much lower than the MIC values for the two strains, while *G. vaginalis* 14019_metR biofilms were inhibited by CAm concentrations well below MIC values. MIC-B_{50} values were not determined, as a sharp cutoff in biofilm formation ability was observed when using a 2-fold serial dilution, such that the MIC-B_{50} values fall between the MIC-B_{90} concentration and the concentration below it.

Lactobacilli biofilms survive CAm concentrations that are bactericidal for *G. vaginalis* biofilms

Lactobacilli biofilms were tolerant to CAm concentrations of 687 to >850 μM, while bactericidal concentrations for *G. vaginalis* biofilms were all below 425.6 μM (Table 3). Interestingly, *G. vaginalis* strain 14018c biofilms were more susceptible to compounds 1b, 1c, 2b and 2c than compounds 1a and 2a. *Gardnerella vaginalis* strain 14019met_R biofilms were most susceptible to compounds 1b and 2c, with MBC-Bs of 24.27 and 24.47 μM, respectively. The MBC-Bs for the other compounds against *G. vaginalis* strain 14019met_R were similar to those for *G. vaginalis* strain 14018c, with the exception of compound 1a.

CAms display an additive effect when combined with either metronidazole or clindamycin

All the CAms tested in this study had a FICI between 0.5 and 1.0, indicating an additive effect following the model used in this study (Table 4). Of the tested CAms, 2c is the only compound that could potentially be defined as having synergy in combination with clindamycin, as the FICI = 0.5. Interestingly, compound 2c did not exhibit any synergy with metronidazole, and the FICI of 1.0 shows near indifference for the combination. Similarly, the synergy that was demonstrated between compounds 1a/2a and metronidazole previously (as determined by isobolographic analysis) was not seen when the compounds were used in combination with clindamycin.

CAms are non-mutagenic and do not impact epithelial tissue viability after 48 h

The AMES-MOD-ISO assay did not detect any mutagenic activity against *Salmonella* TA100 at MIC concentrations for *G. vaginalis* ATCC 14018. Additionally, MIC and MBC-B concentrations for *G. vaginalis* ATCC 14018 showed no impact on epithelial tissue viability when compared to an untreated control using the EpiVaginal ectocervical tissue model after 4, 24 and 48 h exposure times.

Genome sequencing and analysis completed for investigated strains

The *de novo* assemblies containing contigs over 1 kb in size were deposited into NCBI GenBank® under the accession numbers shown in the legend to Table 5. The genome assembly coverage for *G. vaginalis* strains ATCC 14018 and 14018c were 204.72× and 202.28×, respectively. Further details of the assemblies in comparison with published genomes are presented in Table 5.

Mapping reads generated by sequencing of our version of *G. vaginalis* ATCC 14018 onto a published complete (circular) genome sequence of *G. vaginalis* strain ATCC 14018 revealed 17 SNPs (single nucleotide polymorphisms), 14 of which were found within the genes and 3 in the intergenic regions. Importantly,

