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Doctoral Dissertation in Medical Sciences

Non-peptide mimics of antimicrobial peptides and their functionalized gold nanosystems to combat antibiotic resistance

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1. SCIENTIFIC OUTPUT

1.1. List of publications constituting the doctoral dissertation.

Review article

1) Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. *Expression and Function of Host Defense Peptides at Inflammation Sites.* Int J Mol Sci. 2019 Dec 22;21(1):104. doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121.

IF: 5.924; MNiSW: 140

Original article

2) Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. *Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins*. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374.

IF: 6.096; MNiSW: 100

1.2. List of other publications.

1) Fiedoruk K, Daniluk T, Rozkiewicz D, Oldak E, Prasad S, Swiecicka I. *Whole-genome comparative analysis of Campylobacter jejuni strains isolated from patients with diarrhea in northeastern Poland.* Gut Pathog. 2019 Jun 19;11:32. doi: 10.1186/s13099-019-0313-x. PMID: 31244901; PMCID: PMC6582539.

IF: 3.274; MNiSW: 70

2) Piktel E, Wnorowska U, Cieśluk M, Deptuła P, Prasad SV, Król G, Durnaś B, Namiot A, Markiewicz KH, Niemirowicz-Laskowska K, Wilczewska AZ, Janmey PA, Reszeć J, Bucki R. *Recombinant Human Plasma Gelsolin Stimulates Phagocytosis while Diminishing Excessive Inflammatory Responses in Mice with Pseudomonas aeruginosa Sepsis.* Int J Mol Sci. 2020 Apr 7;21(7):2551. doi: 10.3390/ijms21072551. PMID: 32272559; PMCID: PMC7177774.

IF: 5.924; MNiSW: 140

3) Wnorowska U, Fiedoruk K, Piktel E, Prasad SV, Sulik M, Janion M, Daniluk T, Savage PB, Bucki R. *Nanoantibiotics containing membrane-active human cathelicidin LL-37 or synthetic ceragenins attached to the surface of magnetic nanoparticles as novel and innovative therapeutic tools: current status and potential future applications.* J Nanobiotechnology. 2020 Jan 2;18(1):3. doi: 10.1186/s12951-019-0566-z. PMID: 31898542; PMCID: PMC6939332.

IF: 10.435; MNiSW: 140

2. IMPACT FACTOR AND MINISTRY OF SCIENCE POINTS SUMMARY

Type of Publication	Number	Impact factor	MEiN points
Publications included in the doctoral dissertation	2	12.02	240
Publications that are not included in the doctoral dissertation	3	19.633	350
Conference/symposium abstracts	3	–	–
Total	**8**	**31.653**	**590**

3. ABBREVIATIONS

AOM – Acute otitis media

AMPs – Antimicrobial peptides

AMR – Antimicrobial resistance

L-Ara4N – 4-amino-4-deoxy-L-arabinose

AOM – Acute otitis media

AuP NPs – Peanut-shaped gold nanoparticles

CAMH – Cation-Adjusted Mueller Hinton

CAMPs – cationic antimicrobial peptides

CF – Cystic fibrosis

COPD – Chronic obstructive pulmonary disease

CPE – Carbapenem-producing Enterobacteriaceae

CRE – Carbapenem-resistant Enterobacteriaceae

CTAB – Cetrimonium bromide

CSAs – Cationic steroid antimicrobials (ceragenins)

DEGs – Differentially expressed genes

DCFH–DA – 2'-7'dichlorofluorescin diacetate

ESBL – Extended spectrum beta-lactamase

ESKAPE – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*

FDR – False discovery rate

GO – Gene ontology

HAADF – High-angle annular dark field

HGT – Horizontal gene transfer

HDPs – Host defense peptides

HPI – High-pathogenicity island

ICU – Intensive care unit

KEGG – Kyoto encyclopedia of genes and genomes

Log_2FC – Log_2-fold change

LPS – Lipopolysaccharide

MBC – Minimum bactericidal concentration

MDR – Multi-drug resistant

MHDA – Mercaptohexadecanoic acid

MIC – Minimum inhibitory concentration

NPs – Nanoparticles

MRSA – Methicillin-resistant *Staphylococcus aureus*

NPN – N-Phenyl-1-naphthylamine

OM – Otitis media

OME – Otitis media with effusion

PBS – Phosphate buffered saline

PDR – Pan-drug resistant

RNA-seq – RNA sequencing

ROS – Reactive oxygen species

SNPs – Single nucleotide polymorphisms

STEM – Scanning transmission electron microscopy

T6SS – Type VI secretion system

UPEC – Uropathogenic *Escherichia coli*

UTIs – Urinary tract infections

WGS – Whole-genome sequencing

WHO – World Health Organization

XDR – Extensively drug resistant

4. INTRODUCTION

4.1. The global threat of antimicrobial resistance (AMR)

Antimicrobial resistance (AMR) is a public health problem that significantly increases medical expenses and influences treatment quality. In addition, the future implications of unresolved AMR are projected to result in roughly 10 million patient deaths by 2050 [1]. Particularly, immunocompromised and intensive care unit (ICU) patients suffer a significant risk of acquiring drug-resistant infections.

AMR, in general, is an example of the natural adaptation process to challenging environmental conditions that bacteria encounter in their habitats or to the host's defenses in the case of pathogenic species, which ultimately results in the selection of resistant bacterial strains. To cope with a broad range of stress factors, bacteria have evolved response mechanisms that modulate gene expression via regulatory networks, resulting in modifications of their physiology and behavior, known as stress response, e.g., oxidative, acidic, osmotic, temperature and starvation, or more specifically the cell envelope stress response [2]. In addition, the acquired drug resistance can quickly evolve through chromosomal DNA mutations in antibiotics' target genes and plasmid-mediated horizontal gene transfer (HGT) [3]. Furthermore, so-called adaptive resistance may occur transiently in bacteria exposed to subinhibitory concentrations of antimicrobials, e.g., due to poor drug penetration in specific body sites or suboptimal dosing. This phenomenon is commonly linked with changes, e.g., in gene expression, in a fraction of cells resulting in the phenotypically heterogenous resistant population of cells. Notably, the selective pressure exerted by a particular antimicrobial agent may also lead to cross-resistance to other antimicrobials due to multiple pathway-specific alterations [4]. Overall, these processes are responsible for the emergence of multi- (MDR), extensively- (XDR) or even pan-drug resistant (PDR) bacterial pathogens [5].

4.2. Multidrug-resistant bacteria demanding immediate research and development of new antibiotics

Given the AMR threat, the World Health Organization (WHO) has classified medically relevant bacteria requiring urgent research and development of new antimicrobials into three categories: critical, high, and medium priority.

Accordingly, the dissertation aimed to investigate (i) the antibacterial activity of a novel class of cationic antimicrobial agents – ceragenins (CSAs) and their nanosystems, as well as (ii) potential mechanisms of resistance to CSAs in selected pathogens representing these categories. The former issue addressed the leading causative agents of otitis media (OM), namely *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis,* while an emerging gram-negative pathogen – *Enterobacter hormaechei* subsp. *steigerwaltii* (ST89), served as the model organism in experiments with induction of resistance to ceregenins. The choice of this gram-negative rod enteric rod is not accidental as it is a growing problem in Poland, particularly in the context of carbapenem resistance [6].

Since *S. pneumoniae, H. influenzae,* and *M. catarrhalis* are involved in most otitis media cases, they are known as the 'classical otopathogens' [7]. Otitis media is an inflammatory infection of the middle ear commonly affecting children between 3 and 24 months, classified as acute OM (AOM) and chronic OM (COM), including OM with effusion (OME). The clinical manifestations of AOM include severe ear pain, fever, swelling, ear discharge, and acute tympanic membrane perforation, along with elevated levels of proinflammatory cytokines IL-6, IL-1β, TNF-α, IFN-γ, and IL-8 [8].

Additionally, all these otopathogens are responsible for various illnesses, including sinusitis, pneumonia, chronic obstructive pulmonary disease (COPD), meningitis, and bloodstream infections. Implementing a 7-valent protein-polysaccharide pneumococcal vaccine in early 2000 significantly declined the incidence of invasive pneumococcal diseases in children and in not vaccinated adults due to herd immunity [9]. However, the difficulty of addressing nasopharyngeal carriage of pneumococcal serotypes not covered in PCV-7 vaccinations prompted the adoption of PCV-13 and PPV-23 vaccines to counteract the emergence of MDR strains [10]. For instance, a high level of resistance to penicillin, erythromycin, and clindamycin was reported in *S. pneumoniae* strains isolated from the middle ear fluid of pediatric patients with AOM [11]. Similarly, the rate of antibiotic resistance, especially to β-lactams, in *H. influenzae*, including the non-encapsulated and non-typeable strains, and *M. catarrhalis* has rapidly grown [12, 13]. It is particularly worrying since amoxicillin or amoxicillin-clavulanate are considered the gold standard for OM treatment.

It is noteworthy that OM is a biofilm-associated infection, and the otopathogens persist in the nasopharynx and middle ear and as mono- and multispecies biofilms, protecting them from antibiotics and immune responses [14]. Consequently, ineffective pathogen eradication is a risk factor for recurrent and persistent infections [11, 15]. Therefore, targeted therapeutic drugs with antibiofilm and anti-inflammatory activity are required to reduce the severity, local inflammation, and recurrence of this condition.

Enterobacter hormaechei is a member of the ESKAPE group of MDR pathogens (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa*, and ***E****nterobacter* species). Currently, the primary concern associated with gram-negative ESKAPE pathogens, such as *E. hormaechei*, is the global spread of their clones equipped with extended-spectrum β-lactamases (ESβL) and/or carbapenemases, that have been linked to multiple clinical outbreaks [16]. *E. hormaechei,* like other Enterobacterales, is a rod-shaped, gram-negative, facultative anaerobe which inhabits the human gut ecosystem and natural environments. According to Hoffman and Roggenkamp's classification, *E. hormaechei* belongs to the *E. cloacae* complex, i.e., a cluster of 13 phylogenetically related *Enterobacter* species/subspecies: *E. asburiae* (cluster I), *E. kobei* (cluster II), *E. ludwigii* (cluster V), *E. hormaechei* subsp. *oharae* (cluster VI), *E. hormaechei* subsp. *hormaechei* (cluster VII), *E. hormaechei* subsp. *steigerwaltii* (cluster VIII), *E. cloacae* subsp. *cloacae* (cluster XI), *E. cloacae* subsp. *dissolvens* (cluster XII), an unstable sequence crowd (XIII), and three unnamed *E. cloacae* complex clusters (III, IV, IX) [17-19]. Remarkably, *E. cloacae* and *E. hormaechei* subsp. *steigerwaltii*, specifically its ST89 clones, are the most medically relevant ones, frequently isolated from clinical specimens, and are responsible for a variety of infections ranging from urinary tract infections (UTIs) through the surgical site and intravascular device-related infections, including these after organ transplants, to pneumonia and sepsis [6, 20, 21]. Recently, *E. hormaechei* has emerged as a problematic pathogen because it may survive and spread in nosocomial conditions and often demonstrates treatment failure to critical antibiotics [6, 19, 21-23]. In addition, the presence of a high-pathogenicity island (HPI) renders *E. hormaechei* more virulent than other *E. cloacae* complex species [19], that utilizes multiple virulence factors, such as lipopolysaccharide (LPS), capsule, adhesins, and type VI secretion system (T6SS) as well

as outer membrane proteins to establish infections. Since the *E. cloacae* complex members are natural producers of Amp-C β-lactamases, resistance to ampicillin, amoxicillin-clavulanate, first- and second- as well as third-generation cephalosporins, in the case of so-called derepressed AmpC mutants, is their intrinsic characteristic [24]. As a result, they have contributed to the increased consumption of carbapenems and the emergence of carbapenem-resistant, also known as carbapenem-producing, Enterobacteriaceae (CRE, CPE) isolates [25]. Therefore, polymyxin E (colistin) and other 'old' antibiotics, such as fosfomycin, have reemerged as the last resort drugs for treating severe CPE/CRE infections.

Colistin is the cyclic, cationic, polypeptide antibiotic with hydrophobic and lipophilic moieties. Consequently, its primary mode of action involves electrostatic interactions with the anionic lipopolysaccharide (LPS) molecules leading to the outer membrane, resulting in membrane disruption by displacement of magnesium and calcium ions stabilizing LPS molecules [26]. Moreover, colistin may promote cell death by the generation of reactive oxygen species (ROS), including hydroxyl radicals (•OH), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), resulting in physiological and metabolic alterations that eventually lead to oxidative stress.

4.3. Endogenous antimicrobial peptides − role in immunity and therapeutic potential

Endogenous antimicrobial peptides (AMPs), properly known as host defense peptides (HDPs), represent the first line of defense against bacterial, viral, and fungal infections [27]. These small, 12-50 amino acid peptides are produced by organisms from all kingdoms of life as the first line of defense against invading pathogens. Similarly to polymyxins, the positive net charge of +2 to +9, along with hydrophobic residues, allow these cationic AMPs (CAMPs) to engage electrostatically with the bacterial cell membrane, leading to its permeabilization via pore formation. Notable is the fact that AMPs have substantial benefits over conventional antibiotics since their diverse molecular targets make them less prone to resistance development [27, 28]. On the other hand, due to immunomodulatory properties, e.g., neutralization of the proinflammatory stimuli, such as LPS, and various interactions with the adaptive immune system, these peptides contribute to host-microbiome homeostasis [27, 29].

Defensins and cathelicidins represent the major families of human AMPs. The cathelicidin LL-37 is the most intensively explored human AMP [29, 30]. This amphipathic α-helical multifunctional CAMP produced by various immune and epithelial cells is characterized by a broad spectrum of antimicrobial activity as well as a plethora of immunomodulatory properties, ranging from neutralization of bacterial endotoxins through chemotaxis and stimulation of immune cells to wound healing [27, 29, 30]. LL-37 and other eukaryotic CAMPs have shown significant antibacterial efficacy in treating infections caused by antibiotic-resistant biofilm-forming bacteria, such as chronic pneumonia in patients with cystic fibrosis, otitis media, and chronic wound infections [31, 32]. However, implementing AMP-based drugs must overcome many difficulties, including stability, administration route, and safety [29, 30].

4.4. Ceragenins − promising broad-spectrum antimicrobials

Ceragenins have been developed as an alternative to conventional antibiotics to address the impending antimicrobial resistance crisis [33-35]. The ceragenins were designed to mimic the cationic and facially amphiphilic structures of AMPs; thus, these cholic acid-based agents are also referred to as cationic steroid antimicrobials (CSAs) [36]. The structural similarities and distinct chemical composition of ceragenins allow them to retain the mode of action and a broad spectrum of antimicrobial activity of AMPs but without their downsides, such as sensitivity to proteases or reduced activity in the presence of mucin, F-actin, DNA, or other host compounds that may concentrate in high quantities at sites of infection [37-39]. At the same time, CSAs can inhibit the endotoxin-activated host inflammation, hence may be potentially implemented to prevent systemic inflammation, e.g., in patients with cystic fibrosis (CF) lung infection [33-35].

In vitro studies have reported that ceragenins do not induce resistance in bacterial and fungal cells [40]. Moreover, their synthesis is economical, and CSAs are resistant to deterioration even during long-term storage [33, 34, 41]. The substitution of the ceragenins' core structure with various side chains, provided a series of compounds, such as CSA-8, CSA-13, CSA-44, CSA-90, CSA-131, CSA-138, CSA-142 [33-35]. Because of their favorable activity/toxicity profile, CSA-13, CSA-44 and CSA-131 are the most studied ones [35, 36].

The primary mechanism of action of CSAs is associated with preferential interaction with the bacterial cell membranes over the mammalian ones, resulting in instability of the membrane lipid architecture followed by its depolarization [40]. LPS and lipoteichoic acids are the primary targets for ceragenins in gram-negative and gram-positive bacteria, respectively [35, 36]. Furthermore, due to translocation across the outer membranes of gram-negative bacteria, CSAs may also disrupt the cytoplasmic membrane and induce oxidative stress [36]. Remarkably, the antimicrobial activity of CSAs is independent of resistance mechanisms to other antimicrobial agents. For instance, susceptibility to CSAs has been reported for several MDR pathogens, such as (i) various carbapenem-resistant gram-negative rods, *Acinetobacter baumannii*, *P. aeruginosa,* and Enterobacterales species, (ii) colistin-resistant isolates of *Klebsiella pneumoniae*, (iii) ceftazidime-resistant *Stenotrophomonas maltophilia*, (iv) methicillin- and vancomycin-resistant *S. aureus* (MRSA and VRSA), and (v) drug-resistant isolates of *Candida auris* [42-44].

4.5. Application of nanoparticles and ceragenin-based nanosystems to prevent microbial infections

Nanoparticles (NPs) are promising agents for overcoming the problem of antimicrobial resistance in bacterial and fungal pathogens. The physicochemical parameters of NPs, such as (i) small size (< 100 nm at least in one dimension), (ii) a large surface area to volume ratio (SA:V), (iii) surface charge, and (iv) diverse shapes, mediate not only strong interactions with microbial membranes ensuring microbicidal activity at nanogram concentrations, but also enable effective surface functionalization [41, 45]. Therefore, modification of these parameters, e.g., via synthesis of nonspherical NPs, allows for the production NPs with variable antimicrobial activity [46, 47]. For instance, our previous study revealed the high killing efficiency of nonspherical gold nanoparticles (AuNPs), i.e., star-, peanut-, and rod-shaped, against several bacterial and fungal species, including *P. aeruginosa, S. aureus, C. albicans,* and uropathogenic *E. coli* (UPEC) [47-49]. Similarly, their application as anti-biofilm agents has shown promising results [49].

On the other hand, the functionalization of NPs by ceragenins can enhance their antimicrobial activity [47, 49, 50]. For example, conjugating CSA-13 with core-shell magnetic nanoparticles significantly increased its bactericidal properties against planktonic

and biofilm forms of *P. aeruginosa* [49]. Furthermore, attachment of CSA-13 to the surface of rod-, star- and peanut-shaped AuNPs augmented its activity against MRSA and ESβL- and carbapenem-producing gram-negative rods, concurrently decreasing CSA-13 toxicity against human erythrocytes [47]. Therefore, CSA-based nanosystems appear to be a viable strategy for developing the next generation of antimicrobials with enhanced biocompatibility, thereby addressing potential issues associated with the clinical application of CSAs alone [51].

5. DESCRIPTION OF THE RESEARCH STUDIES

5.1. AIMS AND OBJECTIVES

Antimicrobial resistance (AMR) is one of the greatest threats to global public health in the twenty-first century. This problem is especially urgent in terms of bacterial antibiotic resistance. Therefore, I have outlined two goals:

1. To examine the antibacterial activity of ceragenins and ceragenin-conjugated peanut-shaped gold nanoparticles (AuP@CSA) against the leading causative pathogens of otitis media, namely *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis*.

2. To characterize, at the genomic and transcriptomic levels, the development of resistance during experimental adaptation of the nosocomial pathogen *Enterobacter hormachei* subsp. *steigerwaltii* (ST89) to sub-inhibitory concentrations of ceragenins and the polycationic antibiotic – colistin.

5.2. MATERIAL AND METHODS

5.2.1. Bacterial strains, media, and growth conditions

Haemophilus influenzae ATCC 49766, *Moraxella catarrhalis* ATCC 25238, and *Streptococcus pneumoniae* ATCC 49619 (denoted hereinafter as the otopathogens) were purchased from American Type Culture Collection (VA, USA). The bacterial strains were grown from the freezer stocks (-80 °C) on BBL Chocolate II Agar and blood No. 2 LAB-AGAR + 5% KB plates and incubated at $37^{\circ C}$ in 5% CO_2 atmosphere. The Hemophilus test medium [HTM] for antimicrobial susceptibility testing was prepared using previously well-established protocols, which consisted of Mueller-Hinton broth base (Sigma-Aldrich) supplemented with bovine hematin, yeast extract, and NAD. The medium was filter sterilized using a 0.22 μm-pore size membrane filter. Cation-adjusted Mueller Hinton II Broth was purchased from Sigma-Aldrich (MO, USA). The studied *E. hormaechei* 4236 strain (sequence type 89, ST89) was obtained from the collection of clinical isolates in the Department of Medical Microbiology and Nanobiomedical Engineering (Medical University of Bialystok).

5.2.2. Cell culture

Immortalized adult human skin keratinocytes cells (HaCaT) were purchased from CLS Cell Lines Service (Eppelheim, Germany). High-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 μg/mL), and glutamine (2 mM) were obtained from American Type Culture Collection (VA, USA) and Sigma-Aldrich (MO, USA) respectively.

5.2.3. Antimicrobial compounds

Ceragenins CSA-13, CSA-44, and CSA-131 were provided by Professor Paul B. Savage from the Department of Chemistry and Biochemistry, Brigham Young University, USA. The ceragenins stock solutions prepared from dry powder were dissolved in phosphate-buffered saline (PBS) and stored at 4 °C. The reagents for the synthesis of peanut-shaped gold nanoparticles coated with respective ceragenin compounds such as cetrimonium bromide [CTAB], gold(III) chloride hydrate [$HAuCl_4$], silver nitrate

[$AgNO_3$], sodium borohydride [$NaBH_4$], ascorbic acid [$C_6H_8O_6$], as well as colistin sulfate salt ≥ 19,000 IU/mg were purchased from Sigma-Aldrich (MO, USA).

5.2.4. Collection of human cerumen samples

Cerumen (earwax) samples were collected from healthy adult volunteers under the approval of the Bioethics Committee (No. 20/2019) of Jan Kochanowski University in Kielce. Informed written consent was obtained from all participants prior to inclusion into the study.

5.2.5. Synthesis and physiochemical analysis of nonspherical gold nanoparticles functionalized by ceragenins

The synthesis process of these compounds involved the seed-mediated method, as explained in detail in the attached publication. After the peanut-shaped gold nanoparticles (AuP NPs) were synthesized by the cetrimonium bromide (CTAB)-assisted method, they were surface functionalized using mercaptohexadecanoic acid (MHDA) as the linker, allowing to attach ceragenins to AuNPs by forming a covalent bond between -NH_3 group of CSA and -COOH group of MHDA. This approach to nanosystem synthesis is reproducible and highly controllable, thanks to the appropriate selection of reaction times, temperature, and the use of excess MHDA resulting in the collection of uniform nanoparticles and efficient attachment of ceragenins to AuNPs surface. The obtained concentrations of CSAs and AuP NPs in the nanosystems were 2 mg/mL and 2.93 ng/mL, respectively, indicating that the loading efficiency of ceragenin on the AuP NP surface was approximately 1.33×10^5 of CSA/1 AuP NP. To determine the morphology of the prepared gold nanosystems, scanning transmission electron microscopy (STEM) with high-angle annular dark field detector (HAADF) in both conventional and high-resolution mode was used. The derived STEM images were obtained on an aberration-corrected FEI Titan electron microscope operating at 300 kV equipped with a FEG cathode. The crystal microstructure of AuP NPs were analyzed using a two-circle laboratory diffractometer such as Panalytical X'Pert Pro with a lamp using standard θ-2θ geometry and Cu anode working at 40 kV and 30 mA. The emitted X-ray beam from the X-ray tube was converted into a parallel beam by a divergence slit with a constant height 1/2° and a parabolic graded W/Si mirror with an equatorial divergence less than 0.05, 0.04 rad Soller slit collimator and by

a mask of constant width of 20 mm that is used to restrict the width of the beam. The diffracted beam optics composed of the anti-scattered slit with a height of 8.7 mm, 0.04 rad Soller slit collimator, which is a curved graphite monochromator to eliminate the contribution of the Cu *K*β radiation, and a semiconductor silicone stripe detector with an active length of 2.122°. Nanoparticle dispersions were dried using a zero-background holder and placed on a sample spinner with a rotation time of 16 s. The obtained data was in the range between 20 – 80° with a step size of 0.08°, and time per step was about 7000 s. The diffractogram fitting, lattice constants, and coherent scattering length were evaluated using the Fullprof software. The stability of the biofunctionalization and immobilization process was investigated using Fourier transform (FT)-Raman spectra. The spectra were recorded according to previously published protocols with a Nicolet NXR 9650 FT-Raman Spectrometer provided with an Nd:YAG laser (1064 nm and a germanium detector.

Furthermore, Multiskan SkyHigh UV-VIS (Thermo Fisher Scientific) was implemented to explore the CSA molecules anchored on the Au NPs. TA Instruments' DSC 2500 differential scanning calorimeter (DSC) fitted with a liquid nitrogen LN2P pump was used for checking the temperature stability of nanoparticles functionalized by MHDA, as well as MHDA and ceragenins. For this purpose, the analyzed samples were kept in aluminium pans and crimped with hermetic lids. In addition, the thermal behavior of the samples was studied using the given conditions: dry N5.0 pure nitrogen purge (25 ml/min^{-1}), 10-200 °C temperature range, 5 and 10 °C min^{-1} heating rate, and 5 °C min^{-1} cooling rate. The samples were held isothermally at the minimum and maximum temperature for 5 min. TRIOS software was used for calculating the peak temperatures and enthalpy values of the registered thermal events. TA Instruments' TGA 5500 thermogravimetric analyzer with high-temperature platinum pans was used for thermogravimetric analysis (TGA). For these measurements, the samples were placed in open 100-µl platinum pans or previously enclosed inside hermetic aluminium pans to restrict the evaporation rate. Firstly, a 0.9-mm-diameter hole was punched through the lid of the container, samples were heated at a rate of 5 or 10 °C min^{-1} rate up to 500 °C under a flow of N5.0 pure nitrogen (25 ml/min^{-1}), and temperature was calibrated using nickel and alumel standards.

5.2.6. Antibacterial susceptibility testing

The minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of synthesized nanosystems and free molecules of ceragenins against the otopathogens were determined using the broth microdilution method (BMD). To that end, the bacterial inoculum at the logarithmic growth phase was adjusted to ~ 10^5 CFU/mL in Hemophilus test medium and incubated with serial two-fold dilutions of the tested agents ranging from 256 µg/mL to 0.5 µg/mL. The plates were analyzed after 18 – 20 h incubation at 37 °C in 5 % CO_2. Subsequently, samples with ≥ MIC representative values were plated on chocolate or blood agar plates for the measurement of MBC after 48 hours.

Similarly, BMD with Cation-Adjusted Mueller Hinton (CAMH) medium was used to estimate MIC for colistin and ceragenin CSA-13 in *E. hormaechei* 4236 isolate. Briefly, the inoculum of *E. hormaechei* 4236 was taken at the logarithmic growth phase and adjusted to ~10^5 CFU/mL, and 100 µL of this starting inoculum was incubated with serial two-fold dilutions (256 – 0.25 µg/mL) of colistin and CSA-13 for 18 – 20 h at 37 °C. Additionally, a commercial ready-to-use colistin susceptibility test – ComASP panel (Liofilchem, Italy) was used to verify the obtained colistin MIC results [52]. All the experiments were performed in triplicate.

5.2.7. Killing assays

The killing activity of ceragenins and CSA-containing nanosystems was determined against the otopathogens according to previously established protocols. Briefly, bacteria were subjected to different concentrations of free ceragenins and CSA-containing nanosystems ranging from 0.5 – 20 µg/mL in sterile PBS (pH 7.4), and the plates were incubated at 37 °C, 5 % CO_2 for 18 h. Next, the concentrations of the tested agents that are required to limit the viability of the bacterial population to 100 CFU/mL (2 logs CFU/mL) were estimated by interpolation dose-response curves. Additionally, human cerumen, a natural bodily fluid secreted as a waxy substance in the outer ear, was used in the colony counting assay to reflect the *in vivo* conditions and test the performance of the antimicrobial compounds. For this purpose, the action of the tested systems in the range of 0.5 – 5 µg/mL against the bacterial strains was measured in a buffer prepared from the uncontaminated

collected cerumen samples along with sodium bicarbonate ($NaHCO_3$) and 30 % glycerol (pH ~8.2) at a concentration of 10% (weight/volume).

5.2.8. Antibiofilm assay

The formation of biofilm by causative the otopathogens in the presence of 5, 20, and 50 μg/mL of the corresponding antimicrobial compounds was assessed using a colorimetric crystal violet assay. Briefly, overnight cultures of the bacterial strains in brain-heart infusion broth (BHI) were adjusted to 2×10^7 CFU/mL and incubated for 24 h at 37 °C in 5% CO_2. Following the removal of planktonic bacteria, only the attached biofilms were stained using 0.1% (w/v) crystal violet. Finally, after the crystal violet was removed and solubilized in 95% ethanol, the plates were scanned at 570 nm using a microplate reader (Varioscan Lux, Thermo Fisher Scientific, Waltham, MA, USA) in order to measure the optical density of the dye attached to stained biofilms.

5.2.9. Characterization of bactericidal mechanisms

The impact of the tested antimicrobial compounds was analyzed by the measurement of (i) oxidative stress, through ROS generation assessment (DCFH-DA; final concentration of 20 μM), (ii) integrity of the cell membranes, by means of outer membrane permeabilization (NPN; final concentration of 40 μM) and (iii) intracellular protein leakage using Bradford's reagent (final concentration of 20 %). All experiments were performed in 96-well microtiter plates (Sarstedt, Newton, NC, USA) with a final volume of 100 μL using bacterial suspensions (OD_{600} ~0.1) in PBS and exposed to ceragenins in free form and CSA-containing nanosystems in concentrations ranging from 5 to 50 μg/mL. The absorbance of the Bradford reagent was measured at 595 nm. The fluorescence measurement for the NPN assay was performed at an excitation/emission wavelength of 355/405 nm and at 488/535 nm for the generation of ROS using a Varioskan Lux microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

5.2.10. Evaluation of IL-8 release and cytotoxicity

The cytotoxic effect of the tested compounds against human keratinocytes was evaluated using MTT assay. For this purpose, HaCaT cells were seeded into each well of a 96-well flat-bottom microtiter plate to adhere overnight. The cells were treated with the tested compounds, and after 24 h incubation, the absorbance of the solubilized crystal of

formazan was measured at 570 nm. To determine the effect of ceragenins and CSA-based nanosystems on the release of interleukin-8 (IL-8) as a result of the pathogen-induced inflammatory responses, cultured HaCaT cells were exposed to *H. influenzae* infected DMEM (OD_{600} ~0.1) and supplemented with tested agents at concentrations 5, 20 and 50 µg/mL. Plates were incubated for 3 hours at 37 °C, 5 % CO2, then the concentration of IL-8 in harvested cell culture media was assessed using the Human ELISA kit..