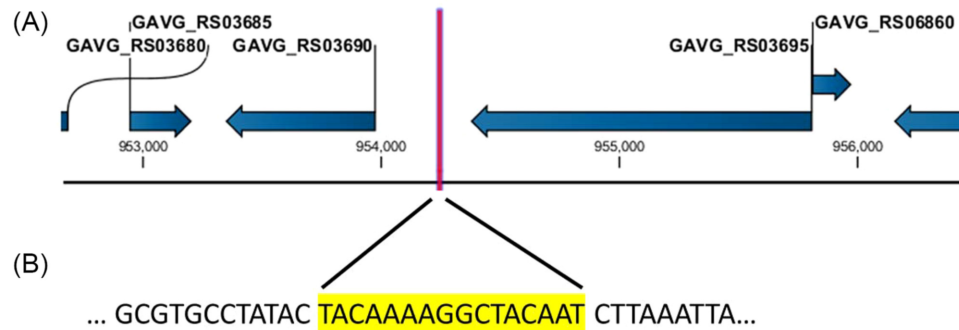
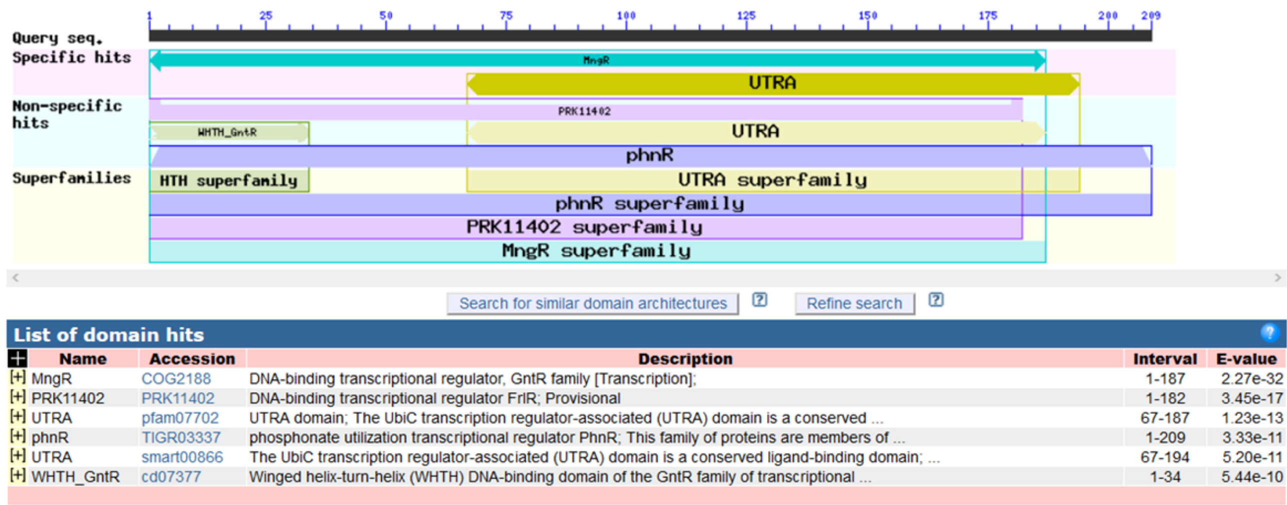


Figure 2. Location of the region (highlighted in yellow) deleted in the 14018 derivative.



Name	Accession	Description	Interval	E-value
[+] MngR	COG2188	DNA-binding transcriptional regulator, GntR family [Transcription];	1-187	2.27e-32
[+] PRK11402	PRK11402	DNA-binding transcriptional regulator FrlR; Provisional	1-182	3.45e-17
[+] UTRA	pfam07702	UTRA domain; The UbiC transcription regulator-associated (UTRA) domain is a conserved ...	67-187	1.23e-13
[+] phnR	TIGR03337	phosphonate utilization transcriptional regulator PhnR; This family of proteins are members of ...	1-209	3.33e-11
[+] UTRA	smart00866	The UbiC transcription regulator-associated (UTRA) domain is a conserved ligand-binding domain; ...	67-194	5.20e-11
[+] WHTH_GntR	cd07377	Winged helix-turn-helix (WHTH) DNA-binding domain of the GntR family of transcriptional ...	1-34	5.44e-10

Figure 3. Conserved domains of a putative regulator GntR identified via NCBI Blast similarity search.

exactly the same SNPs were foundfig in the antibiotic-resistant *G. vaginalis* derivative 14018c, confirming that the latter and the *G. vaginalis* ATCC 14018 wild-type version of this strain used in this study are isogenic.

The availability of the complete genome sequence of the *G. vaginalis* ATCC 14018 strain allows exhaustive comparison of the differences between the wild-type strain and its clindamycin-resistant derivative. Mapping sequencing reads to the reference genome allowed the generation of consensus genome sequences of both strains. The only difference detected by comparing the latter with each other was the presence of a deletion of a 16-nucleotide sequence stretch in an intergenic region (Fig. 2), which may have affected gene expression leading to the phenotypic change. The region is located upstream of a putative GntR family transcriptional regulator, 209 amino acid residues in size (Fig. 3). Analysis of the derived amino acid sequence of this protein using the NCBI Blast search tool and a non-redundant database revealed a large number of hits with putative regulators having unknown functions. A search against Swiss-Prot database, which includes proteins with experimentally determined function, revealed only low similarity (up to 32% identity with 72% coverage) hits with various transcriptional regulators, and the presence of two major domains. One of the latter is a helix-turn-helix domain typical to many DNA binding regulatory proteins, and another one is believed to 'modulate activity of bacterial transcription factors in response to binding small molecules'. A sigma 70 promoter was identified outside the deletion (Fig. 4). The finding does not exclude a possibility of other effects of the deleted region on gene expression, which could be due to the presence of unidentified promoter and/or attenuator sequences.

DISCUSSION

The increasing emergence of resistant strains of pathogenic bacteria is a pressing issue when it comes to treating bacterial infections with traditional antibiotics. This emergence is especially concerning in biofilm-associated infections, as biofilms are inherently more resistant to antimicrobial agents than planktonic cells. In the case of BV, the most frequently prescribed antibiotics are clindamycin and metronidazole, and resistance to these compounds is increasingly common (Nagaraja 2008; Bostwick *et al.* 2016). Recurrence of BV is common within 12 months of initial treatment and 6-month recurrence rates are in excess of 50% (Bradshaw *et al.* 2006; Eschenbach 2007). BV is linked to a decrease in numbers of commensal lactobacilli and an increase in anaerobic pathogens, with *G. vaginalis* being the most studied and predominant species associated with BV (Muzny, Schwebke and Josey 2014; Cerca and Machado 2015; Castro Machado and Cerca 2019).

In this study, a new series of cationic amphiphilic molecules (CAms) was investigated for the ability to control both planktonic and biofilm-associated cells of *G. vaginalis* 14018 and two antibiotic-resistant strains, *G. vaginalis* 14018c and 14019_metR. Since BV is a polymicrobial infection, the CAms were tested against other commonly isolated BV anaerobes, such as *M.*

TACAATTGTGAAGAGTTATTGTAGAAATTAAACCTAAATCACAATTAAAAATAGAA
ACGATAAGTCTAAAAGGTTATAATCTTGAATTTGCGATTAAATAGCAACTTTAATCA
ATAATTTAAGATTGTAGCCTTTTGTAGTATAGGCACGCCTTAATCGTATATTGGCATA
ATATACTGATACTATGTAACTGTTGTGTGTTTTGAAAGCAAATTTGTAAAAATTTTCT
TAAAAGCTTCATTTTTTAAGATTTTTTTTTTAATAGCTTTTAAAAGAAAATTACTATT
TGGA

-35 -10
GAGTGGATTGTGACCGAAGGTGTACCTAAATATATTGCCGTGCGAGACAATCTTAGG
AAACGCATTGACTTAATGGAGCCAGACGAACAGCTTCCTCCATAAGTAGAA

Figure 4. Location of -35 and -10 sigma 70 promoter sequences (underlined) in the intergenic region of strain ATCC 14018. The sequence deleted in 14018c derivative is highlighted in yellow. The sequence shown is a reverse complement of the intergenic area shown in Fig. 2.

curtisii ATCC 35241, *P. bivia* ATCC 29303 and *P. anaerobius* ATCC 27337. CAms were also evaluated against several *Lactobacillus* species commonly found among the healthy vaginal microbiota to determine the effects CAms may have on the commensal bacteria, as they are essential in maintaining the health and stability of the vaginal microbiota (Saunders *et al.* 2007; Petrova *et al.* 2015).