5.2.11. Induction of resistance to colistin and CSA-13 in *E. hormaechei* 4236

In vitro induction of resistance was performed using a serial passage experiment based on previously described methods [40, 53]. Briefly, *E. hormaechei* 4236 strain (denoted hereinafter as Eh4236wt) was exposed to the sub-inhibitory concentrations (0.5x MIC) of colistin (denoted hereinafter as Eh4236_ColR) and CSA-13 (denoted hereinafter as Eh4236_CSA13R). During each subculture, the bacterial cells growing at the highest drug concentration were adjusted to OD_{600} 0.1 and used as inoculum for freshly prepared dilution series in 96-well plates subjected to the next passage at 18-24 h intervals. Throughout the entire experiment, the intermediate samples in the wells were preserved in 20% glycerol freeze stock solution at -80 °C. The experiment was performed in triplicates. After the completion of 40 passages, the MIC values of CSA-13 and colistin were evaluated for Eh4236_CSA13R and Eh4236_ColR isolates using the BMD method.

5.2.12. DNA and RNA isolation

To elucidate the genetic and transcriptomic differences between the Eh4236wt, Eh4236_ColR, and Eh4236_CSA13R isolates, DNA and RNA were extracted using Wizard HMW DNA Extraction Kit (Promega) and RNeasy Protect Bacteria Mini Kit (Qiagen) respectively, according to the manufacturer's recommendations from overnight cultures grown in cation-adjusted Mueller Hinton broth at 37°C.

5.2.13. Whole-genome and RNA sequencing

Sequencing of short DNA reads was performed on the Illumina NovaSeq 6000 platform using paired-end (2 x 150 read length) sequence mode at the sequencing facility. MinION sequencer (Oxford Nanopore, England) was used for sequencing long DNA reads on R9.4.1 FLO-MIN106 flowcell and Rapid Barcoding Sequencing kit (Oxford Nanopore, England) according to the manufacturer's recommended protocol. For RNA library

preparation, TruSeq adapter sequences were used with the NEBNext Ultra II Directional RNA Library Prep Kit for sequencing on Illumina NovaSeq 6000 platform using paired-end (2 x 150 read length) sequence mode.

5.2.14. Bioinformatic data analysis

Hybrid genome *de novo* assembly based on the long and short DNA reads was performed with Unicycler software v0.4.9 [54]. The annotated genome (chromosome and one plasmid) sequences and technical details, such as genome coverage and assembly parameters, are available in the GenBank database (Accession no. CP104401, CP104402, CP104403, CP104404, CP104405, CP104406). Comparative genomic analysis was performed with Geneious software.

The data generated from the samples consisted within the range of 12,847,600 – 19,958,100 sequenced RNA raw reads that had passed the quality check. Prior to subsequent analysis, the adapter sequences were trimmed using Trimmomatic tool v0.39, the reads were independently aligned to Eh4236wt strain as the reference genome using the Rsubread align (version 2.8.2), and the feature counts package was used for the read counts quantification in R (version 4.1.2). For the differential gene expression analysis, the raw read counts were used as an input to the DESeq2 package (version 1.34.0). Only differentially expressed genes (DEGs) with a $\log_2$ fold-change ($\log_2$FC) ≥ 1.5 and ≤ -1.5 and the FDR-adjusted (false discovery rate) p-value of <0.05 were selected for further analyses. Venn diagram analysis of DEGs from Eh4236_ColR and Eh4236_CSA13R isolates was conducted using the online web tool Venny 2.1 [55]. For Gene ontology (GO) and pathway enrichment analysis of the significant DEGs, ShinyGo v0.76 was used with the *E. cloacae* ATCC 13047 as the best matching species [56]. After removing the redundant genes, the most enriched GO-terms and KEGG pathways with the FDR corrected at $p < 0.05$ was considered significant.

5.2.15. Statistical analysis

All statistical analyses were conducted using OriginPro 9.65 software (OriginLab, MA, USA). The collected quantitative data were reported as the mean ± standard deviation (SD) of three replicates. The two-tailed Student's t-test with p-value < 0.05 was used to assess the statistical significance of the observed differences.

5.3. RESULTS

5.3.1. Physicochemical properties of CSA-based gold nanosystems

Extensive analysis characterizing the morphology, chemical structure, and crystallographic properties was performed. The resulting synthesized peanut-shaped AuNPs consisted an average size along the longitudinal axis of around 60 nm and along the transverse axis of ~30 nm. Structural characterization of the synthesized nanoparticles using X-ray diffraction showed that within the detection limit, all the peaks were attributed to the standard Bragg reflections (111), (200), (220), (311), and (222) of Au nanocrystals with the face-centered cubic lattice and the resulting cell parameter of the AuP NPs was 4.078 Å with uncertainties of ~0.001 Å. The evidence for efficient biofunctionalization and immobilization of ceragenins on the surface of AuP NPs, as well as good chemical stability, was determined using the FT-Raman spectra. The disappearance of a peak originating from the thiol group (2743 cm^{-1}) demonstrated successful attachment of the MHDA linker to the gold nanopeanuts. Furthermore, other peaks at 278 and 1680 cm-1 corresponding to Au-S stretching and C=O vibrations were visible. Additionally, the UV-Vis absorption spectra indicated a shift to higher wavelengths of the respective AuP NPs peaks by about 30 nm owing to the increasing nanoparticle particle size and efficient outcome of anchoring ceragenin molecules on the AuP NPs. Collectively, the calorimetric results also exhibited that all samples remained thermally stable up to 100 °C at a 5 °C min-1 heating rate. The DSC thermogram indicated no signs of any notable anomalies up to 150 °C.

5.3.2. Assessment of the antibacterial properties of ceragenins and CSA-based gold nanosystems against the otopathogens

All ceragenins functionalized by peanut-shaped gold nanoparticles demonstrated superior bactericidal properties than ceragenins in free form in the range of 0.5 to 5 μg/mL. Particularly, this effect was prominent for CSA-13 as its AuP NP conjugated counterpart since AuP@CSA-13 was highly effective in eradicating *H. influenzae* at nearly 50-fold lower doses, as evidenced by a decrease of effective doses from 16.11 ± 0.2 μg/mL to 0.313 ± 0.01 μg/mL. Among the different ceragenins, CSA-131 exhibited superior killing activity since they reduced the colony-forming ability by 2 log CFU/mL at doses of 1.60 ± 0.01, 0.62 ± 0.01, and 3.51 ± 0.15 for *H. influenzae, M. catarrhalis, S. pneumoniae* respectively.

In agreement with these results, the MIC and MBC values, measured in the presence of a highly nutritious medium, showed that AuP NPs augmented the antimicrobial properties of ceragenins. In addition, ceragenins and CSA-containing nanosystems showed up to 90 % biofilm mass reduction within 5 – 20 μg/mL concentrations. Furthermore, at a bactericidal dose of 10 μg/mL, i.e., safe for mammalian cells, the formed biofilm mass in the presence of ceragenins and CSA-based nanosystems ranged from 21.09 ± 2.14 – 87.07 ± 22.82 and 19.45 ± 5.18 – 36.17 ± 7.44 % respectively in comparison to the untreated biofilms.

5.3.3. Analysis of bacterial response to the tested agents at a molecular level

The fluorometric and colorimetric assessments of the tested agents showed that all CSA-based nanoformulations exhibited strong membrane permeabilizing properties resulting in the leakage of intracellular proteins from the treated bacterial strains compared to the CSAs alone, in particular at concentrations > 20 μg/mL. Finally, CSA-13 and CSA-44 containing AuP NPs triggered in *H. influenzae* an excessive release of reactive oxygen species (ROS).

5.3.4. Biocompatibility and anti-inflammatory properties of the tested agents

The developed nanosystems showed minimal toxicity on human keratinocytes, i.e., not more than 30 % of the cells metabolic activity was inhibited at bactericidal doses, except for CSA-13 containing AuNPs, which were more toxic than other tested agents. Furthermore, the results demonstrated anti-inflammatory properties of the CSA-based nanosystems, that reduced the release of proinflamotory cytokine IL-8 from 1482 to 477.8 – 657.8 pg/mL.

5.3.5. *In vitro* induction of resistance to colistin and CSA-13

MIC for colistin in Eh4236_ColR isolate increased considerably after 15 passages, and ultimately it raised from 0.125 μg/mL to 128 μg/mL. In contrast, a modest fluctuation in MIC was noted over the first 15 days of Eh4236_CSA13R exposure to CSA-13, and after 40 passages, the MIC varied from 32 to 64 μg/mL, as opposed to the initial MIC of 4 μg/mL (Figure 1). Notably, there was no cross-resistance between colistin and CSA-13 in Eh4236_ColR isolate, whereas Eh4236_CSA13R also developed resistance to colistin (MIC 32 μg/mL).

5.3.6. Whole-genome sequence analysis

The genome of the examined *E. hormaechei* 4236 strain consists of a chromosome (4675 CDSs) and one plasmid (129 CDSs). However, practically all mutations affected the chromosome. Deletion of a 5-kb region chromosomal region involving important for colistin resistance *mgrB* gene along with the six adjacent genes was recorded in Eh4236_ColR isolate. On the contrary, point mutations disrupted the *tetR* and *ompC* genes, encoding TetR-family transcriptional regulator and outer membrane protein OmpC, respectively.

5.3.7. Transcriptome analysis

5.3.7.1. Functional classification of differentially expressed genes (DEGs)

Basic statistics regarding the number of DEGs, including the unique and shared by Eh4236_ColR and Eh4236_CSA13R ones, are shown in Figure 2 and 3. In addition, their functional classification based on gene ontology (GO) is compared in Figure 4. Briefly, genes involved in the 'Lipid A biosynthesis' and 'Lipopolysaccharide biosynthesis pathway', such as the *arnBCADTEF* operon and the *pagP* gene were highly overexpressed in both isolates. Likewise, the genes associated with the bacterial outer membrane biogenesis and cellular biosynthetic processes, such as the cationic antimicrobial peptide (CAMP) resistance KEGG pathway [enc01503]. On the other hand, the 'Flagellar assembly' KEGG pathway [enc02040], comprising *fli* operon with *fliG, fliM*, and *fliN* genes was up-regulated only in Eh4326_CSA13R isolate.

The details regarding the most up- and down-regulated genes are described in separate paragraphs. The potential link between the identified alternations at the genomic and transcriptomic levels and resistance to colistin and CSA-13 is summarized in Figure 5.

5.3.7.2. Expression of the membrane-associated genes

In both isolates the *arnBCADTEF* operon was substantially up-regulated, by a $\log_2$ fold change ($\log_2$FC) in the range of $> 4 – 6$ in Eh4236_ColR and $> 3 – 5$ $\log_2$FC in Eh4236_CSA13R, compared to the control strain (Eh4236wt). This operon covers seven genes involved in the biosynthesis of 4-amino-4-deoxy-l-arabinose (L-Ara4N), its transport through the outer membrane, and the attachment to the lipid A phosphate [57-59].

Ultimately, this process reduces electrostatic interactions between colistin, other CAMPs, and LPS, as well as suppresses the LPS-induced host immune responses via the TLR4 pathway [60, 61]. Although an increased amount of L-Ara4N after exposure to CSAs was reported in other bacteria, such as *P. aeruginosa* and *K. pneumoniae* [40], it appears that this modification may not be the sole mechanism of CSA-13 resistance.

Similarly, both isolates up-regulated the *pagP* gene, > 2 – 5 $\log_2$FC, encoding the LPS modifying enzyme – palmitoyl transferase. This enzyme catalyzes the transfer of a palmitate chain from donor phospholipids to the lipid A component imposing a redistribution of the outer membrane lipid at the cell surface [60]. Furthermore, also overexpression of *ybjG* and *pap2* genes responsible for the synthesis of undecaprenyl phosphate (Und-P) was shared by Eh4236_ColR and Eh4236_CSA13R isolates. Undecaprenyl phosphate is an essential substrate for the biogenesis of the cell surface polymers such as peptidoglycan and LPS [60]. Additionally, YbjG participates in adaptive stress responses and contributes to resistance to bacitracin, a cyclic polypeptide synthesized from *Bacillus licheniformis* [62].

5.3.7.3. Induction of efflux pump systems and transporters

As the drug extrusion process is a key mechanism of resistance to several antibiotics, gram-negative bacteria are equipped with multiple efflux pump families, such as (i) major facilitator superfamily (MFS), (ii) ATP-binding cassette (ABC) superfamily, (iii) resistance-nodulation-division (RND) superfamily, (iv) multidrug and toxic compound extrusion (MATE), (v) small multidrug resistance (SMR) family, and (vi) drug metabolite transporter (DMT) superfamily [63]. Accordingly, up-regulation (3 – 6 $\log_2$FC expression) of various efflux pumps or their components, such as MFS and RND transporter subunits, and MacAB family efflux pump, was observed in Eh4236_ColR isolate. Although the substrates for many of these transporters remain to be identified, the MacAB is associated with transporting structurally dissimilar substrates such as antibiotics, cationic biocides, sugars, and amino acids across the outer membrane. Therefore, it plays a critical role in the development of multidrug resistance to aminoglycosides, colistin, bacitracin, and macrolides [64, 65]. Several of these transporters were also up-regulated in Eh4236_CSA13R isolate, however, their number and expression levels were lower than in colistin-resistant isolate.

On the other hand, colistin and CSA-13 induced expression of DedA family protein/transporter [66]. Indeed, these proteins have been recently linked with resistance to colistin in other gram-negative rods, such as *E. coli, K. pneumoniae*, and *Burkholderia thailandensis* [66-68]. For instance, their regulatory role on the *arnBCADTEF* operon-mediated colistin resistance has been suggested since their deletion in *E. coli* reduced LPS modification by L-Ara4N, and, in turn, increased susceptibility to colistin [66-69]. Interestingly, another *dedA* gene was up-regulated in colistin-resistant *E. hormaechei* isolate.

5.3.7.4. Outer membrane proteins and virulence factors

While the emergence of bacterial resistance depends on their successful adaptation to various external stress environments, the outer membrane proteins also contribute to the modulation of membrane integrity [70]. These outer membrane channels are a selective barrier acting as a point of entry for small molecules and hydrophilic drugs while blocking larger hydrophobic drugs. The expression of *ompX* increased by nearly 2.5 $\log_2$FC in both isolates. The *ompX* gene encodes a small outer membrane protein, and its overexpression may modulate the outer membrane permeability by inhibiting the production of OmpC and OmpC porins, leading to a multidrug resistant phenotype [71, 72]. Interestingly, the *ompC* gene was disrupted by a premature stop codon in Eh4236_CSA13R isolate, which suggests its possible role in the development of resistance to CSA-13 [71, 73]. Another interesting but unexpected observation is the enhanced expression of a periplasmic protein VirK, typically identified as a virulence factor in such pathogens as *Shigella* spp., *Salmonella* spp., and *Campylobacter jejuni*, and only sporadically connected with resistance to CAMPs [60, 61].

5.4. DISCUSSION

Exploring antimicrobial peptides (AMPs) as alternatives to traditional antibiotics is one of the most promising strategies to combat MDR infections. In addition to inhibiting essential cellular processes by direct membrane-binding activity and translocation into the cytoplasm, this naturally occurring diverse collection of peptides has various immunomodulatory features. However, their clinical usage is hindered by susceptibility to endogenous proteolytic degradation, low tolerance to physiological salt concentrations and pH, resulting in a short half-life and poor bioavailability, as well as by their expensive production process. To overcome these drawbacks, ceragenins were developed as synthetic mimics of AMPs, with potent bactericidal and anti-inflammatory properties. Moreover, the antibacterial activity and biocompatibility of ceragenins may be improved by their conjugation with nanomaterials, e.g., gold nanoparticles (AuNPs). Consequently, this research aimed to examine the antibacterial efficiency of ceragenins alone and in combination with peanut-shaped gold nanoparticles (AuP NP@CSA) against three leading bacterial pathogens, i.e., *S. pneumoniae, H. influenzae* and *M. catarrhalis*, responsible for otitis media. Indeed, enhanced antibacterial efficiency of the ceragenin-based nanosystems on both planktonic and biofilm forms of the tested otopathogens was reported. For instance, eradication of *H. influenzae* was obtained with nearly 50-fold lower doses of AuP NP@CSA-131. Furthermore, the CSA-based nanosystems displayed enhanced killing mechanisms involving the generation of reactive oxygen species (ROS), membrane permeabilization, and protein leakage. In addition, suppression of the proinflammatory cytokine IL-8 and improved biocompatibility with the host cells were observed. Therefore, CSAs and their nanosystems may be promising candidates for developing a unique class of locally administered antibacterial medicines to treat ear infections.

In light of the AMR crisis, however, the next generation of antimicrobials must possess not only potent activity but also a low risk of inducing resistance. Therefore, identifying potential molecular mechanisms underlying AMR should be an integral part of the research and development of novel antimicrobial agents. Until recently, their deciphering has mainly been inadequate and challenging due to the complexity of AMR nature, frequently mediated by multiple alterations in the bacterial cell's structural, signaling, and regulatory components. However, implementing high-throughput multi-

omics methods, such as whole-genome sequencing (WGS) and RNA-seq, has revolutionized these studies, allowing for a comprehensive analysis of qualitative (SNPs, DNA deletions/inversions events, etc.) and quantitative, i.e., in gene expression, changes occurring in bacterial cells exposed to antimicrobial agents [74].

In line with this, I have investigated the consequences of prolonged exposition of *E. hormaechei* ST89 to ceragenin CSA-13 and colistin at the genomic and transcriptomic levels. The selection of this gram-negative rod, a member of Enterobacterales, was dictated by its growing importance as a hospital pathogen, especially in Poland, where it is the primary carbapenem-resistant *Enterobacter* species [21, 75, 76]. Remarkably, the mechanisms responsible for colistin resistance, namely (i) incorporation of L-Ara4N to LPS mediated by the *arnABCDEF* operon and regulation of this process by MgrB, (ii) LPS palmitoylation by PagP, and (iii) deacetylation of poly-β-1,6-N-acetylglucosamine by the genes encoded in the *pgaABC* operon [77, 78], are not crucial for CSA-13 resistance in *E. hormaechei.* Since all these processes change the net charge of the outer membrane and, in turn, impede electrostatic interactions with colistin, the antibacterial activity of CSA-13 is possibly additionally mediated via an alternative, i.e., independent of the cationic properties, mechanisms of action. Moreover, the disruption of the genes encoding outer membrane protein OmpC and transcriptional regulator TetR in CSA-13 resistant isolate suggests that the cell envelope permeability and specific regulatory processes may be implicated with resistance to this ceragenin. Furthermore, a number of bacterial transporters and efflux pumps, including recently recognized members of the DedA family proteins, appear to be important for the development of resistance to both tested agents. Therefore, processes like proton motive force (PMF) and membrane potential, along with cytoplasmic pH homeostasis, are likely connected with resistance to colistin and CSA-13 in *E. hormaechei* (Figure 5). Collectively, the obtained results showed that prolonged exposition to both tested agents, in particular ceragenin CSA-13, results in continuous genomic and transcriptomic changes, affecting virtually all elements of the cell machinery, including ribosomal proteins, various stress proteins as well as type VI secretion (T6SS), and fimbriae or flagella subunits. A detailed analysis of the observed changes and their potential impact on resistance to both tested agents was included in a paper that has already been submitted for publication in the Journal of Antimicrobial Chemotherapy.

5.5. CONCLUSIONS

1. Nonspherical gold nanoparticles (AuNPs) as drug carriers (AuNP@CSA) enhance the antibacterial action of ceragenins (CSAs) against planktonic and biofilm forms of *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis,* either under *in vitro,* i.e., in bacteriological growth media, as well as *ex vivo* conditions mimicking pathological lesions in the external ear canal, i.e., in the presence of human cerumen.

2. Ceragenin-containing nanosystems exert satisfactory biocompatibility in bactericidal concentrations and limit the bacterial-induced inflammation augmenting the beneficial effects of tested agents in the treatment of otitis media infections.

3. Despite the common cationic nature of colistin and ceragenin CSA-13, the prolonged exposure of *Enterobacter hormaechei* to CSA-13 induced only moderate level resistance to this ceragenin (increase in MIC value from 4 to 32-64 μg/mL) in comparison to colistin (increase in MIC value from 0.125 to 128 μg/mL).

4. Molecular mechanisms responsible for the high level of resistance to colistin, including well-known colistin-resistant factors, developed by *E. hormaechei* during prolonged exposure to this antibiotic, have no impact on its susceptibility to ceragenin CSA-13.

5. Application of multi-omics methods, whole-genome (WGS) and transcriptome sequencing (RNA-seq), allowed to identify of several novel mechanisms that directly or indirectly may contribute to the development of resistance to colistin and/or ceragenin CSA-13 in *E. hormaechei.*

5.6. LIST OF FIGURES

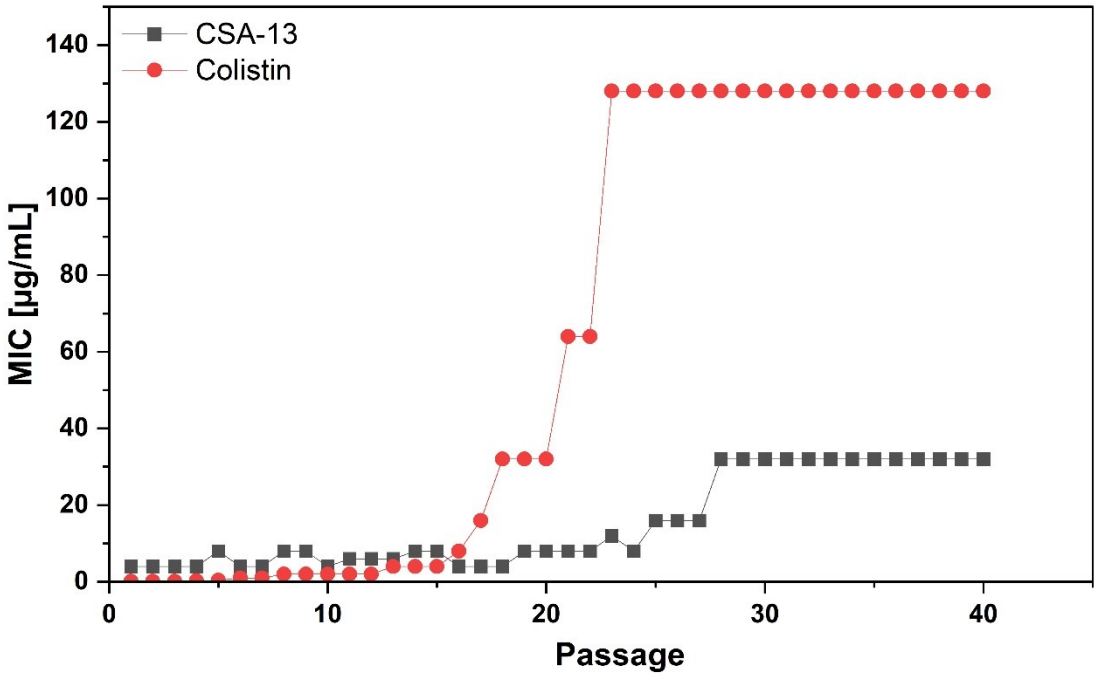

Figure 1. Results of *in vitro* resistance induction to colistin and CSA-13 in serial passages experiment in *E. hormaechei* 4236 strain.

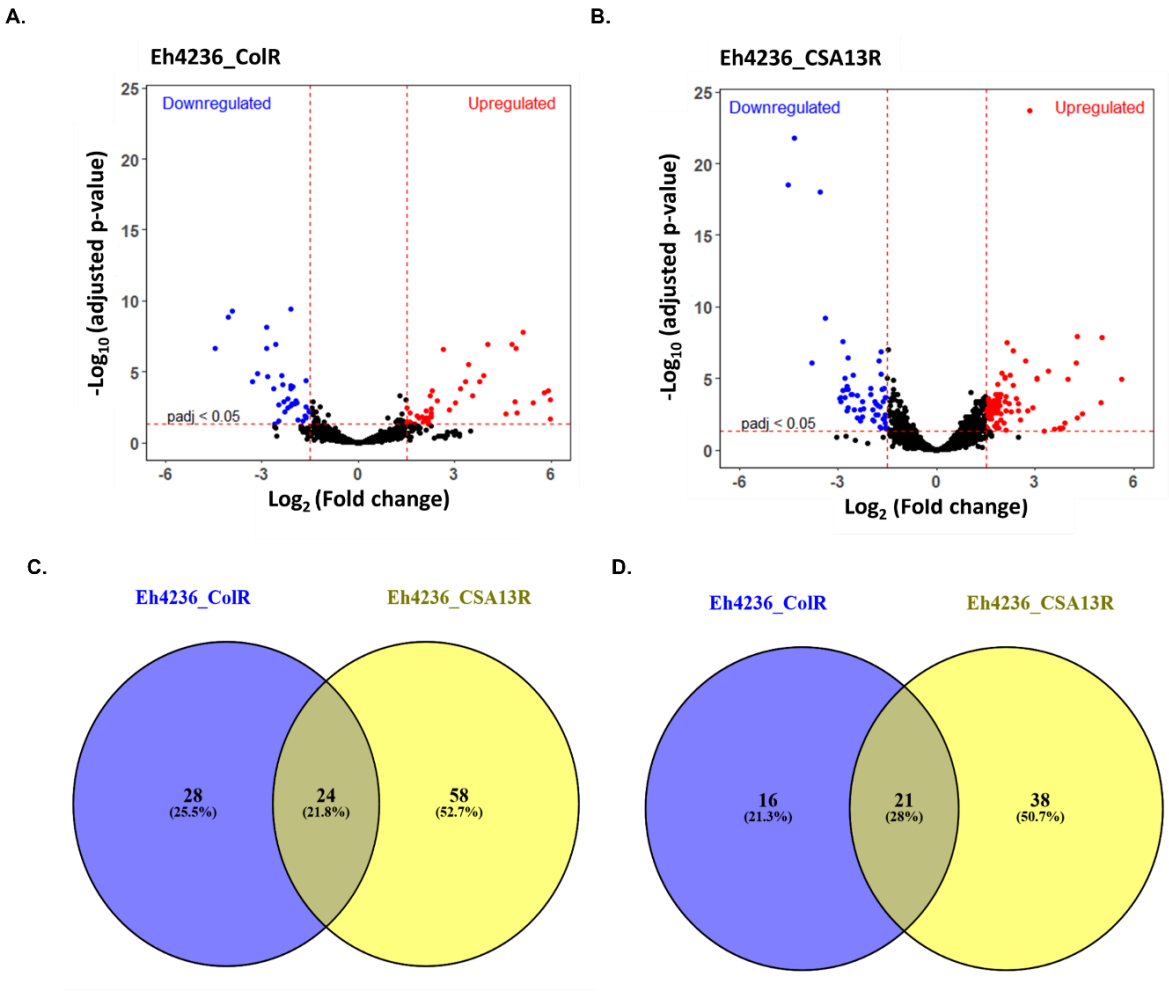

Figure 2. Volcano plots of differentially expressed genes (DEGs) in Eh4326_ColR (panel A) and Eh4326_CSA13R (panel B). Venn diagrams illustrate the number of unique and shared by both isolates dysregulated genes; up- and down-regulated genes are shown in panel C and D, respectively.

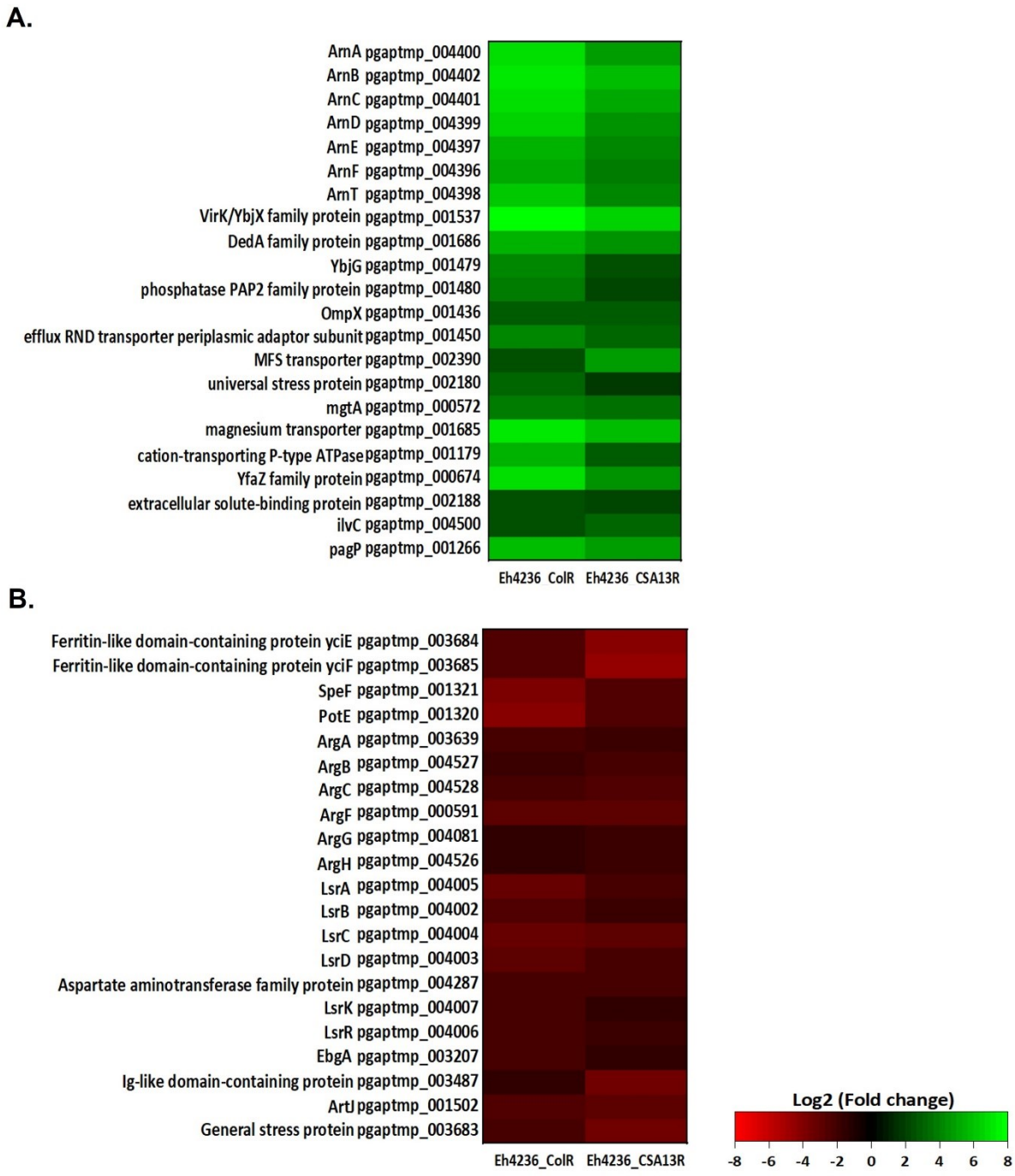

Figure 3. Heatmap illustrating the log_2-fold change expression levels of the most prevalent up- (panel A) and down-regulated genes (panel B) shared by Eh4326_ColR and Eh4326_CSA13R isolates.