Bacterial vaginosis is a polymicrobial biofilm-associated infection, in which opportunistic anaerobic pathogens such as *G. vaginalis, P. bivia*, *P. anaerobius* and *M. curtisii* outgrow and replace the commensal microbiota. Each of these species has shown resistance to either clindamycin or metronidazole (Petrina *et al.* 2017; Tosheva-Daskalova *et al.* 2017; Veloo *et al.* 2018), and our lab was able to develop a clindamycin-resistant variant of *G. vaginalis* ATCC 14018 to investigate in addition to a metronidazole-resistant strain, *G. vaginalis* 14019_metR. When tested against planktonic cells, series 1 compounds (composed of hydrolytically stable ether linkages) were generally more effective than those from series 2 (compounds containing hydrolyzable ester linkages), though the trend was less noticeable and even reversed in some instances with compounds 1c and 2c. For the *G. vaginalis* species, 1a appeared most effective (2.5 μM for *G. vaginalis* ATCC 14018, 5.3 μM for *G. vaginalis* strain 14018c and 1.3 μM for *G. vaginalis* strain 14019_metR). The CAms used in this study represent the next generation of CAms generated based on the results of Algburi *et al.* (2017), and again consist of hydrophilic head groups and non-polar domains on opposite ends of the compound, thus giving an amphiphilic conformation (Zhang *et al.* 2017; Algburi *et al.* 2017). CAms 1a and 2a are in fact the same as the G8 CAms from our earlier investigations, while the newer compounds (1b, 1c, 2b, 2c) bear an incrementally increasing number of carbons between the backbone and cationic end-groups. These changes were made to impart increased conformational flexibility on the compounds (Moretti *et al.* 2019). The previous series of CAms has been demonstrated to act via insertion and disruption of the bacterial membrane, leading to cell rupture and eventual cell death (Algburi *et al.* 2017; Zhang *et al.* 2017). The MIC for compound 1a against *G. vaginalis* strain 14018c was highest among the tested *G. vaginalis* strains, and may be attributed to changes related to acquired clindamycin resistance and/or due to the presence of clindamycin in the media before MIC determination. MICs against the other anaerobic pathogens were considerably higher for the new compounds, especially against Gram-negative species. This observation is in line with previous results, and can be attributed to the lipopolysaccharide (LPS) layer in Gram-negative species that acts as hydrophilic barrier, preventing hydrophobic compounds from coming into contact with the cytoplasmic membrane (Zhang *et al.* 2017).

Mobiluncus curtisii has shown resistance to both metronidazole and clindamycin, and has been implicated as a potential key species in the recurrence of BV, with some estimating that 67.9% of reported recurrences are caused by *M. curtisii* cells that remain after initial treatment (Meltzer, Desmond and Schwebke 2008; Petrina *et al.* 2017). *Prevotella bivia* and *Peptostreptococcus anaerobius* species have both shown resistance to clindamycin, while data on metronidazole resistance can only be found for *P. bivia* (Teng *et al.* 2002; Petrina *et al.* 2017; Veloo *et al.* 2018). *Gardnerella vaginalis* is able to form a multispecies biofilm with *M. curtisii*, *P. anaerobius*, *P. bivia* and other anaerobic pathogens; interactions among the various species may be synergistic and lead to a more robust biofilm than is typically seen in single-species biofilms (Cerca and Machado 2015; Castro, Machado and Cerca 2019; Gilbert *et al.* 2019). *Gardnerella vaginalis* is often described as Gram-variable, as it may exhibit characteristics of either Gram-negative or Gram-positive bacteria, depending on what phase the cells are in. During early exponential phase, *G. vaginalis* cells tend to stain as Gram-positive. However, as the cells age, the thickness of the peptidoglycan layer decreases to the point that it is unable to retain the Gram-stain. Cationic amphiphiles continue to show efficacy against anaerobic bacteria with minimal potential for resistance build-up due to the membrane-based modes of action. Nair *et al.* (2017) have demonstrated the activity of a polycation AMP mimic C12-50 against *Propionibacterium acnes* without the occurrence of resistance build-up. More recently, Tague *et al.* (2019a,b) have shown both broad selection and Gram-positive selective antibacterial activity using cationic biaryl 1,2,3-triazolyl peptidomimetic amphiphiles against *Clostridioides* (*Clostridium*) *difficile*. The series of CAms in this study is based on previously developed CAm compounds that showed efficacy against both Gram-positive and Gram-negative pathogens, and represent a series of strategic structural alterations to increase conformational flexibility (Zhang *et al.* 2017; Moretti *et al.* 2019). Additionally, CAms have been shown to effectively target BV-associated pathogens and *G. vaginalis* biofilms with minimal inhibitory and bactericidal effects on either planktonic lactobacilli or biofilms (Algburi *et al.* 2017).

Lactobacilli species contribute to the stability and maintenance of a healthy vaginal environment. Lactic acid production is a key component in controlling the pH, which when maintained at an appropriately low level (pH < 5.0) prevents the overgrowth of opportunistic pathogens (Petrova *et al.* 2015). The CAms in this study have been evaluated against strains of four

representative species of *Lactobacillus*: *L. rhamnosus* 160, *L. plantarum* ATCC 39268, *L. gasseri* ATCC 33323 and *L. crispatus* ATCC 33197. As reported previously, the most effective compound of the current series (1a) is less active against the tested lactobacilli, especially in the case of *L. rhamnosus* 160 and *L. plantarum* ATCC 39268 (Algburi *et al.* 2017). Results for the new CAm compounds with modified linker length follow a similar pattern, with the 1c and 2c compounds having the highest recorded MIC against *L. rhamnosus* 160 and *L. plantarum* ATCC 39268 at 208 and 204 μM, respectively. These results are in agreement with previous findings, showing that CAm compounds may have selectivity against anaerobic pathogens while leaving the commensal microbiota relatively unaffected.

Studies against planktonic cells, while informative, do not present a full picture of how these compounds may be used to treat BV. That is because persistent infections and recurrence are often due to the presence microbial biofilms (Cerca and Machado 2015; Muzny and Schwebke 2015). Biofilms are communities of bacteria that form on biotic and abiotic surfaces, existing within an extracellular polymeric matrix composed of polysaccharides, proteins and DNA. The extracellular polymeric substances (EPS) that make up the secreted matrix form a physical barrier against potential stressors (antibiotics, other microbes, etc.), allowing the biofilm-associated cells to persist in conditions that may be lethal to planktonic cells (Kumar *et al.* 2017). A promising method for the control of biofilms in persistent infections is the inhibition of biofilm formation, rather than the eradication of existing biofilms. This effect may be achieved by the prevention of initial surface attachment or communication between cells, and has been demonstrated to be more effective than treating existing biofilms (Shah *et al.* 2013). Quorum sensing is an essential means of communication between microbial cells for the initiation or dispersal of biofilms. This communication may be disrupted to prevent biofilm formation (Algburi *et al.* 2017). Biofilm prevention can occur at much lower concentrations than needed for biofilm eradication, potentially even occurring at sub-MIC concentrations as demonstrated in previous studies with CAm compounds (Algburi *et al.* 2017). In these studies, CAms were able to inhibit biofilm formation without affecting bacterial growth. However, it was shown that those CAms did not cause QS inhibition, and as such, the observed prevention of biofilm formation must be occurring through a different mechanism. The newly developed CAms used in this study were also able to inhibit biofilm formation at sub-MIC concentrations (Table 2). This property was somewhat expected due to the structural similarity of the compounds to previous CAms and the assumption that the mechanism of inhibition between the CAm compounds are similar.

There are other mechanisms that may explain biofilm inhibition by these compounds. It is possible that the cationic nature of the CAms leads to the coating of the negatively charged attachment surface, thereby preventing initial attachment of bacterial cells and subsequent biofilm formation. This mechanism has been shown with AMP treatments, where coating of either the attachment surface or bacteria is able to inhibit biofilm formation (Segev-Zarko *et al.* 2015). However, this effect is not consistent with all AMPs. Other peptidomimetics such as meta-phenylene ethynylene (mPE) have been shown to inhibit *Staphylococcus aureus* biofilm formation, but at nearly 40 times the concentration needed to eradicate biofilms (Beckloff *et al.* 2007). Other potential mechanisms for the prevention of biofilm formation include the inhibition of other signalling pathways or of the genes responsible for biofilm formation and transportation of binding proteins (Segev-Zarko *et al.* 2015).