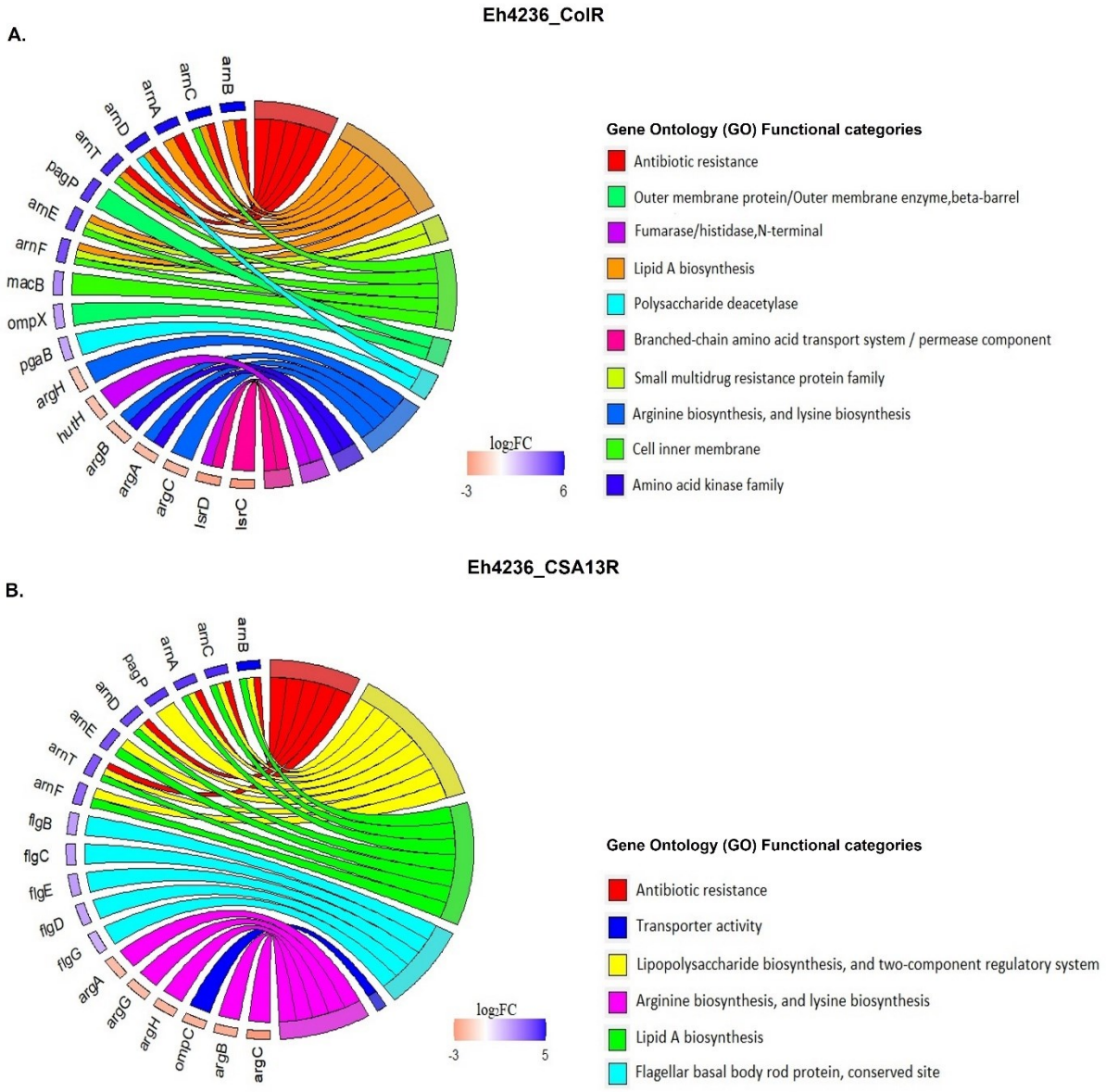

Figure 4. Comparison of the most up- and down-regulated genes and their functional GO categories in Eh4326_ColR (panel A) and Eh4326_CSA13R (panel B) isolates.

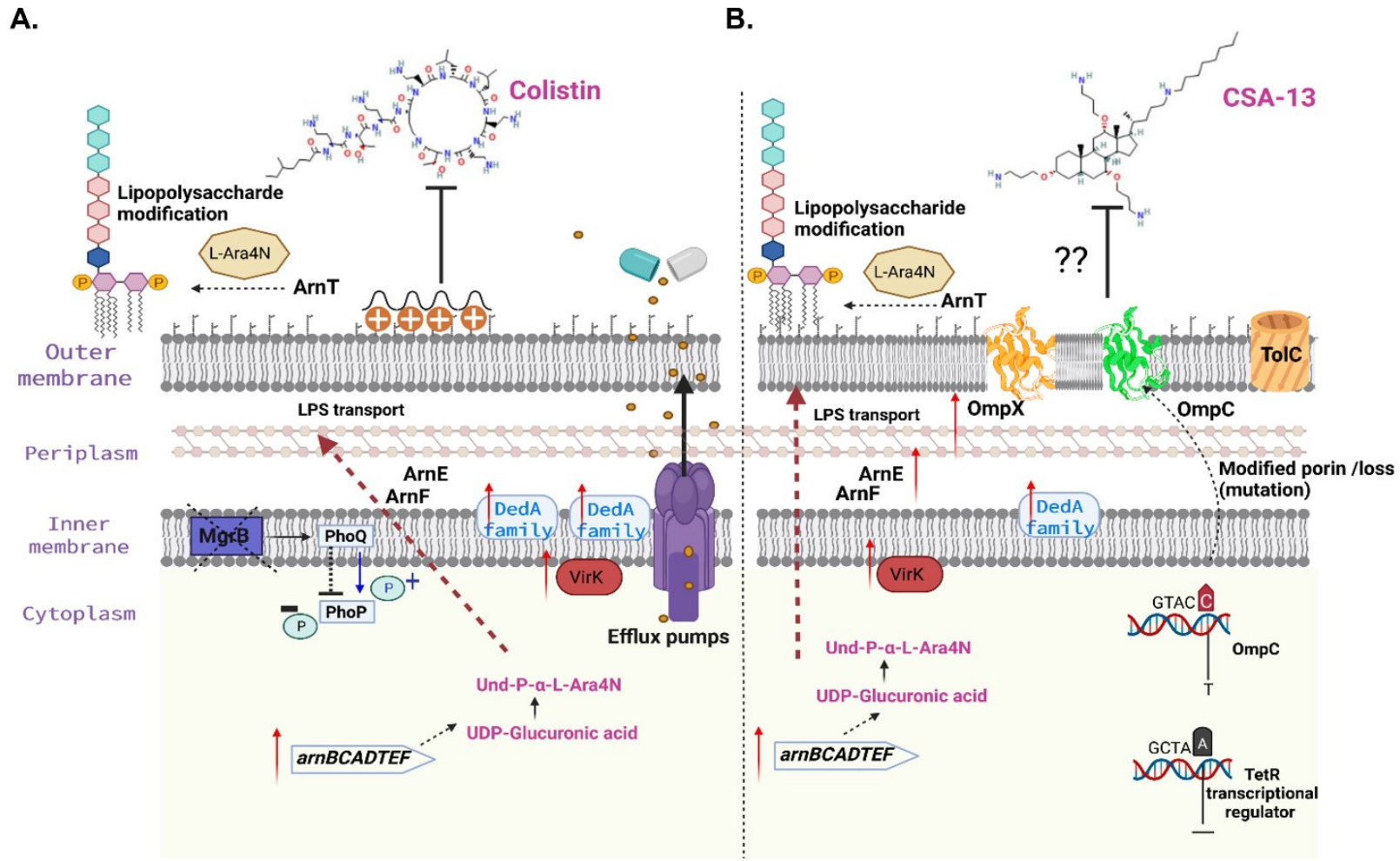

Figure 5. Overview of the alterations at the genomic and transcriptomic levels with confirmed or putative contribution to resistance to colistin and/or other CAMPs, identified in Eh4326_ColR (panel A) and Eh4326_CSA13R (panel B) isolates.

5.7. BIBLIOGRAPHY

1. de Kraker, M.E., A.J. Stewardson, and S. Harbarth, *Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050?* PLoS Med, 2016. **13**(11): p. e1002184.
2. Poole, K., *Bacterial stress responses as determinants of antimicrobial resistance.* J Antimicrob Chemother, 2012. **67**(9): p. 2069-89.
3. Reygaert, W.C., *An overview of the antimicrobial resistance mechanisms of bacteria.* AIMS Microbiol, 2018. **4**(3): p. 482-501.
4. Oz, T., et al., *Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution.* Mol Biol Evol, 2014. **31**(9): p. 2387-401.
5. Magiorakos, A.P., et al., *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.* Clin Microbiol Infect, 2012. **18**(3): p. 268-81.
6. Izdebski, R., et al., *VIM/IMP carbapenemase-producing Enterobacteriaceae in Poland: epidemic Enterobacter hormaechei and Klebsiella oxytoca lineages.* The Journal of antimicrobial chemotherapy, 2018. **73**(10): p. 2675-2681.
7. Massa, H.M., A.W. Cripps, and D. Lehmann, *Otitis media: viruses, bacteria, biofilms and vaccines.* Med J Aust, 2009. **191**(S9): p. S44-9.
8. Kaur, R., J. Casey, and M. Pichichero, *Cytokine, chemokine, and Toll-like receptor expression in middle ear fluids of children with acute otitis media.* Laryngoscope, 2015. **125**(1): p. E39-44.
9. Whitney, C.G., et al., *Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine.* N Engl J Med, 2003. **348**(18): p. 1737-46.
10. Murdoch, D.R., *Assessing the Impact of Pneumococcal Conjugate Vaccines.* Clin Infect Dis, 2020. **70**(8): p. 1589-1590.
11. Korona-Glowniak, I., et al., *Resistant Streptococcus pneumoniae strains in children with acute otitis media- high risk of persistent colonization after treatment.* BMC Infect Dis, 2018. **18**(1): p. 478.
12. Mather, M.W., et al., *A systematic review and meta-analysis of antimicrobial resistance in paediatric acute otitis media.* Int J Pediatr Otorhinolaryngol, 2019. **123**: p. 102-109.
13. Schaar, V., et al., *Moraxella catarrhalis outer membrane vesicles carry β-lactamase and promote survival of Streptococcus pneumoniae and Haemophilus influenzae by inactivating amoxicillin.* Antimicrob Agents Chemother, 2011. **55**(8): p. 3845-53.
14. Donlan, R.M., *Biofilms: microbial life on surfaces.* Emerg Infect Dis, 2002. **8**(9): p. 881-90.
15. Perez, A.C., et al., *Residence of Streptococcus pneumoniae and Moraxella catarrhalis within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence in vivo.* Pathog Dis, 2014. **70**(3): p. 280-8.
16. Diene, S.M. and J.M. Rolain, *Carbapenemase genes and genetic platforms in Gram-negative bacilli: Enterobacteriaceae, Pseudomonas and Acinetobacter species.* Clin Microbiol Infect, 2014. **20**(9): p. 831-8.
17. Paauw, A., et al., *Genomic diversity within the Enterobacter cloacae complex.* PLoS One, 2008. **3**(8): p. e3018.
18. Hoffmann, H. and A. Roggenkamp, *Population genetics of the nomenspecies Enterobacter cloacae.* Appl Environ Microbiol, 2003. **69**(9): p. 5306-18.

19. Davin-Regli, A., J.-P. Lavigne, and J.-M. Pagès, *Enterobacter spp.: Update on Taxonomy, Clinical Aspects, and Emerging Antimicrobial Resistance.* Clinical Microbiology Reviews, 2019. **32**(4): p. e00002-19.
20. Ohad, S., et al., *Rapid identification of Enterobacter hormaechei and Enterobacter cloacae genetic cluster III.* J Appl Microbiol, 2014. **116**(5): p. 1315-21.
21. Wendel, A.F., et al., *Long-Term, Low-Frequency Cluster of a German-Imipenemase-1-Producing Enterobacter hormaechei ssp. steigerwaltii ST89 in a Tertiary Care Hospital in Germany.* Microb Drug Resist, 2018. **24**(9): p. 1305-1315.
22. Roberts, L.W., et al., *Integrating multiple genomic technologies to investigate an outbreak of carbapenemase-producing Enterobacter hormaechei.* Nat Commun, 2020. **11**(1): p. 466.
23. Raun-Petersen, C., et al., *Investigation of an Enterobacter hormaechei OXA-436 carbapenemase outbreak when everything goes down the drain.* Infect Prev Pract, 2022. **4**(3): p. 100228.
24. Annavajhala, M.K., A. Gomez-Simmonds, and A.C. Uhlemann, *Multidrug-Resistant Enterobacter cloacae Complex Emerging as a Global, Diversifying Threat.* Front Microbiol, 2019. **10**: p. 44.
25. Iovleva, A. and Y. Doi, *Carbapenem-Resistant Enterobacteriaceae.* Clin Lab Med, 2017. **37**(2): p. 303-315.
26. Dong, N., et al., *Bioactivity and Bactericidal Mechanism of Histidine-Rich β-Hairpin Peptide Against Gram-Negative Bacteria.* Int J Mol Sci, 2019. **20**(16).
27. Prasad, S.V., et al., *Expression and Function of Host Defense Peptides at Inflammation Sites.* Int J Mol Sci, 2019. **21**(1).
28. Benfield, A.H. and S.T. Henriques, *Mode-of-Action of Antimicrobial Peptides: Membrane Disruption vs. Intracellular Mechanisms.* Front Med Technol, 2020. **2**: p. 610997.
29. Tokajuk, J., et al., *Cathelicidin LL-37 in Health and Diseases of the Oral Cavity.* 2022. **10**(5): p. 1086.
30. De Smet, K. and R. Contreras, *Human antimicrobial peptides: defensins, cathelicidins and histatins.* Biotechnol Lett, 2005. **27**(18): p. 1337-47.
31. Overhage, J., et al., *Human host defense peptide LL-37 prevents bacterial biofilm formation.* Infect Immun, 2008. **76**(9): p. 4176-82.
32. Yasir, M., M.D.P. Willcox, and D. Dutta, *Action of Antimicrobial Peptides against Bacterial Biofilms.* Materials (Basel), 2018. **11**(12).
33. Lai, X.-Z., et al., *Ceragenins: Cholic Acid-Based Mimics of Antimicrobial Peptides.* Accounts of Chemical Research, 2008. **41**(10): p. 1233-1240.
34. Savage, P.B.J.E.J.o.O.C., *Design, synthesis and characterization of cationic peptide and steroid antibiotics.* 2002. **2002**(5): p. 759-768.
35. Surel, U., et al., *Ceragenins–a new weapon to fight multidrug resistant bacterial infections.* 2014. **30**(3): p. 207-213.
36. Hashemi, M.M., B.S. Holden, and P.B.J.F.A.R. Savage, *Ceragenins as non-peptide mimics of endogenous antimicrobial peptides.* 2018. **1**: p. 139-169.
37. Bucki, R., et al., *Salivary mucins inhibit antibacterial activity of the cathelicidin-derived LL-37 peptide but not the cationic steroid CSA-13.* J Antimicrob Chemother, 2008. **62**(2): p. 329-35.

38. Bucki, R., et al., *Resistance of the antibacterial agent ceragenin CSA-13 to inactivation by DNA or F-actin and its activity in cystic fibrosis sputum.* J Antimicrob Chemother, 2007. **60**(3): p. 535-45.
39. Lai, X.Z., et al., *Ceragenins: cholic acid-based mimics of antimicrobial peptides.* Acc Chem Res, 2008. **41**(10): p. 1233-40.
40. Hashemi, M.M., et al., *Susceptibility of Colistin-Resistant, Gram-Negative Bacteria to Antimicrobial Peptides and Ceragenins.* Antimicrob Agents Chemother, 2017. **61**(8).
41. Wnorowska, U., et al., *Nanoantibiotics containing membrane-active human cathelicidin LL-37 or synthetic ceragenins attached to the surface of magnetic nanoparticles as novel and innovative therapeutic tools: current status and potential future applications.* Journal of Nanobiotechnology, 2020. **18**(1): p. 3.
42. Wnorowska, U., et al., *Use of ceragenins as a potential treatment for urinary tract infections.* BMC Infect Dis, 2019. **19**(1): p. 369.
43. Chmielewska, S.J., et al., *NDM-1 Carbapenemase-Producing Enterobacteriaceae are Highly Susceptible to Ceragenins CSA-13, CSA-44, and CSA-131.* Infect Drug Resist, 2020. **13**: p. 3277-3294.
44. Hashemi, M.M., et al., *Preclinical testing of a broad-spectrum antimicrobial endotracheal tube coated with an innate immune synthetic mimic.* J Antimicrob Chemother, 2018. **73**(1): p. 143-150.
45. Piktel, E., et al., *N-Acetyl-Cysteine Increases Activity of Peanut-Shaped Gold Nanoparticles Against Biofilms Formed by Clinical Strains of Pseudomonas aeruginosa Isolated from Sputum of Cystic Fibrosis Patients.* Infect Drug Resist, 2022. **15**: p. 851-871.
46. Wang, L., C. Hu, and L. Shao, *The antimicrobial activity of nanoparticles: present situation and prospects for the future.* Int J Nanomedicine, 2017. **12**: p. 1227-1249.
47. Chmielewska, S.J., et al., *Bactericidal Properties of Rod-, Peanut-, and Star-Shaped Gold Nanoparticles Coated with Ceragenin CSA-131 against Multidrug-Resistant Bacterial Strains.* Pharmaceutics, 2021. **13**(3).
48. Piktel, E., et al., *Varied-shaped gold nanoparticles with nanogram killing efficiency as potential antimicrobial surface coatings for the medical devices.* Sci Rep, 2021. **11**(1): p. 12546.
49. Niemirowicz, K., et al., *Core–shell magnetic nanoparticles display synergistic antibacterial effects against Pseudomonas aeruginosa and Staphylococcus aureus when combined with cathelicidin LL-37 or selected ceragenins.* Int J Nanomedicine, 2016. **11**: p. 5443-5455.
50. Niemirowicz, K., et al., *Bactericidal activity and biocompatibility of ceragenin-coated magnetic nanoparticles.* Journal of Nanobiotechnology, 2015. **13**(1): p. 32.
51. Wnorowska, U., et al., *Nanoantibiotics containing membrane-active human cathelicidin LL-37 or synthetic ceragenins attached to the surface of magnetic nanoparticles as novel and innovative therapeutic tools: current status and potential future applications.* J Nanobiotechnology, 2020. **18**(1): p. 3.
52. Sinirtaş, M., H. Akalin, and S. Gedikoğlu, *Investigation of colistin sensitivity via three different methods in Acinetobacter baumannii isolates with multiple antibiotic resistance.* Int J Infect Dis, 2009. **13**(5): p. e217-20.
53. Pollard, J.E., et al., *In vitro evaluation of the potential for resistance development to ceragenin CSA-13.* J Antimicrob Chemother, 2012. **67**(11): p. 2665-72.

54. Wick, R.R., et al., *Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads.* PLoS Comput Biol, 2017. **13**(6): p. e1005595.
55. JC, O. *Venny. An interactive tool for comparing lists with Venn's diagrams.* (2007–2015); Available from: https://bioinfogp.cnb.csic.es/tools/venny/index.html.
56. Ge, S.X., D. Jung, and R. Yao, *ShinyGO: a graphical gene-set enrichment tool for animals and plants.* Bioinformatics, 2020. **36**(8): p. 2628-2629.
57. Fernández, L., et al., *The two-component system CprRS senses cationic peptides and triggers adaptive resistance in Pseudomonas aeruginosa independently of ParRS.* Antimicrob Agents Chemother, 2012. **56**(12): p. 6212-22.
58. Cheung, C.H.P., et al., *Proteomic Investigation of the Signal Transduction Pathways Controlling Colistin Resistance in Klebsiella pneumoniae.* Antimicrob Agents Chemother, 2020. **64**(8).
59. Cain, A.K., et al., *Morphological, genomic and transcriptomic responses of Klebsiella pneumoniae to the last-line antibiotic colistin.* Sci Rep, 2018. **8**(1): p. 9868.
60. Gogry, F.A., et al., *Current Update on Intrinsic and Acquired Colistin Resistance Mechanisms in Bacteria.* 2021. **8**.
61. Miller, S.I., R.K. Ernst, and M.W. Bader, *LPS, TLR4 and infectious disease diversity.* Nat Rev Microbiol, 2005. **3**(1): p. 36-46.
62. El Ghachi, M., et al., *Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in Escherichia coli.* J Biol Chem, 2005. **280**(19): p. 18689-95.
63. Amaral, L., et al., *Efflux pumps of Gram-negative bacteria: what they do, how they do it, with what and how to deal with them.* 2014. **4**.
64. Shirshikova, T.V., et al., *The ABC-Type Efflux Pump MacAB Is Involved in Protection of Serratia marcescens against Aminoglycoside Antibiotics, Polymyxins, and Oxidative Stress.* mSphere, 2021. **6**(2).
65. Greene, N.P., et al., *Antibiotic Resistance Mediated by the MacB ABC Transporter Family: A Structural and Functional Perspective.* Front Microbiol, 2018. **9**: p. 950.
66. Kumar, S. and W.T. Doerrler, *Members of the conserved DedA family are likely membrane transporters and are required for drug resistance in Escherichia coli.* Antimicrob Agents Chemother, 2014. **58**(2): p. 923-30.
67. Panta, P.R. and W.T. Doerrler, *A Burkholderia thailandensis DedA Family Membrane Protein Is Required for Proton Motive Force Dependent Lipid A Modification.* Front Microbiol, 2020. **11**: p. 618389.
68. Tiwari, V., et al., *A Klebsiella pneumoniae DedA family membrane protein is required for colistin resistance and for virulence in wax moth larvae.* Sci Rep, 2021. **11**(1): p. 24365.
69. Huang, L., Y. Feng, and Z. Zong, *Heterogeneous resistance to colistin in Enterobacter cloacae complex due to a new small transmembrane protein.* J Antimicrob Chemother, 2019. **74**(9): p. 2551-2558.
70. Egan, A.J.F., *Bacterial outer membrane constriction.* 2018. **107**(6): p. 676-687.
71. Dupont, M., et al., *An early response to environmental stress involves regulation of OmpX and OmpF, two enterobacterial outer membrane pore-forming proteins.* Antimicrob Agents Chemother, 2007. **51**(9): p. 3190-8.
72. Davin-Regli, A. and J.M. Pagès, *Enterobacter aerogenes and Enterobacter cloacae; versatile bacterial pathogens confronting antibiotic treatment.* Front Microbiol, 2015. **6**: p. 392.

73. Cardoso, M.H., et al., *Comparative NanoUPLC-MS E analysis between magainin I-susceptible and -resistant Escherichia coli strains.* Sci Rep, 2017. **7**(1): p. 4197.
74. Waddington, C., et al., *Exploiting genomics to mitigate the public health impact of antimicrobial resistance.* Genome Med, 2022. **14**(1): p. 15.
75. Majewski, P., et al., *Altered Outer Membrane Transcriptome Balance with AmpC Overexpression in Carbapenem-Resistant.* Front Microbiol, 2016. **7**: p. 2054.
76. Majewski, P., et al., *Expression of AraC/XylS stress response regulators in two distinct carbapenem-resistant Enterobacter cloacae ST89 biotypes.* J Antimicrob Chemother, 2020. **75**(5): p. 1146-1150.
77. Wang, X., J.F. Preston, 3rd, and T. Romeo, *The pgaABCD locus of Escherichia coli promotes the synthesis of a polysaccharide adhesin required for biofilm formation.* J Bacteriol, 2004. **186**(9): p. 2724-34.
78. Cafiso, V., et al., *Colistin Resistant A. baumannii: Genomic and Transcriptomic Traits Acquired Under Colistin Therapy.* 2019. **9**.

6. DISSERTATION PUBLICATIONS

Review article

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Review

Expression and Function of Host Defense Peptides at Inflammation Sites

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Abstract: There is a growing interest in the complex role of host defense peptides (HDPs) in the pathophysiology of several immune-mediated inflammatory diseases. The physicochemical properties and selective interaction of HDPs with various receptors define their immunomodulatory effects. However, it is quite challenging to understand their function because some HDPs play opposing pro-inflammatory and anti-inflammatory roles, depending on their expression level within the site of inflammation. While it is known that HDPs maintain constitutive host protection against invading microorganisms, the inducible nature of HDPs in various cells and tissues is an important aspect of the molecular events of inflammation. This review outlines the biological functions and emerging roles of HDPs in different inflammatory conditions. We further discuss the current data on the clinical relevance of impaired HDPs expression in inflammation and selected diseases.

Keywords: host defense peptides; human antimicrobial peptides; defensins; cathelicidins; inflammation; anti-inflammatory; pro-inflammatory

1. Introduction

The human body is in a constant state of conflict with the unseen microbial world that threatens to disrupt the host cell function and colonize the body surfaces. The immune system has an arsenal of destructive mechanisms to neutralize the toxic effect of the microbial pathogens. It functions through two layers of defense systems: The innate system and the more intricate adaptive immune system, which closely communicate with each other [1]. Each of those systems form a complex network of immune cells, signaling molecules, and regulatory pathways. Inflammation is a reaction of the host immune system that acts to eliminate the source of inflammatory stimulus, ranging from pathogens to burn injuries [2]. Although microbial infections largely initiate the events of inflammation, we must note that inflammation is also a hallmark feature of various autoimmune, cancer, and systemic diseases [3]. Inflammation is a highly coordinated biochemical sequence of events that commences with the rapid migration of leukocytes to the site of infection, followed by adequate blood supply that transports different inflammatory mediators that control the course of the immune response [4]. However, while the initial events of inflammation are constructive and beneficial to the host, incompetent inflammatory resolution mechanisms, along with inefficient elimination of foreign bodies or pathogens and cellular debris, prompt the onset of chronic inflammation.

Antimicrobial peptides (AMPs) such as defensins and cathelicidins represent a vital part of the human immune system due to their broad spectrum activity against pathogenic bacteria, fungi, protists, and enveloped viruses [5]. Furthermore, in recent years, a growing number of studies have recognized these peptides as potent immune modulators, implicated in multiple pro- and anti-inflammatory responses through (1) neutralization of bacterial toxins, (2) chemoattraction and activation of immune cells, (3) initiation of adaptive immunity, (4) neovascularization and wound healing, as well as (5) anti- or

pro-tumor activity [6]. Actually, AMPs interact with innate and adaptive immune receptors, such as pattern recognition (PRRs) or chemokine receptors (CCRs), as well as inflammasomes and their complement systems, creating a link between innate and adaptive immunity [7–9]. In addition, AMPs may regulate fundamental cellular processes, such as differentiation, proliferation, and programmed cell death, e.g., by stimulating growth factor receptors or as complexes with the host nucleic acids [10,11], hence they resemble cytokines and growth factors. Overall, these activities, by controlling inflammation and/or accelerating repair process of the infected site, appear to support the direct microbicidal function of AMPs in resolving an infection. Therefore, the term host defense peptides (HDPs) was coined to encompass their pleiotropic nature, and association with both infectious as well as non-infectious inflammatory responses [12], although these two terms are used interchangeably. In the latter context, HDPs fit into the definition of "alarmins" or "danger signals", i.e., various endogenous molecules collectively known as DAMPs (damage associated molecular patterns), which are released from damaged or dying cells and initiate a diverse range of physiological and pathophysiological functions [13–15].

At present, HDPs are perceived as multifunctional agents that coordinate diverse immune surveillance functions necessary to maintain homeostasis (Figure 1) [16]. However, if their production is out of the physiological range, they may contribute to an undesirable inflammation in response to local (e.g., periodontal, respiratory, intestinal, and skin) and systemic (e.g., sepsis) infections. They might also function as pathophysiological events of inflammatory diseases, cancers, and even psychiatric disorders (Table 1) [17–23]. In addition, HDPs have been indicated as potential biomarkers in numerous infectious and non-infectious diseases [24].

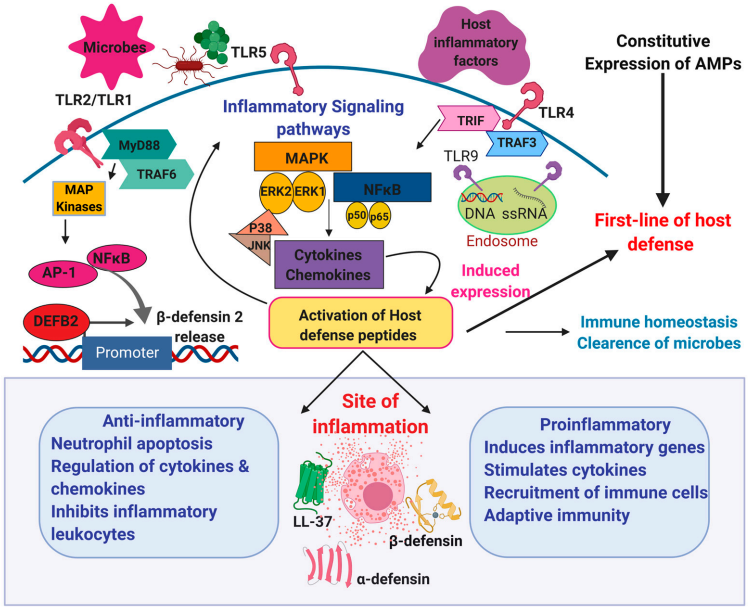

Figure 1. Illustration of the distinct role of host defense peptides at the sites of inflammation. Specific Toll-like receptors TLR-2, TLR-4, TLR-5, and TLR-6 are expressed on the plasma membrane of immune cells, non-immune cells, and intracellular compartments. TLR-7 and TLR-9 within endosomes participate in the host recognition of microbial cellular components and bind to host internal factors. The receptors, along with their adaptor proteins MyD88 and TRIF, can initiate the inflammatory signaling pathways. The host defense peptides promote innate immunity against various pathogens and maintain the immune system homeostasis. These peptides can also be induced in addition to their constitutive expression by the transcriptional modulatory factors NF-κB, AP-1, and intracellular release of cytokines and chemokines. They actively participate in coordinating the host immune signaling mechanisms during inflammation. Furthermore, HDPs can display both pro- and anti-inflammatory properties that may protect against the responses of inflammatory diseases. Abbreviations: AP-1, activator protein; NF-kB, nuclear factor kappa-light-chain enhancer of activated B cells; MAPK, mitogen-activated protein kinase; ERK1,2, extracellular signal-related kinases.

That being said, expanding our knowledge regarding the molecular mechanisms behind expression, processing, and mutual interactions of HDPs with other immune system components is crucial to better understand inflammation processes, and to develop new methods of anti-inflammatory treatment. Certainly, it is a challenging and long-term task, since a single antimicrobial peptide, e.g., human cathelicidin LL-37, may interact with dozens of proteins/receptors and subsequently engage hundreds of secondary effector proteins, as well as modify expression of >900 genes [6]. Considering this, the purpose of this review is to evaluate and summarize recent discoveries considering the functional expression and protective attributes of HDPs/AMPs in the acute inflammatory phase and the detrimental effects of their recruitment in chronic inflammation. Both in vitro and in vivo studies connecting the underlying mechanisms governing the immunoregulatory role of these peptides in the inflammatory microenvironment will be discussed.