The CAms were also evaluated for bactericidal activity against preformed biofilms of *G. vaginalis* and *Lactobacillus* species. Interestingly, the MIC-B values for *G. vaginalis* 14018c were much lower for newer compounds than with compounds 1a and 2a, and the MBC-B values for the resistant strain are also significantly lower than the MBC-B values for *G. vaginalis* 14018 (Table 3). MBC-B values for *G. vaginalis* strain 14019met_R were similar to those observed with strain 14018c, with the exception of compounds 1b and 2c, which had the lowest MBC-B values for any of the tested strains at 24.27 and 21.47 μM, respectively.

All the *Lactobacillus* biofilms were significantly more resistant to the CAms than *G. vaginalis* biofilms. The presence of *Lactobacillus* species is important in maintaining a healthy vaginal environment and helps to lower the risk of BV and other STIs. The beneficial effects of lactobacilli can be attributed to a variety of factors, including lactic acid and bacteriocin production, adhesion to host cells and competitive exclusion, and anti-inflammatory and immunomodulatory activities (Petrova *et al.* 2015). Biofilm formation and persistence by vaginal lactobacilli help secure their place in the environment, and potentiate their antimicrobial and other beneficial properties. For example, *L. reuteri* biofilms have been reported as maintaining the same promoting cytokine and antimicrobial production as seen in planktonic cultures. Moreover, *L. reuteri* biofilms may potentially eradicate and reoccupy *G. vaginalis* biofilms, and recent studies have shown that the probiotic potential of *L. reuteri* may be increased when delivered as a biofilm (Jones and Versalovic 2009; Navarro *et al.* 2017; Mukherjee and Bassler 2019). Considering the importance of the commensal lactobacilli in maintaining a healthy vaginal environment and their potential use as probiotics in the treatment of BV, the tolerance of these lactobacilli to CAm treatment shown here is crucial to their potential use as therapeutic agents.

Metronidazole and clindamycin were chosen for this study as they represent the two most common treatments for BV worldwide (Javed, Parvaiz and Manzoor 2019). Metronidazole and clindamycin resistance are common among BV isolates, and represent a major challenge linked to high levels of recurrence and treatment failure. As such, the ability of metronidazole and clindamycin to synergize with CAm compounds was investigated. FIC data (Table 4) reveals that there is no synergy between any of the tested compounds and the combinations treatments range from having an additive effect to indifference between the two compounds. While synergy was not displayed, an addictive effect is still beneficial, as it may decrease the needed concentrations of antimicrobials and will make it more difficult for the treated pathogens to develop resistance. This aspect is especially true when the compounds have different modes of action (Rybak and McGrath 1996). In addition, the use of naturally derived and/or novel antimicrobials, either alone or in combination with conventional antibiotics, is not associated with the same level of risk seen with the use of conventional antibiotics (i.e. clindamycin/metronidazole) alone (Algburi, Volski and Chikindas 2015). Taking this into account, a growing number of researchers are combating antibiotic resistance by combining conventional antibiotics with complementary methods (National Center for Complementary and Integrative Health 2014). The CAm compounds in this study may effectively be combined with either metronidazole or clindamycin to meet this aim.

To investigate the potential activity of CAms against resistant forms of *G. vaginalis*, and to further understand potential genetic modifications that may lead to the resistance development, two *G. vaginalis* species, 14019_metR and 14018c, were investigated.

Gardnerella vaginalis 14019_metR was previously demonstrated as resistant to metronidazole, and the draft genome is available online in GenBank under the accession number LIYA00000000 (Schuyler *et al.* 2015). *Gardnerella vaginalis* 14018c was made resistant through the gradual introduction of clindamycin, starting at sub-MIC concentrations before increasing beyond the microbiological resistance breakpoint (≥8 μg/ml), as defined by CLSI standards, to a final concentration of 50 μg/ml.

In regard to the draft genome sequence, the differences in single nucleotides (SNPs) when comparing our version of *G. vaginalis* ATCC 14018 with the published genome of this isolate are likely to represent random mutations, which are common in other bacteria. Consistency of variation throughout all reads suggests that either our version is a clonal subpopulation of the strain originally submitted to culture collection or, conversely, the published genome represents a subpopulation of the strain deposited in the culture collection. Despite some limitations of read mapping used for verification of whole genome assemblies (Lehri, Seddon and Karlyshev 2017), the tool is a powerful approach for the identification of SNPs and indels when working with derivatives of bacteria that have complete (circular) sequences available. Although the deletion found in the mutant is outside a putative sigma 70 promoter, it may affect other putative promoters and regulatory regions that may be involved in the expression of the GntR regulator. The latter may take part in a regulatory network affecting expression of the genes involved in enhanced resistance of the derivative to clindamycin. Additional studies including transcriptomics and proteomics are required for the identification of the genes affected.

While the CAms represent a promising alternative treatment for BV, based on the antimicrobial and antibiofilm activity presented above, there are still remaining questions regarding the safety of these compounds as therapeutic treatments that are needed to be answered before *in vivo* and formulation studies can go forward. As such, we performed both a mutagenicity and a vaginal tissue viability assay to determine whether the CAm compounds are potentially carcinogenic or harmful to the vaginal epithelium. The modified AMEs test did not result in any mutations in *Salmonella* TA100 for all tested CAms. However, this does not rule out the possibility for mutations at concentrations well below MIC levels. As such, caution should be taken in interpreting these results. Using the EpiVaginal ectocervical tissue model, the CAms showed no effect on tissue viability, even when administered at relatively high MBC-B concentrations. Low-to-negligible toxicity to human tissues was expected, as the synthesis of the CAms was conducted with materials that are found on the generally recognized as safe (GRAS) list or in FDA-approved formulations for direct addition to food products (Moretti *et al.* 2019). However, previous experiments on hemolytic activity and cytotoxicity against model mammalian membranes showed the CAms as having 100% hemolytic activity at concentrations of 150 μg/ml and higher, and series 1 (hydrolytically stable ether linkages) significantly reduced the viability of mammalian fibroblasts at concentrations of 30 μg/ml and higher, while series 2 (hydrolysable ester linkages) had little to no impact on viability at that concentration (Moretti *et al.* 2019). Thus, it is somewhat surprising that no impact on tissue viability was observed against ectocervical tissues at MBC-B concentrations, which were generally between 150 and 250 μg/ml. This observation may be attributed in part to the negative charge inherent to eukaryotic cell membranes, and further protection may also be provided by secreted glycogen present on the surface of the tissue. Tissue viability testing using the EpiVaginal tissue model has previously been carried out in conjunction with *in vivo* testing of the rabbit vaginal irritation (RVI) system, and further confirmed the safety of the antimicrobial peptide, lactocin 160 (Dover *et al.* 2007). Therefore, we are confident that using the EpiVaginal model instead of animal testing to demonstrate the safety of the CAm compounds provides reliable and valid results.

CONCLUSION

Antibiotic resistance and persistent infections are important challenges in the treatment of BV, and require significant attention from the research community to find alternatives to traditional antibiotic treatments. A new series of CAms and their antimicrobial and antibiofilm activities against BV-associated pathogens have been reported herein, and represent a step toward fine-tuning the physical properties of synthetic CAms with a goal of improved efficacy and specificity against BV-associated species, with a particular focus on antibiotic resistant bacteria. CAms have also been shown to have effectively prevented and eradicated biofilms of *G. vaginalis* at low concentrations (in the micromolar range), without any harmful effects on the protective vaginal microbiota. These same concentrations have been applied to a vaginal epithelial tissue model with not considerable impact on tissue viability, demonstrating that these compounds should be further explored with the objective of developing formulations suitable for clinical use.

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