2. Overview of Human Antimicrobial Peptides

Antimicrobial peptides are widely distributed in all living organisms, representing ancient and primary defense molecules, e.g., innate immune mechanisms conferred by the antimicrobial peptides in insects usually devoid of adaptive responses [25]. Discovery of defensins in rabbit leucocytes, lactoferrin in cow milk, and lysozyme in human saliva are among the first reports of animal-originated antimicrobial molecules, which paved the way for further identification and understanding of the physiological function of other antimicrobial peptides and proteins [26]. At present, 2272 peptides derived from animals, including ~130 of human origin (Figure 2), are recorded in the antimicrobial peptide database (http://aps.unmc.edu/AP), a comprehensive source of naturally existing families of antimicrobial peptides from all form of kingdoms of life [25].

Antimicrobial peptides and proteins contain a short chain of about 12–100 amino acids (Figure 2), and are classified according to their conformational structure (α, β, αβ, and non-αβ), amino acid motifs, and expression pattern [25,27]. For example, the major human AMPs, cathelicidin LL-37 and defensins, are characterized by α-helical and β-sheet structure, respectively. Furthermore, the latter are divided into α- and β-defensins based on the configuration of the disulfide bonds between six cysteine residues. AMPs are characterized by positive charge and substantial proportion (typically 50%) of hydrophobic residues, thus they are also known as cationic antimicrobial peptides (CAPs). However, at present, some negatively charged peptides are also classified as AMPs, e.g., human β-defensin DEFB118, psoriasin, or α-synuclein (Figure 2). Nevertheless, this amphiphilic–cationic organization allows them to selectively associate, and in turn disrupt, highly negatively charged microbial membranes. Hence, it explains their broad spectrum of activity, encompassing all cellular pathogens and enveloped viruses. Additionally, the cationic nature of AMPs may possibly facilitate, via electrostatic forces, their interactions with diverse host receptors, which are behind the immunomodulatory potential of these peptides [28].

Certain AMPs, e.g., cathelicidins, are produced as inactive pro-peptides and must be proteolytically processed for activity. It is noteworthy that this may generate multiple length variants characterized by diverse antimicrobial or immunomodulatory properties. Therefore, the presence of the appropriate proteases and their level is an important factor in regulating the function of the AMPs. Another important activity-related issue is that microbicidal action of AMPs is considerably suppressed by the physiological conditions present in some compartments of the body, including high salt, carbonate, lipoprotein, and polysaccharide concentrations [29–33].

Table 1. Host defense peptides—antimicrobial and immunomodulatory functions and disease association.

Class of Host Defense Peptides	Host Defense Peptides	Gene	Chromosome Location	Site of Expression	Biological Function	Dysregulated Expression of HDPs in Diseases	References
Cathelicidins	LL-37	*CAMP*	3p21.31	Innate immune cells Gut epithelial cells Respiratory system Salivary glands Skin	Wound healing and tissue repair LPS neutralization Recruitment of neutrophils Dendritic cells activation Intestinal barrier integrity Antiviral activity	Chronic intestinal infection↓ Systemic sclerosis↓ Chronic obstructive pulmonary disease↓ Psychological stress (murine CRAMP)↓ Atherosclerosis↑ Psoriasis↑	[18,19,23], [34–37]
α-defensins	HNP-1 HNP-2 HNP-3 HNP-4 HD-5 HD-6	*DEFA1* *DEFA3* *DEFA4* *DEFA5* *DEFA6*	8p23.1	Bone marrow Polymorphonuclear leukocytes Salivary glands Oronasal cavity and nasal mucosa Gastrointestinal and urinary tract Intestinal Paneth cells Bronchial cells Female reproductive system	Chemoattractant Phagocytosis induction Microbicidal activity Gut microbiota homeostasis Antifungal activity	Crohn's disease↓ Graft-versus-host disease↓ Sepsis↑ Coronary heart disease↑ Systemic lupus erythematosus↑ Periodontal infections↓ Colorectal cancer↑	[16,17,21] [38–40]
β-defensins	hBD-1 hBD-2 hBD-3 hBD-4	*DEFB1* *DEFB2* *DEFB3* *DEFB4*	8p 23.1-p23.2 8p23.1-p22 8p23 8p23	Epithelial and blood cells Skin Gut epithelium Respiratory tract Bone marrow Epidermal keratinocytes Gingival epithelium Small intestine	Innate immune defense Wound healing Cytokine enhancement Dendritic cell modulation Neutrophil recruitment Pro-inflammatory mediator Antimicrobial activity	Oral squamous cell carcinoma↓ Liver cancer and colorectal cancer↓ Periodontitis↓ Asthma↑ Esophageal and cervical cancer↑ Interleukin-17A-mediated psoriasis↑ Ulcerative colitis↑ Chronic obstructive pulmonary disorder↑	[20,22], [41–47]
Histatins	His1His3His5	*HTN1* *HTN3* *HTN3* *	4q13.3	Salivary glands	Oral health Wound healing	Aqueous deficient dry eye disease↓ Oral candidiasis↓	[48,49]
RNases	RNase 7	*RNASE7*	14q11.2	Skin Genito-urinary tract	Immunomodulatory	Allergic rhinitis↓ Urinary tract infections↑	[50,51]

Abbreviations: HNP-1, human neutrophil peptide 1; HNP-2, human neutrophil peptide 2; HNP-3, human neutrophil peptide 3; HNP-4, human neutrophil peptide 4; HD-5, human defensin 5; HD-6, human defensin 6; hBD-1, beta-defensin 1; hBD-2, beta-defensin 2; hBD-3, beta-defensin 3; hBD-4, beta-defensin 4; His1, histatin-1; His3, histatin-3; His5, histatin-5; RNase 7, ribonuclease 7; HTN3*, proteolytic variant of HTN3; ↑, upregulated; ↓, downregulated; HDPs, host defense peptides; LPS, lipopolysaccharides.

The sensitivity to environmental factors of these peptides was well illustrated by the inability to reproduce the protective role of insect-derived AMPs, such as drosocin, in a mouse model [52]. Briefly, the authors explained this difference by an unusually high degradation rate of such peptides in mammalian sera (human and mouse) in comparison to insect hemolymph. In contrast, physiological conditions have no impact on the immunomodulatory properties of AMPs, such as chemoattraction or activation of immune cells. In addition, the antimicrobial activity of AMPs estimated in vitro, i.e., MIC (minimal inhibitory concentration) values, is usually observed at micromolar concentrations which are significantly higher than the physiological concentrations of these peptides. For instance, the concentration of LL-37 or β-defensins is less than 2 μg/mL at mucosal sites, and the MIC of LL-37 in vitro against *Escherichia coli* is more than 32 μg/mL [10], whereas modulation of immune responses by AMPs occurs at nanomolar levels [53]. Therefore, it is possible that the other biological functions of AMPs, e.g., as alarmins, may play more prominent roles than their direct microbicidal effects in combating invading pathogens in vivo [6,10,53]. Indeed, several synthetic AMP derivatives, known as innate defense regulator (IDR) peptides, are characterized by potent immunomodulatory activities [54].

In fact, certain human AMPs such as the histone protein H2A (known as buforin I) or ribosomal protein S30 (known as ubiquicidin) were initially known from non-antimicrobial functions, before their antimicrobial potential was recognized. In addition, around 20% of human AMPs (Figure 2) are chemokines, which as cationic and amphipathic molecules are characterized by antimicrobial activity [55]. In addition, the specific chemokine receptor CCR6, expressed by dendritic cells and T cells, is utilized also by human β-defensin-2 peptide [56], supporting the hypothesis that AMPs create a bridge between innate and adaptive immune system.

AMPs protect all human body sites that are continually exposed to microbes, like the skin and mucous membranes, since they are produced by multiple immune and epithelial cells (Table 1). Their expression may be constitutive and some cells (e.g., neutrophils) store a high number of AMPs, or the expression is induced by various microbial or the host stimuli. As a consequence, each tissue has its own profile of different AMPs that may vary significantly depending on the actual host condition. It is tempting to name it as a "peptidiome" using an analogy to microbiome bacteria within a given body habitat. Therefore, (1) a synergism of AMPs activity, supported by their (2) accumulation, e.g., in neutrophil extracellular traps (NETs) (see below), as well as (3) enhanced expression, may explain the insufficient microbicidal concentration issue observed at the basal physiological background. On the other hand, this effect may just be a derivative of inadequate in vitro MIC testing methods. For example, Dorschner et al. [57] showed that cultivation of bacteria, like *Staphylococcus aureus* and *Escherichia coli*, in a medium mimicking the mammalian ionic environment, i.e., carbonate-containing solutions, causes changes in their cell wall thickness and an altered gene expression pattern, that in turn increased susceptibility to AMPs. Furthermore, it is possible that in the skin or inside phagocytic cells, i.e., body niches where the level of the AMPs-inhibiting factors is minor, this antagonism is not significant.

2.1. Human Defensin and Cathelicidin (LL-37) Peptides

Defensins are cysteine-rich peptides classified based on configuration of the disulfide bonds between six cysteine residues into α-, β-, and θ-defensins; however, in humans, the latter exist only as pseudogenes [58]. From the evolutionary perspective, β-defensins are the common ancestor of all vertebrate defensins, and α-defensins are mammalian-specific genes co-located with β-defensin ones on adjacent loci on human chromosome 8p22–p23 [59,60]. Human α-defensins are produced mainly by neutrophils; hence, they are known as human neutrophil peptides 1–4 (HNP-1, HNP-2, HNP-3, and HNP-4), as well as by Paneth cells of the small intestine (HD5 and HD6) (Table 1) [61,62]. Interestingly, the four HNPs are encoded by three genes, since HNP-2 is a truncated variant of HNP-1 or HNP-3 peptides, lacking the first alanine or aspartic acid residue, respectively [63]. HNP1–4 are constitutively expressed and stored in azurophil granules, where they constitute more than 30% of the protein content; however, HNP-4 is the least abundant [61,64].

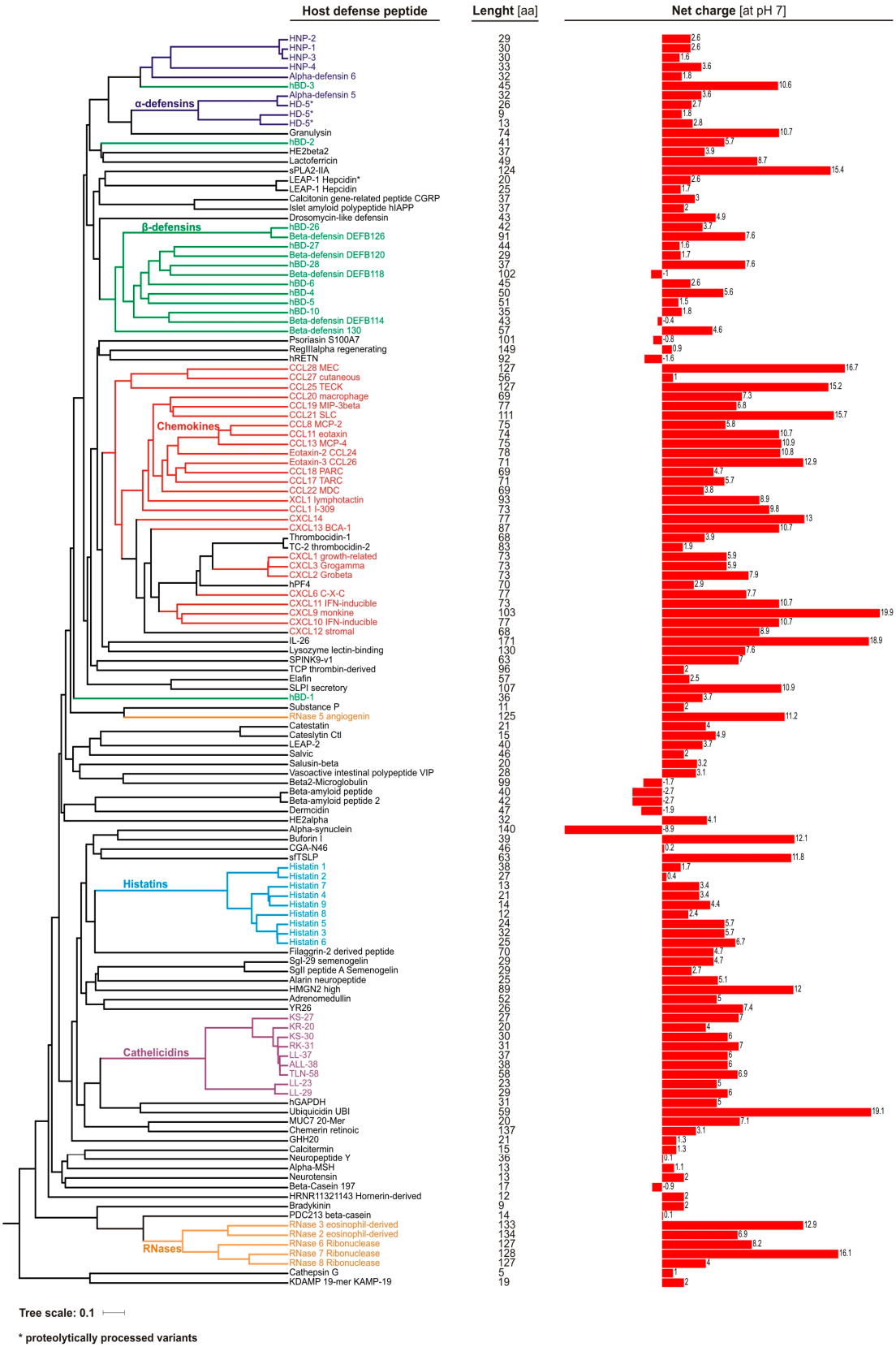

Figure 2. Comparison of human host defense peptides (n = 133) curated in antimicrobial peptide database (http://aps.unmc.edu/AP; accessed in September 2019). The dendrogram was built based on amino acid sequence alignment using MAFFT aligner (https://mafft.cbrc.jp) [65], visualized and annotated with Archaeopteryx [66] and iTOL [67], respectively. Net charge values of the peptides (at pH = 7.0) were estimated using Protein Calculator https://pepcalc.com/protein-calculator.php [68].

In contrast, at least 17 β-defensins (hBDs) have been described, yet hBD1–hBD4 are the best studied [69]. They are produced by various epithelial and mucosal cells, thus protecting body sites directly exposed to microbes, such as respiratory, intestinal, and genitourinary tracts, as well as skin (Table 1), where their expression may be constitutive or inducible. For example, expression of the *hBD-1* gene is essentially constitutive, whereas expression of the *hBD2-4* genes is infection-related or triggered by host-derived stimuli [70]. Remarkably, the microbicidal effect of hBD-3 peptide is not weakened in the presence of the physiological salt concentration found in mucus, which enables it to have a substantially strong anti-HIV effect [71]. In addition, ß-defensin genes *(DEFB4, DEFB103,* and *DEFB104*) have a high degree of copy-number variation (CNV), ranging from 2 to 12 copies per diploid genome [72], which affects their expression level.

Cathelicidins were named based on a conserved cathelin-like domain connected with a C-terminal antimicrobial domain, and are produced mainly by leucocytes and epithelial cells [73,74]. In the human genome, only one cathelicidin gene (*CAMP*) is present. Nevertheless, as the result of proteolytic cleavage by various proteases of its product, i.e., hCAP-18 (human cationic antimicrobial protein 18 kDa), several cathelicidin peptide variants are generated (Figure 2). In detail, in the first step, hCAP-18 is processed by protease 3 to the full-length active peptide LL-37 (leucine–leucine 37 aa), which in turn is cleaved into shorter variants by tissue-specific proteases. In the skin, serine proteases from the kallikrein family, SCTE (stratum corneum tryptic enzyme; kalikrein 5) and SCCE (stratum corneum chymotryptic enzyme; kalikrein 7), generate peptides KS30, KS22, LL29, and RK31 and KR20, respectively [75]. In fact, in the skin, LL-37 accounts for less than 20% of all cathelicidin variants. Interestingly, KS30, KS22, and LL29 are characterized by stronger antimicrobial activity, but lack of chemotactic properties. On the other hand, RK31 and KR20 peptides possess weak antibacterial but strong antifungal activity. Recently, also the TLN-58 variant, possibly generated by neutrophil elastase (ELA2), has been found in the skin palmoplantar pustulosis (PPP) vesicles [76]. Furthermore, since hCAP-18 is present in semen, a longer, by an additional alanine residue, peptide ALL-38 is produced as the result of action of prostate-derived protease, gastricsin, under acid vaginal pH conditions.

2.2. Other Host Antimicrobial Peptides

Besides the classical antimicrobial peptides, there is an array of small proteins regulating immunomodulatory and antimicrobial functions against a broad range of pathogens. For instance, histatin, lysozyme, hepcidin, thrombocidin-1, neuropeptide α-MSH, RNase 7, RNase 5, and dermcidin are inherently expressed in specific tissues and cells (Table 1). Briefly, histatins 1, 3, and 5 belong to a family of salivary peptides that help to maintain the human oral mucosa, along with the β-defensins. An elevated expression of histatin 5 is detected in the saliva of children with a high level of dental cavities harboring specific bacterial species, such as *Streptococcus mutans*, *S. sanguinis*, *S. mitis,* as well as *Lactobacillus rhamnosus* in the oral environment [77,78]. In contrast, RNAse 7 is abundantly found within specialized uroepithelial cells in bladder lining, ureters, and kidneys, protecting the urinary system from invading microbes. This peptide exhibits a significant role in maintaining a bacteria-free bladder, as it inhibits the microbial activity of various drug-resistant microbes, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and vancomycin-resistant *Enterococcus faecium* [79]. Another important antimicrobial peptide synthesized in the liver is hepcidin. While its primary function involves maintenance of iron absorption and transport, hepcidin also exhibits strong antimicrobial activity. During inflammatory conditions, hepcidin mRNA expression is highly stimulated by the cytokines IL-6, IL-1α and IL-1β, which modulates host response [80].

3. Role of Host Defense Peptides in Inflammation

Over the years, our view on antimicrobial peptides (AMPs) has evolved from just endogenous antibiotics into multifunctional agents (HDPs), which execute their antimicrobial tasks at the same time as participating in a pro-inflammatory response and, if required, mediating its suppression. Currently, HDPs are perceived as factors contributing either to efficient clearance of infections or

resolution of the infected sites. To illustrate, these peptides not only attract immune cells, e.g., neutrophils, but also by blocking apoptosis prolong their lifespan, and in turn phagocytic functions [81]. On the other hand, HDPs may function as a "molecular brake" on macrophage-driven inflammation to maximize eradication of pathogens with minimal adverse effects on surrounding tissues [82].

Furthermore, HDPs are essential for proper host–microbiota interactions. In this context, HDPs serve as a buffer, maintaining immune homeostasis via neutralization of pro-inflammatory MAMPs, e.g., lipopolysaccharides (LPS) and lipoteichoic acid (LTA), constantly released by microbiota, as well as a factor shaping its composition, hence protecting from dysbiosis [83]. For example, LL-37 inhibits the expression of specific pro-inflammatory genes up-regulated by NF-κB in the presence of LPS, unlike to LPS-induced genes which antagonize inflammation and certain chemokine genes classically considered pro-inflammatory [84]. On the other hand, the microbiota are a key factor in stimulating production of HDPs, as supported in a classical experiment by Mangoni et al. [85], showing that the presence of HDPs in frog skin (*Rana esculenta*) is microbiota-dependent, and frogs living in a sterile, i.e., the microbiota-free, environment do not synthesize antimicrobial peptides.

Importantly, HDPs inhibit not only pro-inflammatory action of exogenous PAMPs, but also endogenous ones, like DAMPs (also known as "alarmins" or "danger signals"), which are expressed in stressed or dying cells and convey alarm signals to the immune system, including those responsible for autoimmune disorders [13]. For instance, in the skin cathelicidin peptides block release of cytokines induced by the alarmin hyaluronan, thus their low expression may be a risk factor of the development of atopic dermatitis [86]. Initially, the term DAMPs involves factors from various cell/tissue compartments, such as extracellular matrix (hyaluronan, heparan sulfate, eDNA), cytoplasm (heat shock proteins: HSP60, HSP70, HSP90, HSP27; calcium-binding proteins: S100A8, S100A9, S100A12; β-Galactoside binding lectins: Galectin-1, Galectin-3; and uric acid), mitochondria (mitochondrial DNA, ATP, N-formylated peptides), and other subcellular organelles (HMGB1, IL-33, IL-1α, Calreticulin). However, currently, several HDPs, e.g., cathelicidins and α- and β-defensins, are also classified as alarmins [14], which in fact are frequently co-expressed with DAMPs (Figure 3).

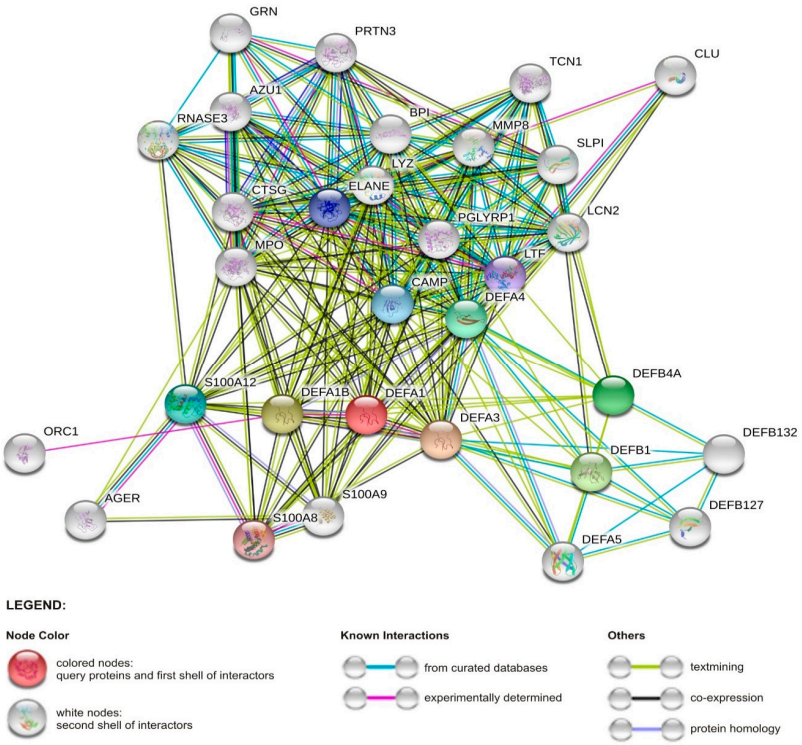

Figure 3. Network of interactions between α-defensin 1 gene (*DEFA-1*) and other human proteins (the network was obtained from STRING v11 database) [87]. Represented proteins are central to antimicrobial

and immunomodulatory activities. Abbreviations: AGER, advanced glycosylation end-product-specific receptor; BPI, bactericidal permeability increasing protein; CAMP, cathelicidin antimicrobial peptide; CLU, clusterin; CTSG, cathepsin; DEFA3, defensin alpha 3; DEFA4, defensin alpha 4; DEFA5, defensin alpha 5; DEFA1B, defensin alpha 1B; DEFB4A, defensin beta 4A; DEFB1, defensin beta 1; DEFB132, defensin beta 132; DEFB127, defensin beta 127; ELANE, neutrophil elastase; GRN, granulin precursor; LCN2, lipocalin 2; LTF, lactotransferrin; LYZ, lysozyme; MPO, myeloperoxidase; MMP8, matrix metallopeptidase 8; ORC1, origin recognition complex subunit 1; PGLYRP1, peptidoglycan recognition protein 1; PRTN3, proteinase 3; RNASE3, ribonuclease A family member 3; SLP I, secretory leukocyte peptidase inhibitor; S100A8, S100 calcium binding protein A8; S100A9, S100 calcium binding protein A9; S100A12, S100 calcium binding protein A12; TCN1, transcobalamin 1.

Indeed, a link between HDPs and multiple autoinflammatory diseases such as skin disease (atopic dermatitis, psoriasis, rosacea) or microbiota-related ones, e.g., IBD (Crohn's disease, colitis ulcerosa), acne vulgaris, and periodontitis, has been established by several studies (see below). An enhancement of Th17 response by HDPs may serve as an example. Briefly, HDPs efficiently attract Th17 (T helper 17 cells), which in turn secrete pro-inflammatory cytokines, IL-17A, IL-17F, IL-21, and IL-22, responsible for mounting mucosal defense against pathogenic microbes in the respiratory or intestinal tract. For instance, IL-17A and IL-22 work synergistically to induce certain β-defensins hBD-1, hBD-3, and hBD-4 in both human and primary mouse gastric epithelial cells (GEC) and gastroids co-cultured with *Helicobacter pylori* [88]. On the other hand, an elevated level of Th17 cells has been connected with various autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, or psoriasis [89].

Also, genetically-mediated deficiency/excess of HDPs, gene sequence polymorphisms, as well as disturbed expression may be a risk factor in inflammatory diseases. For instance, Hollox et al. [90] showed a significant association between higher genomic copy numbers for β-defensin genes, ranging from 2 to 7 copies, and the relative risk of developing psoriasis. Likewise, a lower the *hBD-2* gene copy number in the β-defensin locus predisposes to colonic Crohn's disease [91]. Recently, experimental evidence has highlighted the genetic association between the clinical phenotype of sepsis and *DEFA-1*/*DEFA-3* copy number. Transgenic mice models were engineered to produce a high gene copy number of *DEFA-1*/*DEFA-3*, which manipulated the outcome of sepsis progression [92]. The consequential effect was compared to the low gene copy number wild-type mice models, in that the former showed chronic inflammation, endothelial cell damage, vascular leakage, severe organ injury, and mortality. Thus, treatment of patients with sepsis can be challenging due to the underlying individual genetic associations. However, further research is needed to obtain conclusive data. In addition, single nucleotide polymorphisms (SNPs) of the *hBD-1* gene was connected with the pathogenesis of inflammatory bowel diseases and chronic gastritis [93], as well as oral diseases [94].

In general, expression of HDPs is enhanced during infection or inflammation through transcription factors initialized by pro-inflammatory cytokines or signaling pathways associated with activation of PRRs, e.g., Toll-like receptors (TLRs). For instance, promoter regions of α- and β-defensin genes contain binding sites for major cellular transcription factors, notably nuclear factor κB (NF-κB) and activator protein 1 (AP-1) (Figure 1). It should be noted that NF-κB also plays a crucial role in the pathogenesis of Crohn's disease, along with many other pro-inflammatory molecules that modulate the hBD-2 expression [95], as well as in triggering its production (and IL-6) in severe sepsis [96]. Moreover, the expression of genes encoding LL-37 (and hBD-2) is modulated by vitamin D3 via binding with specific DNA sequences in their promoters, the so-called vitamin D response elements (VDRE) [97]. Additionally, a recent in silico analysis identified a wide range of transcription factors which possibly bind and modulate the gene transcription of many antimicrobial peptides and proteins, such as LL-37, RNAse1, CCL18, CXCL14, and HTN1 [98].

In line with this, it has been established that DNA methylation of the CpG sites in the 5′ flanking region of the *hBD-1* gene contributes to its deficiency in patients with atopic dermatitis [99]. Furthermore, point mutations in the promoter region of *hBD-1* explain a cancer-specific loss of this peptide in 90% and 82% of renal cell carcinomas and prostate [100]. Thus, *hBD-1* was suggested as a

potential tumor suppressor gene for urological cancers. Also, in oral squamous cell carcinoma (OSCC), hBD-1 appears to have anti-tumor properties, while *hBD-2* and *hBD-3* might be proto-oncogenes [101].

Nonetheless, the relation between HDPs and inflammation is not always straightforward, and either their deficiency or overproduction, as well as a balance between pro- or anti-inflammatory effects, may contribute to the pathological inflammatory response. For instance, in atopic dermatitis (AD), despite severe skin inflammation, the expression level of major skin HDPs, dermcidin, LL-37, hBD-2, and hBD-3, is not increased, hence patients with AD are more prone to skin infections and have altered skin colonization patterns. By contrast, in psoriasis, expression of LL-37, hBD-2, and hBD-3 is elevated, hence skin infection is rare. Nevertheless, LL-37 and hBDs are considered as a major driving force of inflammation in psoriasis by mechanisms involving increased production of IFN-α and activation of pDCs, respectively [102]. In addition, these peptides stimulating degranulation of mast cells and increasing production of the pruritogenic cytokine IL-31 may escalate itching (pruritus) manifestation [103]. However, it has been recently observed that LL-37 may also act as an anti-inflammatory agent by blocking the release of inflammatory cytokine IL-1β, depending on its concentration. Interestingly, this observation possibly explains the mechanism underlying the paradoxical effectiveness of vitamin D3, i.e., inducer of LL-37 expression, in treatment of psoriasis [11]. Similarly, hBD-3 may inhibit inflammation by inducing expression of anti-inflammatory cytokine IL-37 in keratinocytes [104]. An elevated level of cathelicidin is also observed in other inflammatory skin conditions, namely rosacea and palmoplantar pustulosis, but instead of the native form of LL-37, its proteolytically cleaved variants drive the inflammation [76,105].

Considering the above, the final contribution of HDPs to inflammatory processes is a derivative of multiple variables related to their expression, processing, concentration, and combination, as well as reciprocal interactions with the remaining components of the host immune system. For instance, pro- and anti-inflammatory effects of cathelicidin LL-37 are concentration-dependent, i.e., the former is visible at >20 μg/mL, whereas the latter at 1–5 μg/mL. Similarly, at the concentration range 1–100 ng/mL, β-defensins can act as chemokines only, since other immunomodulatory functions are not visible [10]. Furthermore, there are more than 20 antimicrobial peptides in the skin characterized by different expression levels (Figure 4) [106]. Therefore, the relationship between HDPs and inflammation appears to be strongly context-dependent (e.g., inflammation site, type of cells, or stimuli), and as such, it should be analyzed on a multidimensional level, rather than as a single action of individual peptides. Otherwise, valid conclusions regarding the ultimate role of HDPs in inflammation may be difficult to draw. In the next paragraph, we discuss mechanisms behind anti- and pro-inflammatory actions of HDPs.

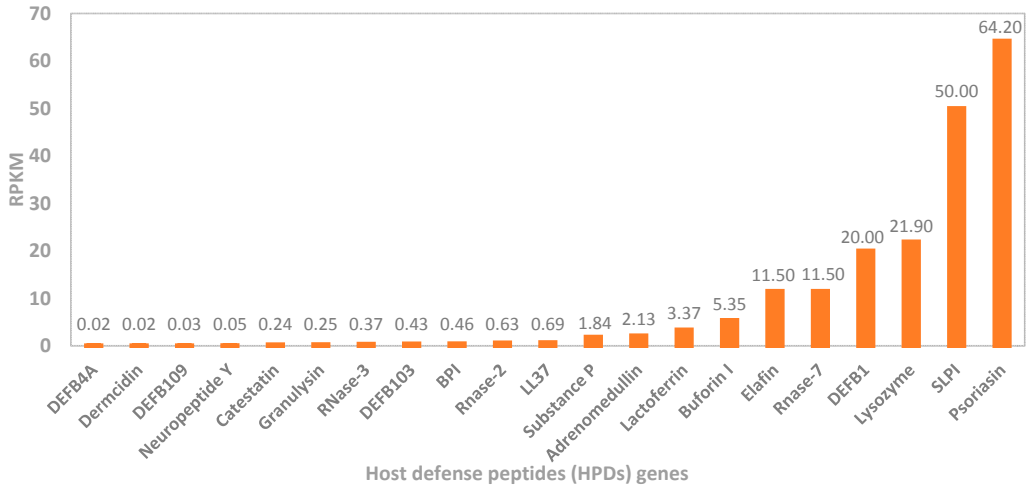

Figure 4. Relative expression of selected host defense peptide (HDP) genes in the human skin, based on RNA-seq analysis of tissue samples from 95 human individuals performed by Fagerberg et al. [107]. The data were obtained from the GenBank Bio Project: PRJEB4337 (RPKM, reads per kilobase per million reads).

4. Molecular Mechanisms of Anti- and Pro-Inflammatory Action of HDPs

The immunomodulatory potential of HDPs is strictly connected with their ability to recruit and activate immune and non-immune cells, as well as a direct or indirect impact on their fate, including maturation, differentiation, degranulation, or apoptosis [10]. This is mediated through interaction with a wide range of membrane-bound and intracellular receptors, followed by stimulation of their downstream signaling pathways. So far, HDPs have been recognized to interact with the following receptors: (1) Pattern recognition receptors (PRRs), (2) purinergic G-protein coupled receptors (formyl peptide receptor like-1), (3) P2X7 receptor, (4) MRGPRX2, (5) chemokine receptors (commonly known CCR2, CCR6), (6) epidermal growth factor receptors (tyrosine kinases), (7) integrin family receptors (macrophage-1 antigen), nucleotide oligomerization domain (NOD) proteins, and NODlike receptors (NLRs), and their number is still growing [108,109]. Hence, HDPs modulate immune responses using the same receptors as MAMPs/PAMPs and DAMPs [110,111]. To illustrate, β-defensins attract cells by interaction mainly with CCR2 and CCR6 receptors, and exert their "alarmin" activity, e.g., induction of cytokine production, via TLRs, EGFR, GPCR, and MrgX2 ones; however, both activities may overlap in one receptor.

It should be noted that TLR receptors may be the root cause of certain HDP-associated diseases. For instance, in individuals with rosacea, a higher expression of TLR-2 sensitizes the facial skin to microbes or environmental stimuli. Under these conditions, enhanced expression of kallikrein-5 proteinase is observed in keratinocytes, and ultimately affects production of cathelicidin peptides, which drives inflammation and abnormal growth of blood vessels [105]. Moreover, the tumor-suppressing effect of hBD-1 is associated with its ability to modulate epidermal growth factor and human epidermal growth factor receptor 2 (EGFR/HER2)-associated signaling pathways [112]. Finally, hBD-3, through deactivation of TLR-4 and TLR-2, may reduce the adverse immune reaction initialized by NF-κB in response to LPS [113].

Furthermore, β-defensins and cathelicidins, in the same manner as PAMPs (e.g., LPS) and DAMPs (e.g., heat shock antigens Hsp60 and Hsp70), are ligands of Toll-like receptor 4 (TLR-4). However, the resulting outcome of the receptor's stimulation may be different for these molecules. For instance, unlike LPS, hBD-3 does not induce production of IL-10, which is an important anti-inflammatory cytokine, e.g., via suppressing function of antigen-presenting cells (APCs), suggesting that hBD-3 can shift the immune response toward pro-inflammatory direction [114]. Similarly, hBD-2 via TLR-4 leads to maturation of dendritic cells (DCs), which consequently exhibit Th1-polarized responses, such as the production of pro-inflammatory cytokines IL-12, IL-1α, IL-1β, and IL-6, which may possibly counter suppressive action of microbial factors by generating more robust host inflammatory and Th1 responses [115]. In contrast, cathelicidin is considered as an inhibitor of TLR-4, and thus can antagonize with other TLR-4 ligands released during skin injury, e.g., hyaluronan [10].

Therefore, HPDs joining properties of MAMPs/PAMPs and DAMPs may operate as central nodes in a network that coordinates immune response to infections as well as non-infectious insults. For instance, it has been shown that synergistic action of MAMPs/PAMPs and DAMPs is necessary for synthesis and subsequent secretion of pro-inflammatory cytokine IL-1β [13]. It is important to note that a lack of IL-1β results in high susceptibility to infections, but its overproduction causes uncontrolled inflammation and tissue damage via T cell-mediated autoinflammatory response [116]. Indeed, overproduction of IL-1β was observed in patients with inflammatory bowel disease, and has been connected with deficiency of α-defensins that serve as regulators of IL-1β maturation [117]. As aforementioned, also in psoriasis, LL-37 may act as an inhibitor of the IL-1β release in keratinocytes by blocking activation of the cytosolic DNA-sensing signaling AIM2, i.e., cytosolic receptor for dsDNA. Hence, cytoplasmic DNA appears to contribute to the pathogenesis of psoriasis via activation of IL-1β in keratinocytes by AIM2-mediated inflammasomes [11].

In this context, it is relevant to mention the relationship of HDPs and self-nucleic acids, and its impact on inflammation. Under normal homeostatic conditions, the host-derived nucleic acids released

from damaged and dying cells do not mediate inflammatory responses because of the systematic regulation and physiological location of nucleic acid sensing TLR7/9. However, several studies have shown that HDPs may disturb immune tolerance to self-nucleic acids, and in turn significantly enhance cell responses—in particular, plasmacytoid dendritic cells (pDCs) [118]. In fact, in the skin, this mechanism has been identified as an important initiator of psoriasis development, where LL-37 and defensins are able to condense self-DNA into particles, which are internalized by pDCs, inducing robust IFN-α response via activation of the TLR-9 signaling pathway [119,120]. This enhances production of large amounts of type I IFN, leading to the functional activation of myeloid dendritic cells (mDCs), monocytes, NK cells, keratinocytes, as well as Th1/Th17 differentiation, which further increase the pro-inflammatory, e.g., IFN-γ, IL-22, and IL-17, cytokine expression [120,121]. In line with this, a novel mechanism of nucleic acid recognition by LL-37 utilizing cell surface RNA scavenger receptors (SRs) has been described [122], which results is enhanced clathrin-dependent endocytosis, facilitating the overproduction of inflammatory cytokines and chemokines. Recently also RNase7 was found to utilize plasmacytoid dendritic cell (pDC) TLR-9 signaling mode of IFN-α activation even more strongly than LL-37, emphasizing its crucial role in autoimmune inflammatory skin diseases [123]. Interestingly, other antimicrobial peptides expressed in the skin, such as psoriasin, elafin, or hBD-1, lack the ability of interacting with the host nucleic acids, which may be related to their lower net charge (Figure 2) [120].

Another interesting consequence of interactions between HDPs and the host nucleic acids is a novel wound healing mechanism, where LL-37 may alter wound repair by modifying the responses to dsRNAs released as a result of skin injury. In detail, LL-37 enhances endosomal uptake of non-coding double stranded RNA in TLR-3-mediated mechanism, that results in activation of several important wound repair growth factors, including fibroblast growth factor (FGF2), and heparin binding EGF-like growth factor (HBEGF) from the dermal keratinocytes and fibroblasts [124]. Inhibition of LL-37/dsRNA relation may contribute to the development of hyperproliferation-based diseases, like psoriasis, whereas its augmentation can lead to increased wound regeneration in pathological conditions of abnormal wound repair (e.g., diabetic ulcers).

Finally, LL-37 actively participates in neutrophil extracellular trap (NET) formation via disruption of the nuclear membrane and promotes their stability [125]. NETs are structures composed of decondensed chromatin and multiple enzymes (elastase, myeloperoxidase, gelatinase, etc.) and proteins, including antimicrobial ones. Thus, NETs act as a mechanical barrier that entraps and subsequently reduces spreading of pathogens and/or their toxic products into the host tissues, where the antimicrobial activity of HPDs is boosted by their accumulation and combination [126]. Moreover, Stephan et al. have shown that complexes of LL-37/DNA formed inside human macrophages may participate in defense against intracellular bacteria, e.g., mycobacteria [127]. Accordingly, a recent study investigated the therapeutic potential of LL-37 in modulating macrophage-mediated excessive inflammatory responses. It was found that LL-37 reduced the severity of tuberculosis by rapidly enhancing the anti-inflammatory cytokine TGF-β, IL-10, and prostaglandin E from the infected macrophages [128]. However, further studies regarding the exogenous effect of LL-37 in severe pulmonary tuberculosis are warranted. Interestingly, administration of vitamin D3 or another potent inducer of LL-37, i.e., 4-phenyl butyrate (PBA), may be an alternative treatment method of tuberculosis [129].

5. Deregulations of HDPs Expression in Selected Diseases

5.1. Periodontal Diseases

An imbalanced unhealthy oral microbiota ushers the entry of various cariogenic, periodontal microbes which engenders oral biofilm formation and periodontal diseases such as gingivitis and periodontitis. The oral epithelial tissues, mainly the gingival epithelium, play a significant role in resisting the colonization of unfavorable oral pathogens. These tissues readily secrete beta-defensin peptides, as well as histatins, which are the major host defense proteins of the saliva that maintain homeostasis of oral microbiota [130]. A significant correlation is observed between elevated levels

of hBD-2, hBD-4, and HNP4 in the oral mucosal epithelial cells of both adults and children with the development of dental caries. They are considered as important clinical biomarkers of periodontal diseases and dental caries. It has been shown that there is a declined expression of beta-defensin 1 mRNA gene in the inflamed gingival tissues and periodontal structures. Conversely, chronic cases of periodontitis manifest an elevated expression of the *hBD-1* gene [131,132]. The severity of periodontal diseases and dental plaques grows, along with a heightened expression of hBD-2 and histatin-5. Higher activity of pro-inflammatory cytokines modulates the progression of the infection, which further stimulates the production of the defensin peptides through various transcription factors [133]. Recently, 89 patients were monitored according to their periodontal status in relation to other clinical parameters [134]. This study identified an increase in the salivary production of hBD-2, triggered by inflammatory processes and pathogen derived metabolites that can be considered as a possible diagnostic biomarker s of periodontal diseases.

Healing of periodontal lesions is initiated by various growth factors, pro-inflammatory mediators, and antimicrobial peptides accumulating at the infected site. In detail, a complex network of highly specialized growth factors, namely, insulin-like growth factor (IGF1, IGF2), transforming growth factor (TGF-α, TGF-β), epidermal growth factor, and platelet-derived growth factor, coordinates the reparative process by rapid differentiation of keratinocytes and fibroblasts [135]. These growth factors also assist the wound healing mechanism by influencing the gene expression pattern of antimicrobial peptides that typically participate in the epithelial cell proliferation, migration, and inhibition of colonizing microbial pathogens at the site of injury. While it is established that wounding influences the expression of HDPs, not all of them function the same way. Recent reports have highlighted the distinct immune responses triggered within the wounded gingival epithelial cells (GECs) and gingival fibroblasts (HGFs) upon treatment with IGF1 and TGF-α. These growth factors enabled efficient wound closure and differently modulated the expression of hBD-2, CCL20, IL-1, and IL-8. The findings indicate that hBD-2 was exclusively enhanced in the gingival epithelial cells measured at set time points of 6 h and 24 h post-wounding, particularly in those cells associated with the keratinocyte differentiation marker involucrin. Additionally, hBD-2 along with CCL20, IL-1, and IL-8 control the invasion of bacterial microbes and impact the neutrophil defense mechanisms [136]. Contrarily, the wounded gingival fibroblasts (HGFs) witnessed a substantially low expression pattern of hBD-2 and CCL20, with or without growth factor treatment, that was suggested as a mechanism protecting fibroblast overgrowth into the epithelial wound [136]. fibroblast overgrowth into the epithelial wound.

5.2. Inflammatory Lung Diseases

Cystic fibrosis (CF) is a life-limiting disease characterized by recurrent respiratory infections and inflammation, connected to altered composition and volume of the airway surface liquid (ASL). For instance, a reduced bicarbonate HCO_3^- secretion resulting in a decrease of airway surface pH (average 6.8–7.5) was observed. Interestingly, it was also found that the acidic pH weakened the action of LL-37 and hBD-3 against invasive *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections by affecting their structural net charge. Therefore, it could be suggested that a similar mechanism of acidic pH-reduced antimicrobial activity may occur in other inflammatory conditions taking place in cerebral spinal fluid, peritoneal fluid, and pleural fluid. Additionally, it has been noted that high ionic strength (Na^+, K^+, Cl^+) may impair the antimicrobial activity of hBD-2, lysozyme, and lactoferrin [137]. Moreover, CF patients suffer from viscous sputum that accumulates and obstructs their airways. The thick mucus is characterized by heterogenous complex aggregates of DNA and F-actin filaments derived from leukocytes that have encountered necrotic death. Thus, the antimicrobial function of LL-37, lysozyme, lactoferrin, and hBD-3 released in the respiratory airways is substantially hindered as they stabilize DNA/F-actin bundles. Additionally, neutralization of the immune function of neutrophil protease and IL-8 take place during DNA/F-actin bundles formation [138,139]. It is also worthwhile to underline that abundant secretion of cysteine cathepsins from the macrophages hinders the functional expression of hBD-2.

Chronic obstructive pulmonary disease (COPD), bronchitis, and asthma are all characterized by inflammation that develops as a consequence of pro-inflammatory mediator secretion. Immune cells distributed throughout the lungs are responsible for sudden exacerbations associated with the production of cytokines, oxidative stress, and protease secretion, including caspases, neutrophil elastase, and matrix metalloproteinases. One study reports the enhanced expression of hBD-2 in the distal airway epithelial cells of COPD patients, but a rather diminished expression of hBD-2 in the central airways, despite the exaggerated expression of TLR-4 receptors [140]. This distinct variation was found to be in correlation with exposure to cigarette smoking. While it is evident that every cell in the body requires ATP for its biological function of energy production and retention, little is known about its possible involvement in the immune system response to bacterial infection and inflammation. In a *P. aeruginosa*-infected rat model, ATP administration led to rapid stimulation of hBD-2 production. The mechanism of ATP action involved NADPH family of oxidases (DUOX 1) via ion channel receptors P2X, P2Y activation, and regulation of multiple signaling pathways ERK1/2 and NF-κB [141]. The released defensin peptide was found to control the inflammatory processes underlying the acute infection of pneumonia by suppression of TNF-α and IL-6. Furthermore, another study detected the potent ability of the IL-17 family of cytokines in the induction mechanism of the *hBD-2* gene. Typically, most of the immune cells, including T helper cells, macrophages, dendritic cells, and natural killer cells, secrete IL-17 family of cytokines. These cytokines act in concordance with the tumor necrosis factor and IL-1 to promote the induction of other inflammatory mediators production, which individually or collectively can stimulate the secretion of beta-defensins via the activation of various signaling pathways [142,143]. For example, the stimulatory functions of IL-17 in the airway epithelial cells promoting transcription of the *hBD-2* gene through the action of JAK and NF-κB signaling have been reported [144]. Moreover, IL-17 has an impact on other cytokines such as IL-1α, IL-β, IL-6, IL-7, and TNF-α, which contribute to the production of hBD-2. On the other hand, the alveolar macrophages and dendritic cells consistently maintain the release of IL-22. According to a recent study, in which alveolar epithelial cells (A549) were screened for the abundant display of IL-22 receptors and subjected to treatment with different doses of IL-22, an increase of *hBD-2* mRNA transcript synthesis via the STAT3 pathway was observed [145]. Thus, this study revealed a new immunomodulatory role of IL-22 in stimulation of the lung defensins in response to exposure to pathogenic bacteria and viruses. Interestingly, hBD-1 has also emerged as a clinical biomarker of COPD and other inflammatory lung diseases, such as asthma [146]. However, in this case, an altered expression of hBD-1 may be aggravated by gene copy number variations.

5.3. Inflammatory Bowel Diseases

The human defensins 5 and 6 (HD5, HD6) are particularly important in preserving the homeostatic equilibrium of the enteric mucosa layer, exhibiting different effects against the essential inducers of their secretion, i.e., various products of the Gram-positive and Gram-negative bacteria [147]. To illustrate, there is a remarkable reduction in the expression levels of HD5 and HD6 by the Paneth cells in inflammatory bowel conditions such as Crohn's disease. This shift in expression could be attributed to the cause by genetic changes in the NOD2 receptor [148]. Furthermore, a recent study suggests the possibility of using other HDPs, such as the level of fecal HNP, as a non-invasive biomarker of intestinal inflammation in patients suffering from colitis ulcerosa [149].

The human beta-defensins are also naturally expressed in the epithelial cells of the gastric mucosa and extensively participate in host defense against *Helicobacter pylori* colonization, a bacterium present in a high proportion (~80%) of people throughout the world [150]. Multilevel signaling pathways promote the molecular mechanism of induction of beta-defensins in response to the initial stages of *H. pylori* infection. In addition, it has been shown that the phosphorylation of a serine residue of EGFR may modulate the release of hBD-3 [151], revealingan underlying interdependent relation between the stimulated transforming growth factor β-activated kinase-1 (TAK1), p38α pathway,

and phosphorylation of EGFR receptor in the amplified release of hBD-3 in the gastric mucosa involved in *H. pylori* infection.

6. Conclusion

The multifunctional host defense peptides provide a link between innate and adaptive immunity against different microorganisms and contribute to inflammation of infected sites. Depending on the cell type and extracellular environment, some of these peptides exert contrasting functions, wherein they promote or suppress inflammatory processes. A strongly compromised action of host defense peptides against intruders and delayed resolution of inflammatory mediators underlies the development of inflammation in different diseases. Some of these peptides may serve as potential clinical biomarkers for a wide range of inflammatory diseases. Evidently, antimicrobial regulation is crucial to limit the exacerbation of inflammatory signaling molecules. While various factors govern the release of HDPs, any dysregulation can favor an imbalanced feedback mechanism between the host-induced anti-inflammatory and pro-inflammatory processes. In summary, a deeper understanding of the diverse functional roles of HDPs in the body's physiological response to inflammation and disease is crucial and represents the first approach to develop new therapeutic strategies based on HDPs aimed at resolving the progression of inflammatory diseases and strengthening the host barrier defenses.

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Abbreviations

AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
BM	Bone morphogenetic protein
CAMP	Cathelicidin antimicrobial peptide
CCR2	C–C chemokine receptor type 2
CCR6	C–C chemokine receptor type 6
CNV	Copy number variation
COPD	Chronic obstructive pulmonary disease
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DUOX1	Dual oxidase 1
EGFR	Epidermal growth factor receptor
ELA2	Neutrophil elastase 2
FGF2	Fibroblast growth factor
GEC	Gingival epithelial cell
HBEGF	Heparin binding EGF like growth factor
HER2	Human epidermal growth factor receptor 2
HGFs	Human gingival fibroblasts
HMGB1	High mobility group box 1
IDR	Innate defense regulator

IGF	Insulin like growth factor
IL-1α	Interleukin-1α
IL-33	Interleukin-33
JAK	Janus Kinase
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MIC	Minimum inhibitory concentration
MIP–3	Macrophage inflammatory protein-3 alpha
MRGPRX2	Mas-related G-protein coupled receptor member X2
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	Nucleotide-binding oligomerization domain
OSCC	Oral squamous cell carcinoma
P2X7	Purinoceptor 7
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription

References

1. Mills, C.D.; Ley, K.; Buchmann, K.; Canton, J. Sequential Immune Responses: The Weapons of Immunity. *J. Innate Immun.* **2015**, *7*, 443–449. [CrossRef] [PubMed]
2. Chen, L.; Deng, H.; Cui, H.; Fang, J.; Zuo, Z.; Deng, J.; Li, Y.; Wang, X.; Zhao, L. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget* **2018**, *9*, 7204–7218. [CrossRef] [PubMed]
3. Libby, P. Inflammatory mechanisms: The molecular basis of inflammation and disease. *Nutr. Rev.* **2007**, *65*, S140–S146. [CrossRef]
4. Muller, W.A. Getting leukocytes to the site of inflammation. *Vet. Pathol.* **2013**, *50*, 7–22. [CrossRef] [PubMed]
5. Gupta, S.; Bhatia, G.; Sharma, A.; Saxena, S. Host defense peptides: An insight into the antimicrobial world. *J. Oral. Maxillofac. Pathol.* **2018**, *22*, 239–244. [CrossRef]
6. Hancock, R.E.; Haney, E.F.; Gill, E.E. The immunology of host defence peptides: Beyond antimicrobial activity. *Nat. Rev. Immunol.* **2016**, *16*, 321–334. [CrossRef]
7. Kopp, Z.A.; Jain, U.; Van Limbergen, J.; Stadnyk, A.W. Do antimicrobial peptides and complement collaborate in the intestinal mucosa? *Front. Immunol.* **2015**, *6*, 17. [CrossRef]
8. Bhat, S.; Song, Y.H.; Lawyer, C.; Milner, S.M. Modulation of the complement system by human beta-defensin 2. *J. Burns Wounds* **2007**, *5*, e10.
9. Lee, E.Y.; Lee, M.W.; Wong, G.C.L. Modulation of toll-like receptor signaling by antimicrobial peptides. *Semin. Cell Dev. Biol.* **2019**, *88*, 173–184. [CrossRef]
10. Lai, Y.; Gallo, R.L. AMPed up immunity: How antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* **2009**, *30*, 131–141. [CrossRef]
11. Dombrowski, Y.; Schauber, J. Cathelicidin LL-37: A defense molecule with a potential role in psoriasis pathogenesis. *Exp. Dermatol.* **2012**, *21*, 327–330. [CrossRef] [PubMed]
12. Haney, E.F.; Straus, S.K.; Hancock, R.E.W. Reassessing the Host Defense Peptide Landscape. *Front. Chem.* **2019**, *7*, 43. [CrossRef] [PubMed]
13. Saïd-Sadier, N.; Ojcius, D.M. Alarmins, inflammasomes and immunity. *Biomed. J.* **2012**, *35*, 437–449. [CrossRef] [PubMed]
14. Pouwels, S.D.; Heijink, I.H.; ten Hacken, N.H.; Vandenabeele, P.; Krysko, D.V.; Nawijn, M.C.; van Oosterhout, A.J. DAMPs activating innate and adaptive immune responses in COPD. *Mucosal. Immunol.* **2014**, *7*, 215–226. [CrossRef] [PubMed]
15. Roh, J.S.; Sohn, D.H. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune. Netw.* **2018**, *18*, e27. [CrossRef]
16. Fruitwala, S.; El-Naccache, D.W.; Chang, T.L. Multifaceted immune functions of human defensins and underlying mechanisms. *Semin. Cell Dev. Biol.* **2019**, *88*, 163–172. [CrossRef]

17. Jourdain, M.L.; Pierrard, L.; Kanagaratnam, L.; Velard, F.; Sergheraert, J.; Lefèvre, B.; Gangloff, S.C.; Braux, J. Antimicrobial peptide gene expression in periodontitis patients: A pilot study. *J. Clin. Periodontol.* **2018**, *45*, 524–537. [CrossRef]
18. Uysal, P.; Simsek, G.; Durmus, S.; Sozer, V.; Aksan, H.; Yurt, S.; Cuhadaroglu, C.; Kosar, F.; Gelisgen, R.; Uzun, H. Evaluation of plasma antimicrobial peptide LL-37 and nuclear factor-kappaB levels in stable chronic obstructive pulmonary disease. *Int. J. Chron Obstruct. Pulmon. Dis* **2019**, *14*, 321–330. [CrossRef]
19. Muniz, L.R.; Knosp, C.; Yeretssian, G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front. Immunol.* **2012**, *3*, 310. [CrossRef]
20. Kolbinger, F.; Loesche, C.; Valentin, M.A.; Jiang, X.; Cheng, Y.; Jarvis, P.; Peters, T.; Calonder, C.; Bruin, G.; Polus, F.; et al. β-Defensin 2 is a responsive biomarker of IL-17A-driven skin pathology in patients with psoriasis. *J. Allergy Clin. Immunol.* **2017**, *139*, 923–932. [CrossRef]
21. Xie, G.H.; Chen, Q.X.; Cheng, B.L.; Fang, X.M. Defensins and sepsis. *Biomed. Res. Int.* **2014**, *2014*, 180109. [CrossRef] [PubMed]
22. Joly, S.; Compton, L.M.; Pujol, C.; Kurago, Z.B.; Guthmiller, J.M. Loss of human beta-defensin 1, 2, and 3 expression in oral squamous cell carcinoma. *Oral Microbiol. Immunol.* **2009**, *24*, 353–360. [CrossRef] [PubMed]
23. Aberg, K.M.; Radek, K.A.; Choi, E.H.; Kim, D.K.; Demerjian, M.; Hupe, M.; Kerbleski, J.; Gallo, R.L.; Ganz, T.; Mauro, T.; et al. Psychological stress downregulates epidermal antimicrobial peptide expression and increases severity of cutaneous infections in mice. *J. Clin. Invest.* **2007**, *117*, 3339–3349. [CrossRef] [PubMed]
24. Silva, O.N.; Porto, W.F.; Ribeiro, S.M.; Batista, I.; Franco, O.L. Host-defense peptides and their potential use as biomarkers in human diseases. *Drug Discov. Today* **2018**, *23*, 1666–1671. [CrossRef]
25. Wang, G.; Li, X.; Wang, Z. APD3: The antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.* **2016**, *44*, D1087–D1093. [CrossRef]
26. Pasupuleti, M.; Schmidtchen, A.; Malmsten, M. Antimicrobial peptides: Key components of the innate immune system. *Crit. Rev. Biotechnol.* **2012**, *32*, 143–171. [CrossRef]
27. Nakatsuji, T.; Gallo, R.L. Antimicrobial peptides: Old molecules with new ideas. *J. Invest. Dermatol.* **2012**, *132*, 887–895. [CrossRef]
28. Semple, F.; Dorin, J.R. beta-Defensins: Multifunctional modulators of infection, inflammation and more? *J. Innate Immun.* **2012**, *4*, 337–348. [CrossRef]
29. Sieprawska-Lupa, M.; Mydel, P.; Krawczyk, K.; Wójcik, K.; Puklo, M.; Lupa, B.; Suder, P.; Silberring, J.; Reed, M.; Pohl, J.; et al. Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases. *Antimicrob. Agents Chemother.* **2004**, *48*, 4673–4679. [CrossRef]
30. Barańska-Rybak, W.; Sonesson, A.; Nowicki, R.; Schmidtchen, A. Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids. *J. Antimicrob. Chemother.* **2006**, *57*, 260–265. [CrossRef]
31. Wang, G. Human antimicrobial peptides and proteins. *Pharmaceuticals* **2014**, *7*, 545–594. [CrossRef] [PubMed]
32. Tomita, T.; Hitomi, S.; Nagase, T.; Matsui, H.; Matsuse, T.; Kimura, S.; Ouchi, Y. Effect of ions on antibacterial activity of human beta defensin 2. *Microbiol. Immunol.* **2000**, *44*, 749–754. [CrossRef] [PubMed]
33. Goldman, M.J.; Anderson, G.M.; Stolzenberg, E.D.; Kari, U.P.; Zasloff, M.; Wilson, J.M. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell* **1997**, *88*, 553–560. [CrossRef]
34. Sun, L.; Wang, W.; Xiao, W.; Yang, H. The Roles of Cathelicidin LL-37 in Inflammatory Bowel Disease. *Inflamm. Bowel. Dis.* **2016**, *22*, 1986–1991. [CrossRef] [PubMed]
35. Dürr, U.H.; Sudheendra, U.S.; Ramamoorthy, A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim. Biophys. Acta* **2006**, *1758*, 1408–1425. [CrossRef] [PubMed]
36. Scott, M.G.; Davidson, D.J.; Gold, M.R.; Bowdish, D.; Hancock, R.E. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol.* **2002**, *169*, 3883–3891. [CrossRef]
37. Edfeldt, K.; Agerberth, B.; Rottenberg, M.E.; Gudmundsson, G.H.; Wang, X.B.; Mandal, K.; Xu, Q.; Yan, Z.Q. Involvement of the antimicrobial peptide LL-37 in human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **2006**, *26*, 1551–1557. [CrossRef]
38. Cerrillo, E.; Moret, I.; Iborra, M.; Ramos, D.; Busó, E.; Tortosa, L.; Sáez-González, E.; Nos, P.; Beltrán, B. Alpha-defensins (α-Defs) in Crohn's disease: Decrease of ileal α-Def 5 via permanent methylation and increase in plasma α-Def 1-3 concentrations offering biomarker utility. *Clin. Exp. Immunol.* **2018**, *192*, 120–128. [CrossRef]

39. Sthoeger, Z.M.; Bezalel, S.; Chapnik, N.; Asher, I.; Froy, O. High alpha-defensin levels in patients with systemic lupus erythematosus. *Immunology* **2009**, *127*, 116–122. [CrossRef]
40. Lisitsyn, N.A.; Bukurova, Y.A.; Nikitina, I.G.; Krasnov, G.S.; Sykulev, Y.; Beresten, S.F. Enteric alpha defensins in norm and pathology. *Ann. Clin. Microbiol. Antimicrob.* **2012**, *11*, 1. [CrossRef]
41. Bonamy, C.; Sechet, E.; Amiot, A.; Alam, A.; Mourez, M.; Fraisse, L.; Sansonetti, P.J.; Sperandio, B. Expression of the human antimicrobial peptide β-defensin-1 is repressed by the EGFR-ERK-MYC axis in colonic epithelial cells. *Sci. Rep.* **2018**, *8*, 18043. [CrossRef] [PubMed]
42. Ling, Y.M.; Chen, J.Y.; Guo, L.; Wang, C.Y.; Tan, W.T.; Wen, Q.; Zhang, S.D.; Deng, G.H.; Lin, Y.; Kwok, H.F. β-defensin 1 expression in HCV infected liver/liver cancer: An important role in protecting HCV progression and liver cancer development. *Sci. Rep.* **2017**, *7*, 13404. [CrossRef] [PubMed]
43. Dilek, F.; Emin, Ö.; Gültepe, B.; Yazıcı, M.; Çakır, E.; Gedik, A.H. Evaluation of nasal fluid β-defensin 2 levels in children with allergic rhinitis. *Turk. Pediatri. Ars.* **2017**, *52*, 79–84. [CrossRef] [PubMed]
44. Andresen, E.; Günther, G.; Bullwinkel, J.; Lange, C.; Heine, H. Increased expression of beta-defensin 1 (DEFB1) in chronic obstructive pulmonary disease. *PLoS ONE* **2011**, *6*, e21898. [CrossRef] [PubMed]
45. Shi, N.; Jin, F.; Zhang, X.; Clinton, S.K.; Pan, Z.; Chen, T. Overexpression of human β-defensin 2 promotes growth and invasion during esophageal carcinogenesis. *Oncotarget* **2014**, *5*, 11333–11344. [CrossRef] [PubMed]
46. Xu, D.; Zhang, B.; Liao, C.; Zhang, W.; Wang, W.; Chang, Y.; Shao, Y. Human beta-defensin 3 contributes to the carcinogenesis of cervical cancer via activation of NF-κB signaling. *Oncotarget* **2016**, *7*, 75902–75913. [CrossRef]
47. Abe, S.; Miura, K.; Kinoshita, A.; Mishima, H.; Miura, S.; Yamasaki, K.; Hasegawa, Y.; Higashijima, A.; Jo, O.; Sasaki, K.; et al. Copy number variation of the antimicrobial-gene, defensin beta 4, is associated with susceptibility to cervical cancer. *J. Hum. Genet.* **2013**, *58*, 250–253. [CrossRef]
48. Kalmodia, S.; Son, K.N.; Cao, D.; Lee, B.S.; Surenkhuu, B.; Shah, D.; Ali, M.; Balasubramaniam, A.; Jain, S.; Aakalu, V.K. Presence of Histatin-1 in Human Tears and Association with Aqueous Deficient Dry Eye Diagnosis: A Preliminary Study. *Sci. Rep.* **2019**, *9*, 10304. [CrossRef]
49. Khan, S.A.; Fidel, P.L.; Thunayyan, A.A.; Varlotta, S.; Meiller, T.F.; Jabra-Rizk, M.A. Impaired Histatin-5 Levels and Salivary Antimicrobial Activity against. *J. AIDS Clin. Res.* **2013**, *4*. [CrossRef]
50. Bogefors, J.; Kvarnhammar, A.M.; Millrud, C.R.; Georén, S.K.; Cardell, L.O. LEAP-2, LL-37 and RNase7 in tonsillar tissue: Downregulated expression in seasonal allergic rhinitis. *Pathog. Dis.* **2014**, *72*, 55–60. [CrossRef]
51. Spencer, J.D.; Schwaderer, A.L.; Wang, H.; Bartz, J.; Kline, J.; Eichler, T.; DeSouza, K.R.; Sims-Lucas, S.; Baker, P.; Hains, D.S. Ribonuclease 7, an antimicrobial peptide upregulated during infection, contributes to microbial defense of the human urinary tract. *Kidney Int.* **2013**, *83*, 615–625. [CrossRef] [PubMed]
52. Hoffmann, R.; Bulet, P.; Urge, L.; Otvos, L., Jr. Range of activity and metabolic stability of synthetic antibacterial glycopeptides from insects. *Biochim. Biophys. Acta* **1999**, *1426*, 459–467. [CrossRef]
53. Weinberg, A.; Jin, G.; Sieg, S.; McCormick, T.S. The yin and yang of human Beta-defensins in health and disease. *Front. Immunol.* **2012**, *3*, 294. [CrossRef] [PubMed]
54. Haney, E.F.; Hancock, R.E. Peptide design for antimicrobial and immunomodulatory applications. *Biopolymers* **2013**, *100*, 572–583. [CrossRef]
55. Yang, D.; Chen, Q.; Hoover, D.M.; Staley, P.; Tucker, K.D.; Lubkowski, J.; Oppenheim, J.J. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J. Leukoc. Biol.* **2003**, *74*, 448–455. [CrossRef]
56. Vongsa, R.A.; Zimmerman, N.P.; Dwinell, M.B. CCR6 regulation of the actin cytoskeleton orchestrates human beta defensin-2- and CCL20-mediated restitution of colonic epithelial cells. *J. Biol. Chem.* **2009**, *284*, 10034–10045. [CrossRef]
57. Dorschner, R.A.; Lopez-Garcia, B.; Peschel, A.; Kraus, D.; Morikawa, K.; Nizet, V.; Gallo, R.L. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. *FASEB J.* **2006**, *20*, 35–42. [CrossRef]
58. Venkataraman, N.; Cole, A.L.; Ruchala, P.; Waring, A.J.; Lehrer, R.I.; Stuchlik, O.; Pohl, J.; Cole, A.M. Reawakening retrocyclins: Ancestral human defensins active against HIV-1. *PLoS Biol.* **2009**, *7*, e95. [CrossRef]

59. Liu, L.; Zhao, C.; Heng, H.H.; Ganz, T. The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: Two peptide families with differing disulfide topology share a common ancestry. *Genomics* **1997**, *43*, 316–320. [CrossRef]
60. Schneider, J.J.; Unholzer, A.; Schaller, M.; Schäfer-Korting, M.; Korting, H.C. Human defensins. *J. Mol. Med.* **2005**, *83*, 587–595. [CrossRef]
61. Ganz, T.; Selsted, M.E.; Szklarek, D.; Harwig, S.S.; Daher, K.; Bainton, D.F.; Lehrer, R.I. Defensins. Natural peptide antibiotics of human neutrophils. *J. Clin. Invest.* **1985**, *76*, 1427–1435. [CrossRef] [PubMed]
62. Bevins, C.L. Paneth cell defensins: Key effector molecules of innate immunity. *Biochem. Soc. Trans.* **2006**, *34*, 263–266. [CrossRef] [PubMed]
63. Lehrer, R.I.; Lichtenstein, A.K.; Ganz, T. Defensins: Antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* **1993**, *11*, 105–128. [CrossRef] [PubMed]
64. Faurschou, M.; Kamp, S.; Cowland, J.B.; Udby, L.; Johnsen, A.H.; Calafat, J.; Winther, H.; Borregaard, N. Prodefensins are matrix proteins of specific granules in human neutrophils. *J. Leukoc. Biol.* **2005**, *78*, 785–793. [CrossRef] [PubMed]
65. Katoh, K.; Rozewicki, J.; Yamada, K.D. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* **2019**, *20*, 1160–1166. [CrossRef]
66. Czech, L.; Huerta-Cepas, J.; Stamatakis, A. A Critical Review on the Use of Support Values in Tree Viewers and Bioinformatics Toolkits. *Mol. Biol. Evol.* **2017**, *34*, 1535–1542. [CrossRef]
67. Letunic, I.; Bork, P. Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* **2016**, *44*, W242–W245. [CrossRef]
68. Lear, S.; Cobb, S.L. Pep-Calc.com: A set of web utilities for the calculation of peptide and peptoid properties and automatic mass spectral peak assignment. *J. Comput. Aided Mol. Des.* **2016**, *30*, 271–277. [CrossRef]
69. Suarez-Carmona, M.; Hubert, P.; Delvenne, P.; Herfs, M. Defensins: "Simple" antimicrobial peptides or broad-spectrum molecules? *Cytokine Growth Factor Rev.* **2015**, *26*, 361–370. [CrossRef]
70. Ganz, T. Defensins: Antimicrobial peptides of innate immunity. *Nat. Rev. Immunol* **2003**, *3*, 710–720. [CrossRef]
71. Dhople, V.; Krukemeyer, A.; Ramamoorthy, A. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochim. Biophys. Acta* **2006**, *1758*, 1499–1512. [CrossRef] [PubMed]
72. Hollox, E.J.; Armour, J.A.; Barber, J.C. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. *Am. J. Hum. Genet.* **2003**, *73*, 591–600. [CrossRef] [PubMed]
73. Bucki, R.; Leszczynska, K.; Namiot, A.; Sokolowski, W. Cathelicidin LL-37: A multitask antimicrobial peptide. *Arch. Immunol Ther. Exp.* **2010**, *58*, 15–25. [CrossRef] [PubMed]
74. Kościuczuk, E.M.; Lisowski, P.; Jarczak, J.; Strzałkowska, N.; Jóźwik, A.; Horbańczuk, J.; Krzyżewski, J.; Zwierzchowski, L.; Bagnicka, E. Cathelicidins: Family of antimicrobial peptides. A review. *Mol. Biol. Rep.* **2012**, *39*, 10957–10970. [CrossRef] [PubMed]
75. Murakami, M.; Lopez-Garcia, B.; Braff, M.; Dorschner, R.A.; Gallo, R.L. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. *J. Immunol.* **2004**, *172*, 3070–3077. [CrossRef]
76. Murakami, M.; Kameda, K.; Tsumoto, H.; Tsuda, T.; Masuda, K.; Utsunomiya, R.; Mori, H.; Miura, Y.; Sayama, K. TLN-58, an Additional hCAP18 Processing Form, Found in the Lesion Vesicle of Palmoplantar Pustulosis in the Skin. *J. Invest. Dermatol.* **2017**, *137*, 322–331. [CrossRef]
77. Gornowicz, A.; Tokajuk, G.; Bielawska, A.; Maciorkowska, E.; Jabłoński, R.; Wójcicka, A.; Bielawski, K. The assessment of sIgA, histatin-5, and lactoperoxidase levels in saliva of adolescents with dental caries. *Med. Sci. Monit.* **2014**, *20*, 1095–1100. [CrossRef]
78. Güncü, G.N.; Yilmaz, D.; Könönen, E.; Gürsoy, U.K. Salivary Antimicrobial Peptides in Early Detection of Periodontitis. *Front. Cell Infect. Microbiol.* **2015**, *5*, 99. [CrossRef]
79. Spencer, J.D.; Schwaderer, A.L.; Dirosario, J.D.; McHugh, K.M.; McGillivary, G.; Justice, S.S.; Carpenter, A.R.; Baker, P.B.; Harder, J.; Hains, D.S. Ribonuclease 7 is a potent antimicrobial peptide within the human urinary tract. *Kidney Int.* **2011**, *80*, 174–180. [CrossRef]
80. Lee, P.; Peng, H.; Gelbart, T.; Wang, L.; Beutler, E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc. Natl Acad Sci. USA* **2005**, *102*, 1906–1910. [CrossRef]
81. Nagaoka, I.; Suzuki, K.; Niyonsaba, F.; Tamura, H.; Hirata, M. Modulation of neutrophil apoptosis by antimicrobial peptides. *ISRN Microbiol.* **2012**, *2012*, 345791. [CrossRef] [PubMed]

82. Brook, M.; Tomlinson, G.H.; Miles, K.; Smith, R.W.; Rossi, A.G.; Hiemstra, P.S.; van 't Wout, E.F.; Dean, J.L.; Gray, N.K.; Lu, W.; et al. Neutrophil-derived alpha defensins control inflammation by inhibiting macrophage mRNA translation. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 4350–4355. [CrossRef] [PubMed]
83. Meade, K.G.; O'Farrelly, C. beta-Defensins: Farming the Microbiome for Homeostasis and Health. *Front. Immunol.* **2018**, *9*, 3072. [CrossRef] [PubMed]
84. Agier, J.; Efenberger, M.; Brzezinska-Blaszczyk, E. Cathelicidin impact on inflammatory cells. *Cent. Eur. J. Immunol.* **2015**, *40*, 225–235. [CrossRef] [PubMed]
85. Mangoni, M.L.; Miele, R.; Renda, T.G.; Barra, D.; Simmaco, M. The synthesis of antimicrobial peptides in the skin of Rana esculenta is stimulated by microorganisms. *FASEB J.* **2001**, *15*, 1431–1432. [CrossRef] [PubMed]
86. Morioka, Y.; Yamasaki, K.; Leung, D.; Gallo, R.L. Cathelicidin antimicrobial peptides inhibit hyaluronan-induced cytokine release and modulate chronic allergic dermatitis. *J. Immunol.* **2008**, *181*, 3915–3922. [CrossRef]
87. Szklarczyk, D.; Gable, A.L.; Lyon, D.; Junge, A.; Wyder, S.; Huerta-Cepas, J.; Simonovic, M.; Doncheva, N.T.; Morris, J.H.; Bork, P.; et al. STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* **2019**, *47*, D607–D613. [CrossRef]
88. Dixon, B.R.; Radin, J.N.; Piazuelo, M.B.; Contreras, D.C.; Algood, H.M. IL-17a and IL-22 Induce Expression of Antimicrobials in Gastrointestinal Epithelial Cells and May Contribute to Epithelial Cell Defense against *Helicobacter pylori*. *PLoS ONE* **2016**, *11*, e0148514. [CrossRef]
89. Zambrano-Zaragoza, J.F.; Romo-Martinez, E.J.; Duran-Avelar Mde, J.; Garcia-Magallanes, N.; Vibanco-Perez, N. Th17 cells in autoimmune and infectious diseases. *Int. J. Inflam.* **2014**, *2014*, 651503. [CrossRef]
90. Hollox, E.J.; Huffmeier, U.; Zeeuwen, P.L.; Palla, R.; Lascorz, J.; Rodijk-Olthuis, D.; van de Kerkhof, P.C.; Traupe, H.; de Jongh, G.; den Heijer, M.; et al. Psoriasis is associated with increased beta-defensin genomic copy number. *Nat. Genet.* **2008**, *40*, 23–25. [CrossRef]
91. Fellermann, K.; Stange, D.E.; Schaeffeler, E.; Schmalzl, H.; Wehkamp, J.; Bevins, C.L.; Reinisch, W.; Teml, A.; Schwab, M.; Lichter, P.; et al. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. *Am. J. Hum. Genet.* **2006**, *79*, 439–448. [CrossRef] [PubMed]
92. Chen, Q.; Yang, Y.; Hou, J.; Shu, Q.; Yin, Y.; Fu, W.; Han, F.; Hou, T.; Zeng, C.; Nemeth, E.; et al. Increased gene copy number of *DEFA1/DEFA3* worsens sepsis by inducing endothelial pyroptosis. *Proc. Natl. Acad Sci. USA* **2019**, *116*, 3161–3170. [CrossRef] [PubMed]
93. Huang, Y.P.; Wang, T.Y.; Wang, W.; Sun, H.Z. Association between Genetic Polymorphisms in DEFB1 and Susceptibility to Digestive Diseases. *Med. Sci. Monit.* **2015**, *21*, 2240–2250. [CrossRef] [PubMed]
94. Polesello, V.; Zupin, L.; Di Lenarda, R.; Biasotto, M.; Ottaviani, G.; Gobbo, M.; Cecco, L.; Alberi, G.; Pozzato, G.; Crovella, S.; et al. Impact of DEFB1 gene regulatory polymorphisms on hBD-1 salivary concentration. *Arch. Oral Biol.* **2015**, *60*, 1054–1058. [CrossRef] [PubMed]
95. Atreya, I.; Atreya, R.; Neurath, M.F. NF-kappaB in inflammatory bowel disease. *J. Intern. Med.* **2008**, *263*, 591–596. [CrossRef]
96. Mookherjee, N.; Rehaume, L.M.; Hancock, R.E. Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin. Ther. Targets* **2007**, *11*, 993–1004. [CrossRef]
97. Wang, T.T.; Nestel, F.P.; Bourdeau, V.; Nagai, Y.; Wang, Q.; Liao, J.; Tavera-Mendoza, L.; Lin, R.; Hanrahan, J.W.; Mader, S.; et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* **2004**, *173*, 2909–2912. [CrossRef]
98. Mandal, S.M.; Manna, S.; Mondal, S.; Ghosh, A.K.; Chakraborty, R. Transcriptional regulation of human defense peptides: A new direction in infection control. *Biol. Chem.* **2018**, *399*, 1277–1284. [CrossRef]
99. Noh, Y.H.; Lee, J.; Seo, S.J.; Myung, S.C. Promoter DNA methylation contributes to human β -defensin-1 deficiency in atopic dermatitis. *Anim. Cells Syst.* **2018**, *22*, 172–177. [CrossRef]
100. Sun, C.Q.; Arnold, R.; Fernandez-Golarz, C.; Parrish, A.B.; Almekinder, T.; He, J.; Ho, S.M.; Svoboda, P.; Pohl, J.; Marshall, F.F.; et al. Human beta-defensin-1, a potential chromosome 8p tumor suppressor: Control of transcription and induction of apoptosis in renal cell carcinoma. *Cancer Res.* **2006**, *66*, 8542–8549. [CrossRef]

101. Winter, J.; Pantelis, A.; Reich, R.; Martini, M.; Kraus, D.; Jepsen, S.; Allam, J.P.; Novak, N.; Wenghoefer, M. Human beta-defensin-1, -2, and -3 exhibit opposite effects on oral squamous cell carcinoma cell proliferation. *Cancer Invest.* **2011**, *29*, 196–201. [CrossRef] [PubMed]
102. Takahashi, T.; Gallo, R.L. The Critical and Multifunctional Roles of Antimicrobial Peptides in Dermatology. *Dermatol. Clin.* **2017**, *35*, 39–50. [CrossRef] [PubMed]
103. Niyonsaba, F.; Ushio, H.; Hara, M.; Yokoi, H.; Tominaga, M.; Takamori, K.; Kajiwara, N.; Saito, H.; Nagaoka, I.; Ogawa, H.; et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J. Immunol.* **2010**, *184*, 3526–3534. [CrossRef] [PubMed]
104. Smithrithee, R.; Niyonsaba, F.; Kiatsurayanon, C.; Ushio, H.; Ikeda, S.; Okumura, K.; Ogawa, H. Human β-defensin-3 increases the expression of interleukin-37 through CCR6 in human keratinocytes. *J. Dermatol. Sci.* **2015**, *77*, 46–53. [CrossRef]
105. Yamasaki, K.; Gallo, R.L. Rosacea as a disease of cathelicidins and skin innate immunity. *J. Investig. Dermatol. Symp. Proc.* **2011**, *15*, 12–15. [CrossRef]
106. Schauber, J.; Gallo, R.L. Antimicrobial peptides and the skin immune defense system. *J. Allergy Clin. Immunol.* **2008**, *122*, 261–266. [CrossRef]
107. Fagerberg, L.; Hallström, B.M.; Oksvold, P.; Kampf, C.; Djureinovic, D.; Odeberg, J.; Habuka, M.; Tahmasebpoor, S.; Danielsson, A.; Edlund, K.; et al. Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol. Cell Proteomics* **2014**, *13*, 397–406. [CrossRef]
108. Suresh, R.; Mosser, D.M. Pattern recognition receptors in innate immunity, host defense, and immunopathology. *Adv. Physiol. Educ.* **2013**, *37*, 284–291. [CrossRef]
109. Skovbakke, S.L.; Holdfeldt, A.; Forsman, H.; Bylund, J.; Franzyk, H. The Role of Formyl Peptide Receptors for Immunomodulatory Activities of Antimicrobial Peptides and Peptidomimetics. *Curr. Pharm. Des.* **2018**, *24*, 1100–1120. [CrossRef]
110. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* **2009**, *22*, 240–273. [CrossRef]
111. Schaefer, L. Complexity of danger: The diverse nature of damage-associated molecular patterns. *J. Biol. Chem.* **2014**, *289*, 35237–35245. [CrossRef] [PubMed]
112. Sun, C.Q.; Arnold, R.S.; Hsieh, C.L.; Dorin, J.R.; Lian, F.; Li, Z.; Petros, J.A. Discovery and mechanisms of host defense to oncogenesis: Targeting the beta-defensin-1 peptide as a natural tumor inhibitor. *Cancer Biol. Ther.* **2019**, *20*, 774–786. [CrossRef] [PubMed]
113. Cui, D.; Lyu, J.; Li, H.; Lei, L.; Bian, T.; Li, L.; Yan, F. Human β-defensin 3 inhibits periodontitis development by suppressing inflammatory responses in macrophages. *Mol. Immunol.* **2017**, *91*, 65–74. [CrossRef] [PubMed]
114. Funderburg, N.; Lederman, M.M.; Feng, Z.; Drage, M.G.; Jadlowsky, J.; Harding, C.V.; Weinberg, A.; Sieg, S.F. Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 18631–18635. [CrossRef] [PubMed]
115. Biragyn, A.; Ruffini, P.A.; Leifer, C.A.; Klyushnenkova, E.; Shakhov, A.; Chertov, O.; Shirakawa, A.K.; Farber, J.M.; Segal, D.M.; Oppenheim, J.J.; et al. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **2002**, *298*, 1025–1029. [CrossRef] [PubMed]
116. Carta, S.; Semino, C.; Sitia, R.; Rubartelli, A. Dysregulated IL-1β Secretion in Autoinflammatory Diseases: A Matter of Stress? *Front. Immunol.* **2017**, *8*, 345. [CrossRef] [PubMed]
117. Shi, J.; Aono, S.; Lu, W.; Ouellette, A.J.; Hu, X.; Ji, Y.; Wang, L.; Lenz, S.; van Ginkel, F.W.; Liles, M.; et al. A novel role for defensins in intestinal homeostasis: Regulation of IL-1beta secretion. *J. Immunol.* **2007**, *179*, 1245–1253. [CrossRef]
118. Tewary, P.; de la Rosa, G.; Sharma, N.; Rodriguez, L.G.; Tarasov, S.G.; Howard, O.M.; Shirota, H.; Steinhagen, F.; Klinman, D.M.; Yang, D.; et al. β-Defensin 2 and 3 promote the uptake of self or CpG DNA, enhance IFN-α production by human plasmacytoid dendritic cells, and promote inflammation. *J. Immunol.* **2013**, *191*, 865–874. [CrossRef]
119. Lande, R.; Gregorio, J.; Facchinetti, V.; Chatterjee, B.; Wang, Y.H.; Homey, B.; Cao, W.; Wang, Y.H.; Su, B.; Nestle, F.O.; et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **2007**, *449*, 564–569. [CrossRef]

120. Lande, R.; Chamilos, G.; Ganguly, D.; Demaria, O.; Frasca, L.; Durr, S.; Conrad, C.; Schroder, J.; Gilliet, M. Cationic antimicrobial peptides in psoriatic skin cooperate to break innate tolerance to self-DNA. *Eur. J. Immunol.* **2015**, *45*, 203–213. [CrossRef]
121. McAleer, J.P.; Kolls, J.K. Mechanisms controlling Th17 cytokine expression and host defense. *J. Leukoc. Biol.* **2011**, *90*, 263–270. [CrossRef] [PubMed]
122. Takahashi, T.; Kulkarni, N.N.; Lee, E.Y.; Zhang, L.J.; Wong, G.C.L.; Gallo, R.L. Cathelicidin promotes inflammation by enabling binding of self-RNA to cell surface scavenger receptors. *Sci. Rep.* **2018**, *8*, 4032. [CrossRef] [PubMed]
123. Kopfnagel, V.; Wagenknecht, S.; Harder, J.; Hofmann, K.; Kleine, M.; Buch, A.; Sodeik, B.; Werfel, T. RNase 7 Strongly Promotes TLR9-Mediated DNA Sensing by Human Plasmacytoid Dendritic Cells. *J. Invest. Dermatol.* **2018**, *138*, 872–881. [CrossRef] [PubMed]
124. Adase, C.A.; Borkowski, A.W.; Zhang, L.J.; Williams, M.R.; Sato, E.; Sanford, J.A.; Gallo, R.L. Non-coding Double-stranded RNA and Antimicrobial Peptide LL-37 Induce Growth Factor Expression from Keratinocytes and Endothelial Cells. *J. Biol. Chem.* **2016**, *291*, 11635–11646. [CrossRef] [PubMed]
125. Neumann, A.; Vollger, L.; Berends, E.T.; Molhoek, E.M.; Stapels, D.A.; Midon, M.; Friaes, A.; Pingoud, A.; Rooijakkers, S.H.; Gallo, R.L.; et al. Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases. *J. Innate Immun.* **2014**, *6*, 860–868. [CrossRef]
126. Delgado-Rizo, V.; Martinez-Guzman, M.A.; Iniguez-Gutierrez, L.; Garcia-Orozco, A.; Alvarado-Navarro, A.; Fafutis-Morris, M. Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. *Front. Immunol.* **2017**, *8*, 81. [CrossRef]
127. Stephan, A.; Batinica, M.; Steiger, J.; Hartmann, P.; Zaucke, F.; Bloch, W.; Fabri, M. LL37:DNA complexes provide antimicrobial activity against intracellular bacteria in human macrophages. *Immunology* **2016**, *148*, 420–432. [CrossRef]
128. Torres-Juarez, F.; Cardenas-Vargas, A.; Montoya-Rosales, A.; González-Curiel, I.; Garcia-Hernandez, M.H.; Enciso-Moreno, J.A.; Hancock, R.E.; Rivas-Santiago, B. LL-37 immunomodulatory activity during *Mycobacterium tuberculosis* infection in macrophages. *Infect. Immun.* **2015**, *83*, 4495–4503. [CrossRef]
129. Mily, A.; Rekha, R.S.; Kamal, S.M.; Arifuzzaman, A.S.; Rahim, Z.; Khan, L.; Haq, M.A.; Zaman, K.; Bergman, P.; Brighenti, S.; et al. Significant Effects of Oral Phenylbutyrate and Vitamin D3 Adjunctive Therapy in Pulmonary Tuberculosis: A Randomized Controlled Trial. *PLoS ONE* **2015**, *10*, e0138340. [CrossRef]
130. Khurshid, Z.; Naseem, M.; Sheikh, Z.; Najeeb, S.; Shahab, S.; Zafar, M.S. Oral antimicrobial peptides: Types and role in the oral cavity. *Saudi. Pharm. J.* **2016**, *24*, 515–524. [CrossRef]
131. Gursoy, U.K.; Könönen, E. Understanding the roles of gingival beta-defensins. *J. Oral Microbiol.* **2012**, 4. [CrossRef] [PubMed]
132. Costa, L.C.M.; Soldati, K.R.; Fonseca, D.C.; Costa, J.E.; Abreu, M.H.N.G.; Costa, F.O.; Zandim-Barcelos, D.L.; Cota, L.O.M. Gingival crevicular fluid levels of human beta-defensin 1 in individuals with and without chronic periodontitis. *J. Periodontal Res.* **2018**, *53*, 736–742. [CrossRef] [PubMed]
133. Jurczak, A.; Kościelniak, D.; Papież, M.; Vyhouskaya, P.; Krzyściak, W. A study on β-defensin-2 and histatin-5 as a diagnostic marker of early childhood caries progression. *Biol. Res.* **2015**, *48*, 61. [CrossRef] [PubMed]
134. Pereira, A.L.; Franco, G.C.; Cortelli, S.C.; Aquino, D.R.; Costa, F.O.; Raslan, S.A.; Cortelli, J.R. Influence of periodontal status and periodontopathogens on levels of oral human β-defensin-2 in saliva. *J. Periodontol.* **2013**, *84*, 1445–1453. [CrossRef] [PubMed]
135. Werner, S.; Grose, R. Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* **2003**, *83*, 835–870. [CrossRef]
136. Dommisch, H.; Winter, J.; Götz, W.; Miesen, J.; Klein, A.; Hierse, L.; Deschner, J.; Jäger, A.; Eberhard, J.; Jepsen, S. Effect of growth factors on antimicrobial peptides and pro-inflammatory mediators during wound healing. *Clin. Oral. Investig.* **2015**, *19*, 209–220. [CrossRef]
137. Abou Alaiwa, M.H.; Reznikov, L.R.; Gansemer, N.D.; Sheets, K.A.; Horswill, A.R.; Stoltz, D.A.; Zabner, J.; Welsh, M.J. pH modulates the activity and synergism of the airway surface liquid antimicrobials β-defensin-3 and LL-37. *Proc. Natl. Acad Sci. USA* **2014**, *111*, 18703–18708. [CrossRef]
138. Weiner, D.J.; Bucki, R.; Janmey, P.A. The antimicrobial activity of the cathelicidin LL37 is inhibited by F-actin bundles and restored by gelsolin. *Am. J. Respir. Cell Mol. Biol.* **2003**, *28*, 738–745. [CrossRef]

139. Bucki, R.; Byfield, F.J.; Janmey, P.A. Release of the antimicrobial peptide LL-37 from DNA/F-actin bundles in cystic fibrosis sputum. *Eur. Respir. J.* **2007**, *29*, 624–632. [CrossRef]
140. Liao, Z.; Dong, J.; Hu, X.; Wang, T.; Wan, C.; Li, X.; Li, L.; Guo, L.; Xu, D.; Wen, F. Enhanced expression of human β-defensin 2 in peripheral lungs of patients with chronic obstructive pulmonary disease. *Peptides* **2012**, *38*, 350–356. [CrossRef]
141. Dalcin, D.; Ulanova, M. The Role of Human Beta-Defensin-2 in *Pseudomonas aeruginosa* Pulmonary Infection in Cystic Fibrosis Patients. *Infect. Dis. Ther.* **2013**, *2*, 159–166. [CrossRef] [PubMed]
142. Archer, N.K.; Adappa, N.D.; Palmer, J.N.; Cohen, N.A.; Harro, J.M.; Lee, S.K.; Miller, L.S.; Shirtliff, M.E. Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of *Staphylococcus aureus* Nasal Colonization. *Infect. Immun.* **2016**, *84*, 3575–3583. [CrossRef] [PubMed]
143. Altieri, A.; Piyadasa, H.; Recksiedler, B.; Spicer, V.; Mookherjee, N. Cytokines IL-17, TNF and IFN-γ Alter the Expression of Antimicrobial Peptides and Proteins Disparately: A Targeted Proteomics Analysis using SOMAscan Technology. *Vaccines* **2018**, *6*. [CrossRef] [PubMed]
144. Kusagaya, H.; Fujisawa, T.; Yamanaka, K.; Mori, K.; Hashimoto, D.; Enomoto, N.; Inui, N.; Nakamura, Y.; Wu, R.; Maekawa, M.; et al. Toll-like receptor-mediated airway IL-17C enhances epithelial host defense in an autocrine/paracrine manner. *Am. J. Respir Cell Mol. Biol.* **2014**, *50*, 30–39. [CrossRef]
145. Li, A.; Gan, Y.; Wang, R.; Liu, Y.; Ma, T.; Huang, M.; Cui, X. IL-22 Up-Regulates β-Defensin-2 Expression in Human Alveolar Epithelium via STAT3 but Not NF-κB Signaling Pathway. *Inflammation* **2015**, *38*, 1191–1200. [CrossRef]
146. Baines, K.J.; Wright, T.K.; Simpson, J.L.; McDonald, V.M.; Wood, L.G.; Parsons, K.S.; Wark, P.A.; Gibson, P.G. Airway β-Defensin-1 Protein Is Elevated in COPD and Severe Asthma. *Mediators Inflamm.* **2015**, *2015*, 407271. [CrossRef]
147. Nakamura, K.; Sakuragi, N.; Takakuwa, A.; Ayabe, T. Paneth cell α-defensins and enteric microbiota in health and disease. *Biosci. Microbiota Food Health* **2016**, *35*, 57–67. [CrossRef]
148. Wehkamp, J.; Salzman, N.H.; Porter, E.; Nuding, S.; Weichenthal, M.; Petras, R.E.; Shen, B.; Schaeffeler, E.; Schwab, M.; Linzmeier, R.; et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc. Natl. Acad. Sci. USA.* **2005**, *102*, 18129–18134. [CrossRef]
149. Kanmura, S.; Hamamoto, H.; Morinaga, Y.; Oda, K.; Fujita, T.; Arima, S.; Nasu, Y.; Sasaki, F.; Hashimoto, S.; Taguchi, H.; et al. Fecal Human Neutrophil Peptide Levels Correlate with Intestinal Inflammation in Ulcerative Colitis. *Digestion* **2016**, *93*, 300–308. [CrossRef]
150. Pero, R.; Coretti, L.; Nigro, E.; Lembo, F.; Laneri, S.; Lombardo, B.; Daniele, A.; Scudiero, O. β-Defensins in the Fight against *Helicobacter pylori*. *Molecules* **2017**, *22*, 424. [CrossRef]
151. Muhammad, J.S.; Zaidi, S.F.; Zhou, Y.; Sakurai, H.; Sugiyama, T. Novel epidermal growth factor receptor pathway mediates release of human β-defensin 3 from *Helicobacter pylori*-infected gastric epithelial cells. *Pathog. Dis.* **2016**, *74*. [CrossRef]

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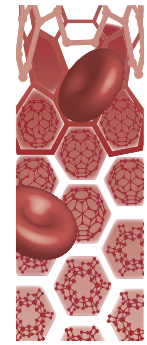

Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins

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Aim: To evaluate the antibacterial and antibiofilm activity of ceragenin-conjugated nonspherical gold nanoparticles against the most common agents of otitis media. **Methods:** Minimal inhibitory and bactericidal concentrations and colony-counting assays, as well as colorimetric and fluorimetric methods, were used to estimate the antibacterial activity of compounds in phosphate-buffered saline and human cerumen. The nanosystems' biocompatibility and ability to decrease IL-8 release was tested using keratinocyte cells. **Results:** The tested compounds demonstrated strong antimicrobial activity against planktonic and biofilm cultures at nontoxic doses due to the induction of oxidative stress followed by the damage of bacterial membranes. **Conclusion:** This study indicates that ceragenin-conjugated nonspherical gold nanoparticles have potential as new treatment methods for eradicating biofilm-forming pathogens associated with otitis media.

Lay abstract: Middle-ear infections can be painful and cause hearing difficulties. If untreated, they can lead to hearing loss. These infections are usually treated with antibiotic drugs. However, the microbes causing the infection can gain drug resistance. This article reports research into a new way of delivering antibiotics to kill the microbes and the communities they form (biofilms). The authors developed tiny gold particles loaded with the antimicrobial drug ceragenin and tested the drug-loaded particles on three common middle-ear infection-causing bacteria. Compared with ceragenin alone, the ceragenin-loaded particles were better at killing the bacteria and their biofilm communities.

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Keywords: ceragenins • cerumen • nanosystems • nonspherical nanoparticles • otitis media

Otitis media (OM) is a clinical condition characterized by the presence of an inflammatory reaction in the middle ear caused by a variety of infectious agents. It can be classified as acute OM (AOM) or chronic OM (COM), including OM with effusion (OME) distinguished by the presence of liquid in the cavities of the middle ear without signs of acute infection [1]. Approximately 50% of children have at least one episode of AOM before school age, with the highest incidence between 3 and 24 months of age, as a consequence of a shorter and more horizontal eustachian tube, which predisposes to ascending route of the middle ear infection by pathogens from the nasopharynx [2]. Aside from the anatomical causes, a defective immune system favoring the colonization of potential pathogens pose a risk in the pathogenesis of AOM [3]. A recent study revealed that 98% of children aged ≥2 years with AOM received antibiotic treatment with amoxicillin being routinely prescribed [4]. Clinically, AOM is characterized by ear pain, fever and temporary hearing difficulties. Unresolved and recurrent OM episodes

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may lead to severe complications, speech development problems, prolonged hearing loss, learning disabilities and decreased performance in educational skills [5,6].

Although the etiology of AOM is multifactorial, encompassing bacteria, viruses or mixed infections, the 'classical otopathogens' (i.e., *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*), are still of the greatest clinical importance [7,8]. These pathogens persist in the form of mono- and multispecies biofilms, and their location in the extracellular matrix of the middle ear or nasopharynx leaves them protected from antibiotics due to poor penetration. The clinical importance of an untreated biofilm mass is implicated in recurrent AOM and COM [8–10]. Interestingly, although the introduction in 2000 of heptavalent *S. pneumoniae* vaccine (PCV7) followed by a 13-valent pneumococcal conjugate vaccine (PCV13) initially reduced the relative prevalence of this pathogen in AOM, an increase of nonvaccine serotypes was observed, and a similar trend may be expected for serotypes not included in emerging pneumococcal vaccines [1,11–13]. It is also noteworthy that viral upper respiratory tract infections, caused mainly by respiratory syncytial virus, cytomegalovirus, influenza viruses, adenoviruses and coronaviruses, often precede or coincide with episodes of AOM. In particular, adenoviruses inhabit the nasopharyngeal niche and possibly gain entry to the middle ear upon interaction with other bacterial otopathogens [14]. To date, a spectrum of effective treatment options is available for AOM, based on systemic administration of beta-lactam antibiotics, such as amoxicillin or amoxicillin/clavulanate and cephalosporins as the first-line therapy or macrolides and lincosamides as alternative drugs [1]. Nevertheless, several issues in OM therapy must be addressed, including the growing resistance of otopathogens to these antibiotics, in particular cephalosporins, and a risk that they pose for development of resistance in bacteria [13]; the aforementioned implication of viral etiology; and difficulties with treatment of polymicrobial biofilms. Moreover, the prevalence of AOM and the continuous emergence of drug-resistant organisms highlight the need for development of new treatment options and their alternative methods of delivery to the middle ear, such as transtympanic or intratympanic pathways, which would potentially improve the outcome of treatment and prevent the systemic administration of antibiotics to mitigate a selection of resistant pathogens [15,16].

Ceragenins represent a novel class of low molecular weight antimicrobial agents designed as nonpeptide and low-cost mimics of endogenous antimicrobial peptides (AMPs) [17]. Chemically, ceragenins are positively charged derivatives of cholic acid, and therefore also called cationic steroid antimicrobials (CSAs); they are distinguished by various number and distribution of amine groups (e.g., CSA-13, CSA-44 or CSA-144). Like AMPs, ceragenins are broad-spectrum antimicrobials with potent killing action against multiple bacterial, fungal, protozal as well as viral pathogens, including multidrug-resistant organisms [18]. For example, we previously reported the activity of CSAs against various oral and upper respiratory tract infection pathogens, including the classical otopathogens, for which minimal inhibitory (MIC) and bactericidal (MBC) concentrations were below 2 μg/ml [19]. Similarly, Moscoso *et al.* reported a high activity of CSA-13 against both planktonic cultures and biofilms of *S. pneumoniae* [20]. In addition, ceragenins exhibit potent microbicidal properties against polymicrobial *Candida albicans* and *Staphylococcus aureus* biofilms [21]. CSA's antimicrobial action is exerted through a direct interaction with the negatively charged bacterial membrane components and lipid bilayers in the case of viruses, leading to morphological changes and disruption [18,22,23]. Importantly, a high level of resistance of ceragenins to inhibitory action of the host compounds present at high concentrations in inflamed sites such as mucin, DNA or F-actin [24–26], makes them a strong candidate for novel agents to treat OM. Finally, the fact that bacteria do not easily develop resistance to ceragenins makes them suitably attractive as long-term antimicrobial agents for treatment of various resistant pathogens [27,28].

Nanoparticles have recently emerged as promising antimicrobial agents themselves and as carriers for other antimicrobials owing to their unique physicochemical properties, in particular a large surface area-to-volume ratio that ensures their strong interactions with microbial membranes [29]. Among a spectrum of nanomaterials available to date, metallic nanoparticles, including those made of gold, have gained considerable interest due to their stability, good biocompatibility, favorable pharmacokinetic parameters [30], potent antimicrobial activity [31,32], anti-inflammatory potential [33] and synergistic interactions with commonly used antibiotics [34]. An ever-growing number of studies also demonstrate high applicability of gold nanoparticles (AuNPs) as drug nanocarriers. In such applications, in recent years AuNPs were functionalized with aminoglycosides, amoxicillin or auranofin to achieve satisfactory therapeutic efficiency against burn wound-isolated *Acinetobacter baumannii* [35], MRSA [36] or *S. pneumoniae* and *S. pyogenes* [37], which legitimates the validity of such approach. Nevertheless, these studies were performed using spherical AuNPs. A thorough analysis of antimicrobial activity of AuNPs performed to date clearly indicates that biological interactions of AuNPs are governed by a spectrum of physicochemical features – in particular, their size, shape (spherical, rod, cube, square, flake, irregular etc.) – and surface charge or type of

material [38,39]. Therefore, one of the contemporary trends in the nanoantibiotic field is to explore the functional properties and effects of different geometrical shapes of nanoparticles for the treatment of resistant pathogens [40] and our recent studies confirm that nonspherical nanomaterials should be considered as the potent antimicrobials [41,42]. Data collected to date also demonstrate the possibility of employing those nanomaterials as drug nanocarriers. Most recently, we discovered that AuNPs in the shape of rods, peanuts or stars are effective nanocarriers of ceragenin CSA-131 and through increased ceragenin density on the surface of AuNPs, improved therapeutic efficiency against multidrug-resistant strains can be accomplished [43]. Nevertheless, there are still limited data on the activity of nonspherical, ceragenin-containing nanosystems in the presence of body fluids or their ability to decrease inflammatory burden. For this reason, the current study is aimed at investigating the antibacterial activity of ceragenins CSA-13, CSA-131 and CSA-44 alone and conjugated with peanut-shaped AuNPs (AuP NPs) against planktonic cells and biofilms produced by *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and to investigate the anti-inflammatory activities of these compounds. To that end, microbiological assays were performed in artificial media as well as in the presence of human cerumen to estimate the potential utility of these compounds for the local treatment of ear infections. Simultaneously, bacterial responses to the tested agents were captured by reactive oxygen species (ROS) generation, outer membrane permeability and protein leakage assays. Moreover, their anti-inflammatory potential was estimated by measurement of IL-8 production by bacteria-stimulated cells of keratinocyte cell line.

Materials & methods

Materials

Reagents required for ceragenin-attached gold nanosystems [43] were purchased from Sigma-Aldrich (MO, USA). Laboratory strains of OM-causing pathogens: *H. influenzae* ATCC 49766, *M. catarrhalis* ATCC 25238, *Streptococcus pneumoniae* ATCC 49619 were from American Type Culture Collection (VA, USA). Growth media were as follows: chocolate II Agar, blood No 2 LAB-AGAR+ 5% KB, brain heart infusion broth medium and required supplements: β-nicotinamide adenine dinucleotide (NAD) and hematin porcine were obtained from Becton Dickson (NJ, USA), BioMaxima (Lublin, Poland) and Sigma-Aldrich, respectively. Brain heart infusion broth medium was purchased from Sigma-Aldrich. Reagents for colorimetric and fluorimetric analysis: 1-*N*-phenylnaphtylamine (NPN), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), Bradford reagent and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich. IL-8 Human ELISA Kit was purchased from Thermo Fisher Scientific (MA, USA).

Synthesis & physicochemical analysis of ceragenin-decorated nanosystems

Ceragenins (CSA-13, CSA-131, CSA-44) were synthesized as previously reported [44], dissolved in phosphate-buffered saline (PBS) and stored at 4°C. Ceragenin-containing nanosystems consisting of CSA-13, CSA-44 or CSA-131 attached to the surface of peanut-shaped AuNPs (AuP@CSA-13, AuP@CSA-44 and AuP@CSA-131, respectively) were synthetized according to the protocols presented in our previous papers [39,41,43]. In general, peanut-shaped AuNPs, prepared via cetrimonium bromide (CTAB)-assisted method were surface functionalized with tested ceragenins using mercaptohexadecanoic acid (MHDA) as linker, allowing in following steps to form a covalent bonding between the –COOH group of MHDA and –NH3 groups of ceragenins. The morphology and composition of synthetized nanosystem was ensured due to the reproducible and highly controllable synthesis method of nanoparticles; appropriate selection of reaction times and temperature, which allows for collection of the suspension of uniform nanoparticles; and using MHDA in excess, thanks to which it can guaranteed that all ceragenins molecules are attached to the surface of the AuNP. Final concentration of CSAs and AuP NPs in prepared nanosystems were 2 mg/ml and 2.93 ng/ml, respectively. On this basis, loading efficiency of ceragenin on the surface of AuP NP was estimated as 1.33×10^5 molecules of CSA/1 AuP NP.

Scanning transmission electron microscopy (STEM) with a high-angle annular dark field detector (HAADF), in conventional and high-resolution mode, was used to determine the AuP NPs morphology. The STEM images were acquired on an aberration-corrected FEI Titan electron microscope operating at 300 kV equipped with a FEG cathode. A two-circle laboratory diffractometer, Panalytical X'Pert Pro, was implemented for analyzing the crystal microstructure of AuP NPs. The measurements were recorded using a lamp with standard θ-2θ geometry and Cu anode working at 40 kV and 30 mA. The x-ray beam emitted by the x-ray tube was converted into a parallel beam by a divergence slit with a constant height 1/2°, a parabolic graded W/Si mirror with an equatorial divergence less than 0.05, 0.04 rad Soller slit collimator and by a mask of constant width of 20 mm, which restricts the width

of the beam. The diffracted beam optics consisted of the antiscattered slit with a height of 8.7 mm, 0.04 rad Soller slit collimator, a curved graphite monochromator to eliminate the contribution of the Cu K_β radiation, and a semiconductor silicone stripe detector with an active length of 2.122°. The dispersion of the nanoparticles was dried on a zero-background holder and was placed on a sample spinner with rotation time of 16 s. The data was obtained in the range between 20–80° and step size of 0.08°. The time per step was equal to 7000 s. The Fullprof software was used for the evaluation of the diffractogram fitting, lattice constants and coherent scattering length [45]. By collection of Fourier transform (FT)-Raman spectra the success and stability of the biofunctionalization and immobilization processes was investigated. Nicolet NXR 9650 FT-Raman Spectrometer equipped with an Nd:YAG laser (1064 nm) and a germanium detector was used for recording the spectra with the settings as described in our previous work [43]. UV-visible spectroscopy was used to investigate the CSA molecules anchored on Au NPs. Accordingly Multiskan SkyHigh UV-VIS (Thermo Fisher Scientific) was used, with a resolution of 1 nm and scan speed of 240 nm/min. Calorimetric measurements were employed to investigate the temperature stability of nanoparticles functionalized by MHDA, as well as MHDA and ceragenins. This experiment was performed using TA Instruments' DSC 2500 differential scanning calorimeter (DSC) equipped with a liquid nitrogen LN2P pump. The analyzed samples were placed in aluminium pans and crimped with hermetic lids. To study the thermal behavior of the samples, the given conditions was followed: dry N5.0 pure nitrogen purge (25 ml/min^{-1}) in a temperature range from 10–200°C with heating rate at 5 and 10°C min^{-1} and cooling rate at 5°C min^{-1}. The samples were held isothermally at the minimum and maximum temperature for 5 min. The peak temperatures and enthalpy values of the registered thermal events were calculated using TRIOS software. The thermogravimetric analysis (TGA) measurements were recorded using a TA Instruments' TGA 5500 thermogravimetric analyzer with high-temperature platinum pans. The samples were either directly placed in open 100-µl platinum pans or previously enclosed inside hermetic aluminum pans to restrict the evaporation rate. Before starting the experiments with aluminium pans, a 0.9-mm-diameter hole was punched through the lid of the container. The samples were heated at a rate of 5 or 10°C min^{-1} rate up to 500°C under a flow of N5.0 pure nitrogen (25 ml/min^{-1}). The temperature was calibrated using nickel and alumel standards.

Bacterial strains, media & experimental settings

Laboratory strains of OM-causing pathogens (*H. influenzae* ATCC 49766, *M. catarrhalis* ATCC 25238, *S. pneumoniae* ATCC 49619) were cultured using BBL Chocolate II Agar and 5% sheep blood agar plates at 37°C in 5% CO_2 atmosphere. Hemophilus test medium supplemented with β-NAD and hematin porcine was prepared according to previously published protocols [46]. For the purpose of antibiofilm activity assessment, brain–heart infusion broth medium was used.

All experiments assessing antibacterial activity of the tested compounds (discussed subsequently) were performed on 96-well microtiter plates in a final volume of 100 µl, with the exception of MIC/MBC measurement, which was carried out in a final volume of 200 µl. The absorbance or fluorescence measurements were recorded with Varioskan Lux microplate reader (Thermo Fisher Scientific).

Killing assay

Estimation of bacterial survival upon treatment with free ceragenins and CSA-containing nanosystems was performed using the colony counting (killing assay) method according to the protocol published previously [47]. The plates were incubated at 37°C, 5% CO_2 for further 18 h to determine the number of viable colonies. Survival of bacteria upon treatment with tested agents was calculated using the appropriate dilution factors and presented as decrease of log (CFU/ml). Concentrations of the tested agents required to limit the viability of bacterial population to 2 log CFU/ml, that is, 100 CFU/ml, was estimated by interpolation of dose–response curves.

Antimicrobial susceptibility testing

Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of CSA-13, CSA-131 and CSA-44, in a free form and conjugated with AuP NPs, for the otopathogens were determined using the broth microdilution method. Briefly, the bacterial inoculum was adjusted to 4×10^5 CFU/ml using the Hemophilus test medium and incubated with serial twofold dilutions of the tested agents ranging from 256 µg/ml to 0.5 µg/ml. Microbial growth was measured after 18–20 h of incubation at 37°C in 5% CO_2. The MBCs were evaluated after 48 h of growth by plating the ≥ MIC representative values on chocolate or blood agar plates.

Activity of the tested compounds in the presence of human cerumen

In another set of experiments, all otopathogens were subjected to free ceragenins and CSA-containing nanosystems in the presence of human cerumen. Cerumen samples were collected from adult healthy volunteers under approval of the Bioethics Committee at the Jan Kochanowski University in Kielce, Faculty of Medicine and Health Sciences (no. 20/2019). Informed written consent has been obtained from the participants involved. In detail, human cerumen samples were collected with a sterile earwax hook from six healthy individuals and kept in a sterile tube at -80°C until use. All collected samples were free from bacterial or fungal contamination, as confirmed by seeding of cerumen samples on chocolate agar plates. The pooled cerumen was mixed thoroughly, weighed and suspended in a buffer containing of 5% of sodium bicarbonate ($NaHCO_3$) and 30% glycerol (pH ~ 8.2) at a concentration of 10% (weight/volume) [48]. Bactericidal activity of the tested compounds was performed using the colony-counting method [47] using a final concentration of antimicrobials of 0.5, 1 and 5 μg/ml and 30% glycerol and 5% $NaHCO_3$ buffer.

Antibiofilm assay

Overnight cultures of *H. influenzae* ATCC 49766, *M. catarrhalis* ATCC 25238 and *S. pneumoniae* ATCC 49619 were brought in brain–heart infusion broth (BHI) to 2×10^7 CFU/ml, incubated with three concentrations (5, 20 and 50 μg/ml) of the tested compounds for 24 h at 37°C in 5% CO_2 and stained using crystal violet as previously described [49].

Investigation of mechanism of killing activity

Mechanisms by which developed nanosystems exert killing activity against AOM pathogens were investigated using fluorimetric and colorimetric probes recording permeability of outer membrane (NPN; final concentration of 40 μM), formation of reactive oxygen species (DCFH-DA; final concentration of 20 μM) and leakage of intracellular content from damaged bacterial cells (Bradford reagent; final concentration of 20%) according to previously published protocols [42,43]. Because the NPN assay is used for only Gram-negative bacteria, which is related to the need to enter the probe into the periplasmatic space, this test was not performed in relation to *S. pneumoniae*. To perform the preceding experiments, overnight cultures of bacteria were brought to OD_{600} ~0.1 and exposed to the tested compounds in concentrations ranging from 5 to 50 μg/ml at 37°C, 5% CO_2 for 10 min (for NPN assay) or 1 h (for ROS generation and protein leakage measurements). To clarify, the larger number of bacteria, and in turn the concentrations of agents, were adopted to the detection limit, that is, 10^7 CFU/ml of these methods. The incorporation of NPN in CSA-treated bacteria was detected at an excitation wavelength of 355 nm and emission wavelength of 405 nm. Generation of ROS was recorded fluorimetrically using excitation/emission wavelengths of 488/535 nm. Absorbance of Bradford reagent-incubated samples was measured at 595 nm.

Cell culture

Immortalized adult human skin keratinocytes cells (HaCaT), purchased from CLS Cell Lines Service (Eppelheim, Germany), were grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM/l), penicillin (50 U/ml) and streptomycin (50 μg/ml) and maintained at 37°C in an atmosphere containing 5% CO_2 with saturated humidity.

Determination of cell cytotoxicity

The cell cytotoxicity effects of tested compounds against human keratinocytes were determined using MTT assay. Previously reported methods have been implemented [50].

Measurement of IL-8 concentration

To elucidate whether free ceragenins and CSA-based nanoformulations are able to decrease pathogen-induced inflammatory responses, release of IL-8 from bacteria-stimulated keratinocytes was measured using enzyme-linked immunosorbent assay (ELISA) [50]. For this purpose, HaCaT cells at 1×10^4 density were seeded onto each well of 96-well plates and cultured until confluence of ~85% was reached. In the next step, culture medium was replaced with *H. influenzae* ATCC 49766-infected DMEM (bacteria brought to OD_{600} ~0.1) supplemented with tested agents at doses of 5 and 10 μg/ml. IL-8 secretion from bacteria-infected cells was measured in supernatant collected after 3 h incubation at 37°C, 5% CO_2 using IL-8 Human ELISA Kit.

Statistical analysis

Data are reported as a mean ± standard deviation. Statistical significance was determined by two-tailed Student's t-test using OriginPro 9.65 software (OriginLab, MA, USA).

Results

Synthesis & physicochemical analysis of developed ceragenin-based nanosystems

Nanosystems consisting of CSA-13, CSA-44 or CSA-131 attached to the surface of AuP NPs were developed using MHDA as a surface linker and investigated in terms of morphology, crystallographic properties and chemical structure using STEM, x-ray diffraction (XRD), FT-Raman technique as well as UV-Vis spectroscopy and calorimetric methods (Figure 1). The scheme of the biofunctionalization and immobilization process of AuP NPs is shown in Figure 1A. Bright field STEM images of AuP NPs showed that the synthesized nanoparticles have a peanut shape with an average size along the longitudinal axis around 60 nm and along the transverse axis ~30 nm (Figure 1B) [41]. The size spread of the AuP NPs is negligible, as determined from measurements of 100 randomly selected nanopeanuts [41]. Structural characterization of the synthesized nanoparticles was acquired using x-ray diffraction (Figure 1C). The XRD pattern of the AuNPs was refined using Rietveld method. Within the detection limit, all peaks were attributed to the standard Bragg reflections (111), (200), (220), (311) and (222) of Au nanocrystals with face-centered cubic lattice [51,52] and the calculated cell parameter of the AuP NP was 4.078 Å with uncertainties of ~0.001 Å. Successful AuP NP biofunctionalization and immobilization by ceragenin, as well as chemical stability of the nanosystem combined with AuP NPs and ceragenin (AuP@CSA-13, AuP@CSA-44, AuP@CSA-131, respectively), was determined by FT-Raman spectroscopy (Figure 1D). Gold nanopeanuts underwent a reaction with the thiol group from MHDA resulting in AuP NP functionalization with a COOH group, which was further used to attach ceragenin to the AuP NPs. Therefore, in the FT-Raman spectra of AuP NP + MHDA and nanosystems, the disappearance of the peak originating from the thiol group (2743 cm^{-1}) confirms a successful attachment of the MHDA to the surface of gold nanopeanuts [4]. Furthermore, in the FT-Raman spectra presented in Figure 1D, peak at 278 cm^{-1} corresponding to Au-S stretching vibrations were visible. These bonds are responsible for creating a connection in between surface of AuNPs and sulphur from MHDA [53]. Moreover, in the FT-Raman spectra of AuP NP + MHDA + ceragenin, peaks corresponding to the C=O vibrations (1680 cm^{-1}) were observed [54]. This group is responsible for linking ceragenin with biofunctional surfactants on the AuP NP surface [55]. Both observations provide evidence of the success of AuP NP biofunctionalization and ceragenin immobilization. Figure 1E shows SPR peaks of ~725 nm (black spectrum) in the UV-Vis absorption spectra. Comparing the UV-VIS spectra of synthesized AuP NPs with the nanoparticles functionalized with ceragenins (spectra red, blue and green), a shift to higher wavelengths of the respective AuP NPs peaks by about 30 nm was observed. This shift indicates that the immobilization of ceragenins on the nanoparticles surface indeed occurred, because the size of the AuP NPs increased as a result of anchoring ceragenins molecules on the nanoparticles. Indeed, it is well known that with increasing particle size, the band gap of the nanogold decreases, resulting in a shift of the SPR peak toward higher wavelength values [56]. Taken together the results from calorimetric experiments indicated that all the samples remained thermally stable up to 100°C at a 5°C min^{-1} heating rate (Figure 1F). Moreover, the DSC thermogram displayed no signs of any significant thermal anomalies up to 150°C. The anomalies describe earlier indicate that temperature, as well as the baseline shift, are due to the rapid boiling and sample decomposition or destabilization of MHDA [57].

Conjugation of ceragenins with Au NPs augments their bactericidal & antibiofilm activity

The colony-counting method (killing assay) was employed to estimate bactericidal activities of ceragenins and ceragenin-containing nanosystems against *H. influenzae* ATCC 49766, *M. catarrhalis* ATCC 25238 and *S. pneumoniae* ATCC 49619 strains. As demonstrated in Figure 2, all ceragenins exert bactericidal activity against *H. influenzae* ATCC 49766, *M. catarrhalis* ATCC 25238 and *S. pneumoniae* ATCC 49619 (Figure 2, panels A1, B1 & C1, respectively) in a concentration range from 0.5 to 10 mg/ml, with the exception of CSA-13, which was able to eradicate *H. influenzae* bacteria at 20 μg/ml. Importantly, conjugation of the ceragenins with Au NPs strongly enhanced their killing efficiency, as all CSA-based nanosystems (AuP@CSA-13, AuP@CSA-44 and AuP@CSA-131) eradicated the tested otopathogens in doses ranging from 0.5 to 5 μg/ml. To more quantitively compare the efficiency of tested agents against AOM pathogens, we estimated doses that are required to limit the survival of bacterial population to 100 CFU/ml (2 log [CFU/ml], i.e., colony-counting assay detection limit) (Figure 2, panels A2–C2). Accordingly, CSA-131 was recorded as the most potent ceragenin, limiting the colony-forming

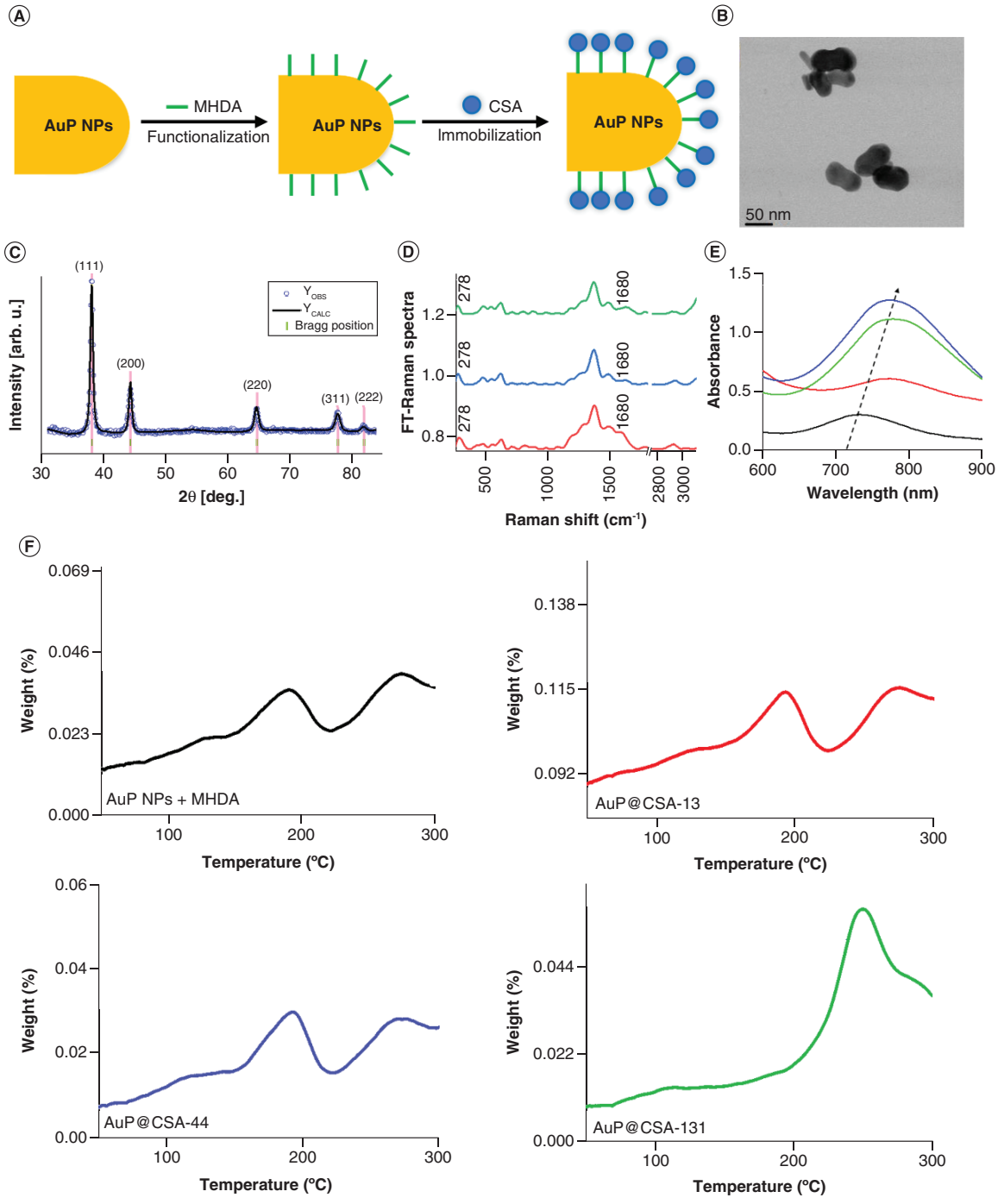

Figure 1. Physicochemical properties of developed nanosystems. (A) Scheme of the AuP NPs biofunctionalization and ceragenin (CSA) immobilization process. **(B)** Overview scanning transmission electron microscopy image. **(C)** X-ray diffraction (XRD) taken for AuP NPs. Experimental (blue dots) and calculated (solid black line) Rietveld refinement plot of the XRD pattern for the AuP NPs. The red line (YOBS–YCALC) represents the difference between the observed and calculated data. Tick marks show allowed Bragg reflections. **(D)** FT-Raman spectra of nanosystems: AuP NPs + MHDA + CSA-13 (red spectrum); AuP NPs + MHDA + CSA-44 (blue spectrum); AuP NPs + MHDA + CSA-131 (green spectrum). **(E)** Ultraviolet-Vis spectra of AuP NPs (black spectrum) and AuP NPs + MHDA + CSA-13 (red spectrum); AuP NPs + MHDA + CSA-44 (blue spectrum); AuP NPs + MHDA + CSA-131 (green spectrum). **(F)** Thermogravimetric analysis and differential scanning calorimetry data of AuP NPs + MHDA and AuP@CSA-13 (red curve), AuP@CSA-44 (blue curve), AuP@CSA-131 (green curve), showing the solvent removal and decomposition of the product.
AuP NP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial; FT: Fourier transform; MHDA: Mercaptohexadecanoic acid.

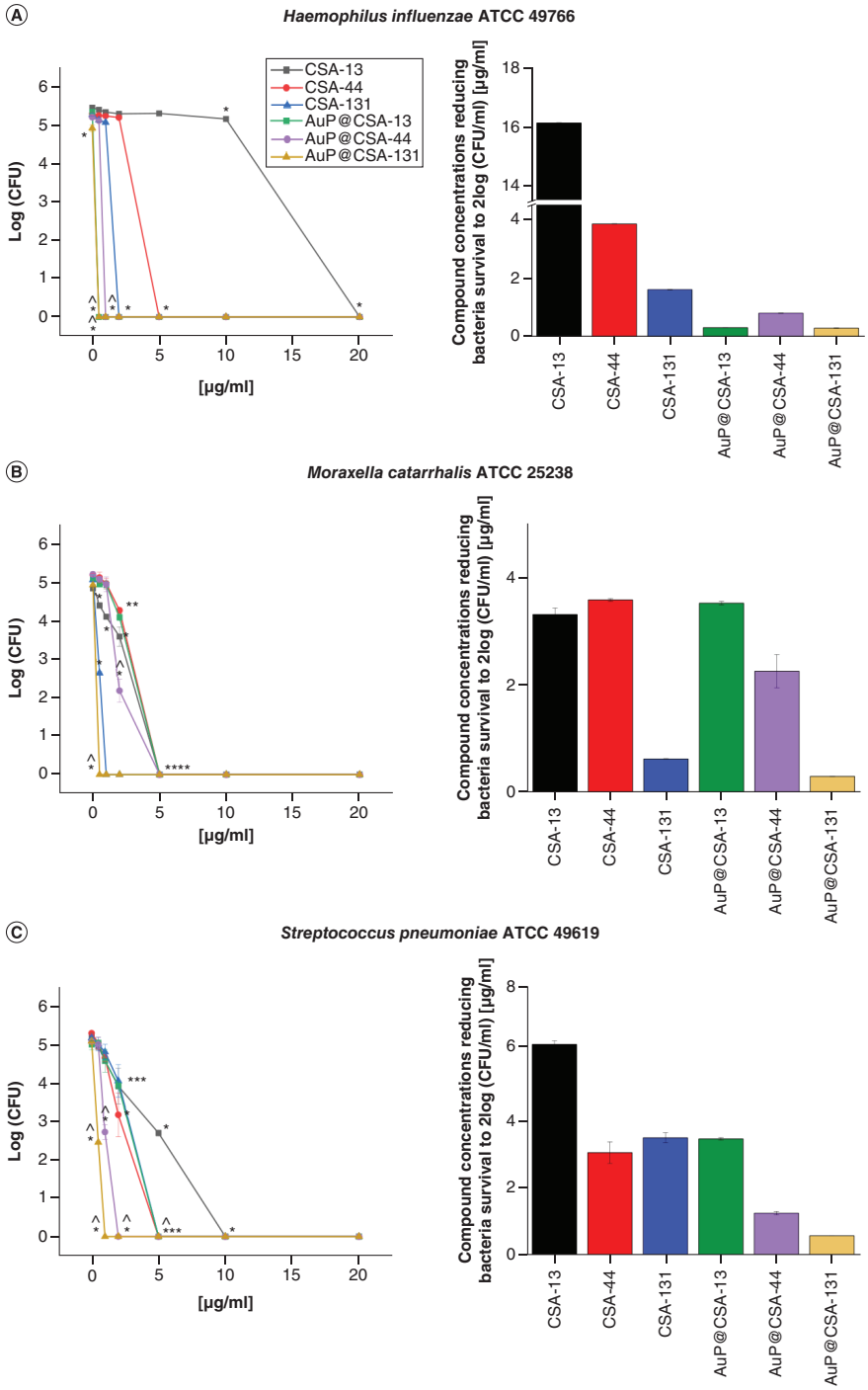

Figure 2. Bactericidal activity of tested compounds against otitis media-causing pathogens. Activity of ceragenins CSA-13 (black squares), CSA-44 (red circles), CSA-131 (blue triangles) and ceragenin-containing nanosystems, AuP@CSA-13 (green squares), AuP@CSA-44 (purples circles) and AuP@CSA-131 (gold triangles) against **(A)** *Haemophillus influenzae* ATCC 49766, **(B)** *Moraxella catarrhalis* ATCC 25238 and **(C)** *Streptococcus pneumoniae* ATCC 49619 strains was evaluated. For each strain, logarithm of number of colonies outgrown [log (CFU)] (panels A1, B1 and C1) and concentration of compound reducing bacteria survival to 2 log (CFU/ml) was calculated (panels A2, B2 and C2). Results are presented as mean ± standard deviation from three replicates.
* and ^ indicates statistical significance $p < 0.05$ when compared to untreated control and free ceragenins, respectively. (Where there is more than one *, the values overlap).
ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CFU: Colony-forming unit; CSA: Cationic steroid antimicrobial.

Table 1. MIC and MBC of ceragenins CSA-13, CSA-44 and CSA-131 in free form and attached to the surface of AuPs against laboratory strains of *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*.

Tested agents	*H. influenzae* ATCC 49766	*M. catarrhalis* ATCC 25238	*S. pneumoniae* ATCC 49619
	MIC/MBC (μg/ml)	MIC/MBC (μg/ml)	MIC/MBC (μg/ml)
CSA-13	0.5/0.5	1/4	0.5/1
CSA-131	1/1	2/8	1/1
CSA-44	2/4	4/16	2/4
AuP@CSA-13	0.5/0.5	0.25/1	1/1
AuP@CSA-131	2/2	2/4	2/2
AuP@CSA-44	2/4	0.5/2	0.5/2

ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial; MBC: Minimal bactericidal concentration; MIC: Minimal inhibitory concentration.

ability of tested bacteria at doses of 1.60 ± 0.01, 0.62 ± 0.01 and 3.51 ± 0.15 μg/ml (for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*, respectively). Notably, in nearly all comparisons, CSA-based nanosystems were far more active than free ceragenins. This effect was particularly prominent for CSA-13 because treatment of *H. influenzae* with AuP@CSA-131 allowed to decrease effective dose of agent nearly 50-fold, from 16.11 ± 0.2 μg/ml to 0.313 ± 0.01 μg/ml (Figure 2, panel A2). A similar tendency was maintained in the presence of high nutritious medium. As presented in Table 1, MIC and MBC values ranged for free ceragenins from 0.5 to 4 μg/ml and 0.5 to 16 μg/ml, respectively. In that respect, the nanosystems were at least equally effective or more potent than ceragenins alone, except AuP@CSA-131 against *H. influenzae* ATCC 49766 and *S. pneumoniae* ATCC 49619, which showed one dilution higher MIC/MBC values (1/1 μg/ml vs 2/2 μg/ml).

Furthermore, substantial antibiofilm activities were recorded for the tested agents in concentrations from 5 to 20 μg/ml, resulting in a decrease of biofilm mass up to 90%. Importantly, when comparing the formation of biofilm at the lowest doses tested, that is, 5 μg/ml, it is prominent that a mass of biofilm formed in the presence of ceragenins varied between 95.96 ± 18.58% and 21.61 ± 2.28%, whereas for CSA-based nanosystems, this effect was considerably stronger because detected biofilm mass ranged from 42.51 ± 7.05% to 22.31 ± 2.01% (Figure 3). To more accurately elucidate how nontoxic doses of the developed nanomaterials affect the formation of biofilms, we estimated viability of biofilms on treatment with 10 μg/ml of CSA/AuP@CSA – that is, the doses demonstrating antibacterial activity in killing assay, but still safe for mammalian cells (Figure 3, panels A2–C2). As demonstrated, biofilm mass at this endpoint ranged from 21.09 ± 2.14 to 87.07. ± 22.82% (for ceragenin-treated samples) and from 19.45 ± 5.18 to 36.71 ± 7.44% (for nanosystem-treated biofilms) compared with untreated samples. This indicates that ceragenin-containing nanosystems effectively inhibit the formation of biofilms by AOM pathogens at doses significantly lower than free ceragenins and are able to exert therapeutic effect at doses which are recognized as nontoxic for host cells.

Bactericidal activity of CSAs & CSA-containing nanosystems is not affected by human cerumen

As shown in Figure 4, the bactericidal effects of the tested compounds are not only preserved but even augmented in the presence of the human cerumen. In detail, in a great majority of tested CSA/AuP@CSA comparisons at concentrations ranging from 0.5 to 5 μg/ml, reduction of bacterial growth log (CFU/ml) values were significantly lower in 10% cerumen-containing samples, compared with controls without cerumen. In accordance with previous results, all tested bacterial strains, in particular *H. influenzae* ATCC 49766 (Figure 4A) and *S. pneumoniae* ATCC 49619 (Figure 4C), were more susceptible to AuP@CSAs than to ceragenins alone.

Mechanism of AuP@CSA-mediated killing involves generation of oxidative stress followed by permeabilization of bacterial membranes

For the purpose of killing mechanism determination, fluorimetric and colorimetric methods were employed. As demonstrated in Figure 5, the tested agents induced intracellular ROS production in *H. influenzae* ATCC 49766 and *S. pneumoniae* ATCC 49619, but not by *M. catarrhalis* ATCC 25238 strains. This effect was significantly more pronounced for CSA-based nanosystems than free CSAs, in particular for *S. pneumoniae*, where CSA-13 and CSA-44 in doses >20 μg/ml triggered substantially less ROS production than their counterparts: AuP@CSA-13 and AuP@CSA-44 (Figure 5C). As expected, AuP NP-based nanosystems more effectively interacted with bacterial membranes, as evidenced by the NPN (Figure 6) and Bradford assay (Figure 7). By measurement

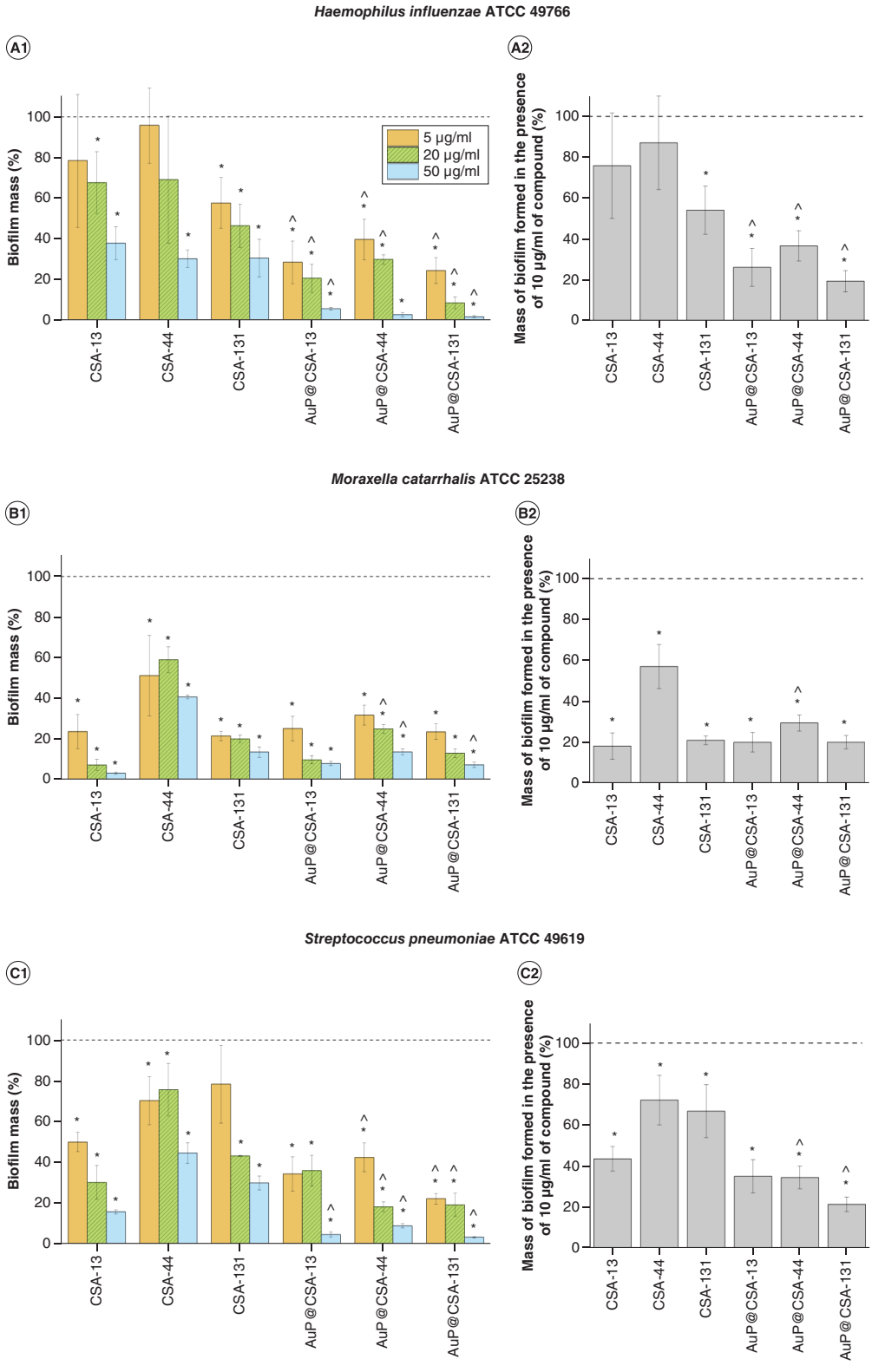

Figure 3. Antibiofilm properties of ceragenins and ceragenin-containing nanosystems. Prevention of biofilm formation by **(A)** *Haemophillus influenzae* ATCC 49766, **(B)** *Moraxella cattarhalis* ATCC 25238 and **(C)** *Streptococcus pneumoniae* ATCC 49619 bacteria treated with ceragenins CSA-13, CSA-44, CSA-131 and CSA-based nanosystems (AuP@CSA-13, AuP@CSA-44, AuP@CSA-131) at concentrations of 5 (gold bars), 20 (green stripped bars) and 50 µg/ml (blue bars) (panels A1, B1 and C1). On the basis of the recorded dose–response curves, mass of biofilm formed in the presence of 10 µg/ml of compound was calculated (panels A2, B2 and C2). Results are presented as mean ± standard deviation from three replicates.

* and ^ indicate statistical significance ($p < 0.05$) compared with untreated control and free ceragenins, respectively.

ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial.

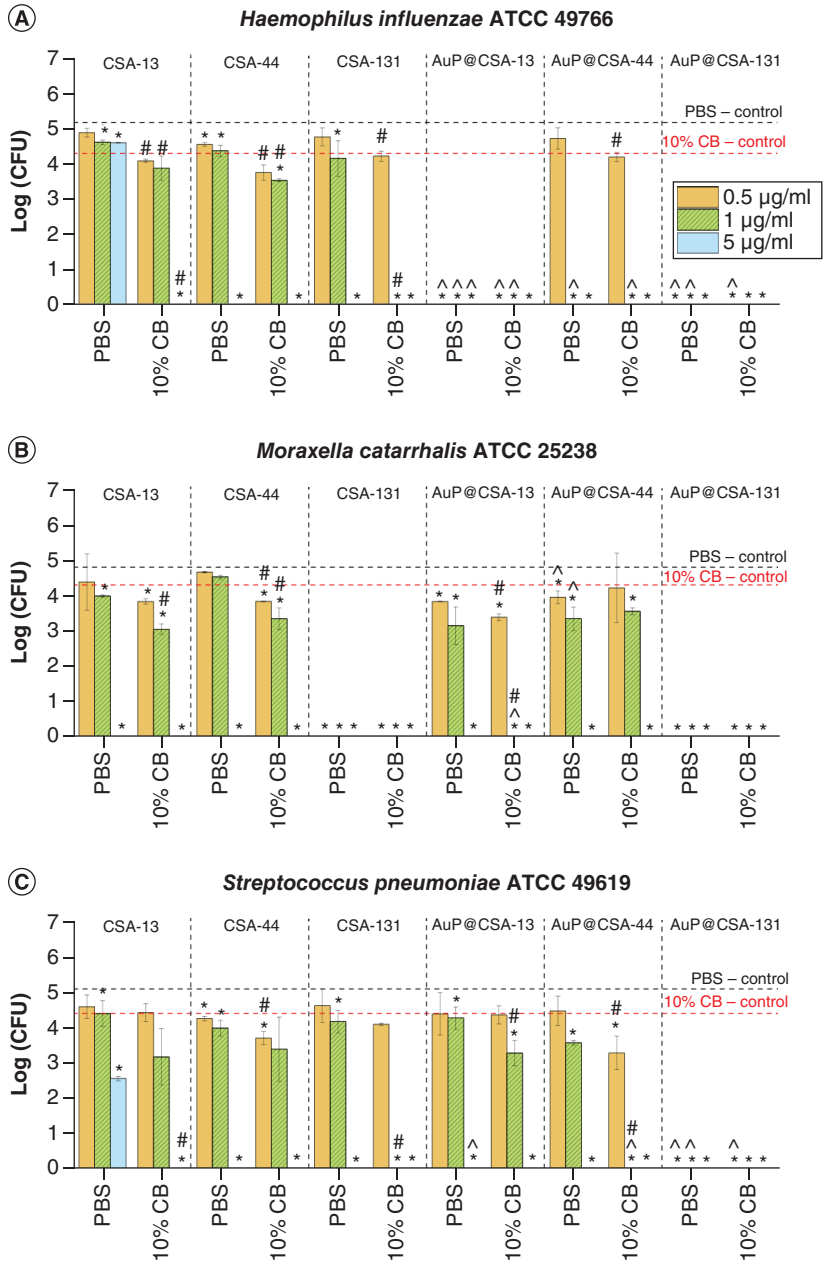

Figure 4. Bactericidal activity of tested compounds against otitis media-causing pathogens in the presence of 10% human cerumen buffer. Activity of ceragenins CSA-13, CSA-44, CSA-131 and ceragenin-containing nanosystems AuP@CSA-13, AuP@CSA-44 and AuP@CSA-131 at doses of 0.5, 1 and 5 µg/ml was tested against **(A)** *Haemophillus influenzae* ATCC 49766, **(B)** *Moraxella catarrhalis* ATCC 25238 and **(C)** *Streptococcus pneumoniae* ATCC 49619 (panel C). Results are presented as mean ± standard deviation from three replicates.
* and ^ indicate statistical significance $p < 0.05$ compared with untreated controls and free ceragenins, respectively.
#indicates statistical significance $p < 0.05$ compared with viability of bacteria in 10% CB and phosphate-buffered saline.
ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CB: Cerumen buffer; CSA: Cationic steroid antimicrobial; CFU: Colony-forming unit; PBS: Phosphate-buffered solution.

of entry of NPN into periplasmic space of Gram-negative bacteria, it is possible to estimate the membrane-permeabilizing properties of tested compounds [43]. As demonstrated in Figure 6, in both tested Gram-negative pathogens incubation of bacteria with nanopeanuts decorated with CSA-13 and CSA-44 resulted in a far stronger permeabilization of the outer membrane than CSAs alone, indicating increased interaction with the bacterial

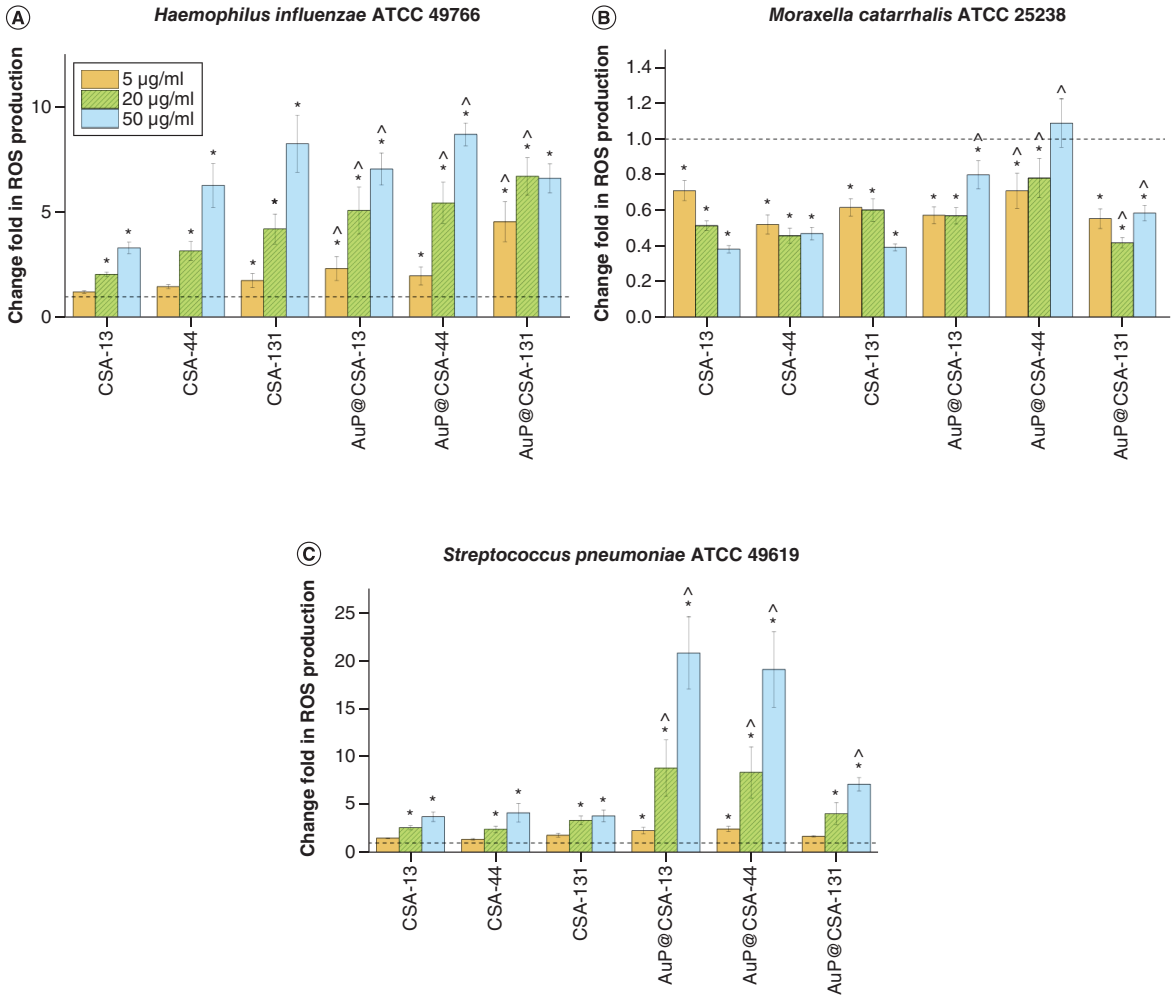

Figure 5. Generation of reactive oxygen species in otitis media-associated pathogens. ROS-inducing abilities of ceragenins CSA-13, CSA-44, CSA-131 and ceragenin-containing nanosystems AuP@CSA-13, AuP@CSA-44 and AuP@CSA-44 at a dose of 5 μg/ml (gold bars), 20 μg/ml (green stripped bars) and 50 μg/ml (blue bars) were tested against **(A)** *Haemophillus influenzae* ATCC 49766, **(B)** *Moraxella catarrhalis* ATCC 25238 and **(C)** *Streptococcus pneumoniae* ATCC 49619. Results are presented as mean ± standard error of the mean from three to six individual experiments with three replicates each.
* and ^ indicate statistical significance ($p < 0.05$) compared with untreated control and free ceragenins, respectively.
ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial; ROS: Reactive oxygen species.

surface. Although at the 5 μg/ml concentration there were no significant differences between free ceragenins and CSA-based nanoformulations, a strong increase in NPN fluorescence was observed at a dose of >20 μg/ml (Figure 6). Likewise, CSA-based nanosystems exerted more intense protein leakage from the treated bacterial strains, highlighting better efficiency of CSA-based nanosystems than ceragenin alone (Figure 7).

CSAs & CSA-containing nanosystems display promising biocompatibility & anti-inflammatory activity in bactericidal concentrations

As presented in Figure 8A, the bactericidal concentrations (i.e., 1–10 μg/ml) of ceragenin-based compounds exerted an acceptable toxicity on human keratinocytes, leading to decrease of metabolic activity in no more than 30% of these cells (with the exception on AuP@CSA-13, which was more toxic than other nanoformulations). Simultaneously, due to the immobilization of ceragenins on the surface of Au NPs, decreased inflammatory response in keratinocytes stimulated by *H. influenzae* compared with free CSAs was observed (Figure 8B). In detail, the former reduced IL-8 release from 1482 to 477.8–657.8 pg/ml, whereas for CSAs this reduction was less pronounced

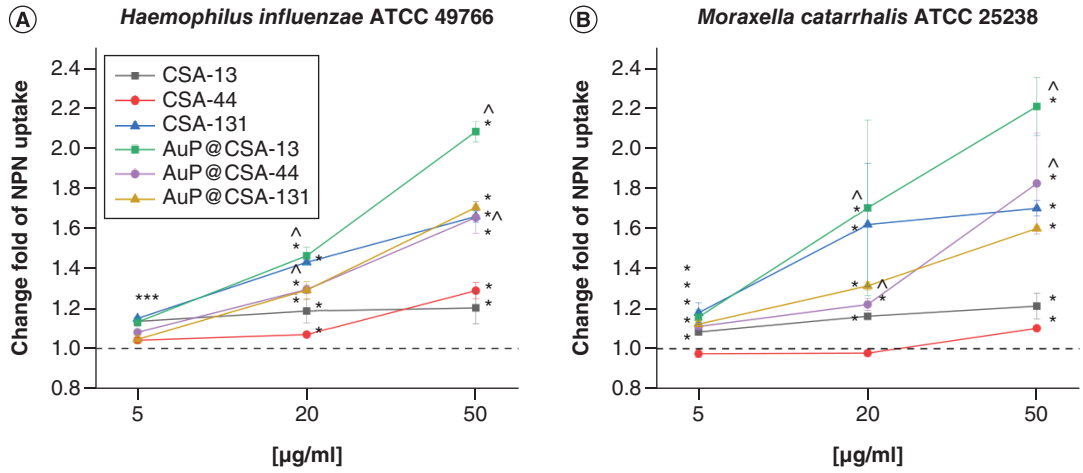

Figure 6. Membrane-permeabilizing properties of ceragenins and cationic steroid antimicrobial-containing nanosystems. Disruption of bacterial membrane in **(A)** *Haemophillus influenzae* ATCC 49766 and **(B)** *Moraxella catarrhalis* ATCC 25238 bacteria treated with ceragenins CSA-13, CSA-44, CSA-131 and CSA-based nanosystems (AuP@CSA-13, AuP@CSA-44 and AuP@CSA-131) at concentrations of 5 (gold bars), 20 (green stripped bars) and 50 µg/ml (blue bars). Results are presented as mean ± standard deviation from two individual experiments with three replicates each.
* and ^ indicate statistical significance $p < 0.05$ compared with untreated control and free ceragenins, respectively.
ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial; NPN: *N*-phenyl-1-naphthylamine.

(782.8–1112.5 pg/ml). These results suggest that the developed nanosystems exert both potent bactericidal and antiinflammatory activity, which would be favorable in the treatment of OM infections.

Discussion

Given the common occurrence of persistent acute OM infections and relatively high administration of antibiotics in the pediatric population [58,59], novel approaches for treatment OM infections are needed. In line with these, we assessed the efficacy of ceragenins and their corresponding Au NP formulations against the three leading pathogens of OM.

Due to their favorable physical features, including size range and high surface-to-volume ratio, Au NPs, particularly nonspherical NPs, should be considered as antimicrobials with clinical potential. Recently, we demonstrated that Au NPs with morphology other than spheres (i.e., rod-, peanut- and star-shaped) display potent killing activity against a spectrum of bacterial and fungal pathogens, regardless of their resistance to conventional antibiotics, at concentrations as low as ng/ml, which is determined by their membrane-permeabilizing capabilities and ROS-promoting activities [41]. Moreover, we demonstrated that gold nanorods are highly effective against *Candida* strains isolated from patients suffering from hemato-oncological conditions and filamentous fungi from *Aspergillus*, *Cladosporium* or *Fusarium* genera [42]. Importantly, nonspherical Au NPs were noted to decrease the formation and viability of pathogenic biofilms, which results from Au-NP-mediated ability to decrease the adhesion of microbes to abiotic surfaces [41,42]. Those reports strongly justify the further research on usefulness of these nanomaterials in therapy of drug-resistant pathogens. To date, the application of Au NPs in middle-ear infections has not been explored. With the advancement of nanomedicine, targeted drug-delivery systems and nanotherapeutics incorporating silver NPs with bactericidal activity in middle-ear prosthesis have been reported [60,61]. Also, silver and magnetic nanoparticles were tested as contrast agents for ear imaging including MRI and CT scans. In the majority of previous studies, the potency of differently shaped Au NPs was implemented for the eradication of *Pseudomonas aeruginosa* and *S. aureus* biofilms which commonly associate with otitis externa (swimmer's ear) [40,41,62]. Notably, according to our best knowledge, there is no literature on the bactericidal effects and toxicity of Au NP application to the middle-ear mucosa. However, studies on the use of Au NPs for inner-ear imaging investigated its efficacy with no toxicity to mouse cochlear cell lines (HEI-OC1), which encourages the future tests in this aspect [63].

In addition to aforementioned research, a compelling number of studies provide the evidence on utility of Au NPs as platforms for immobilization of therapeutics and other functional ligands on their surface. To date,

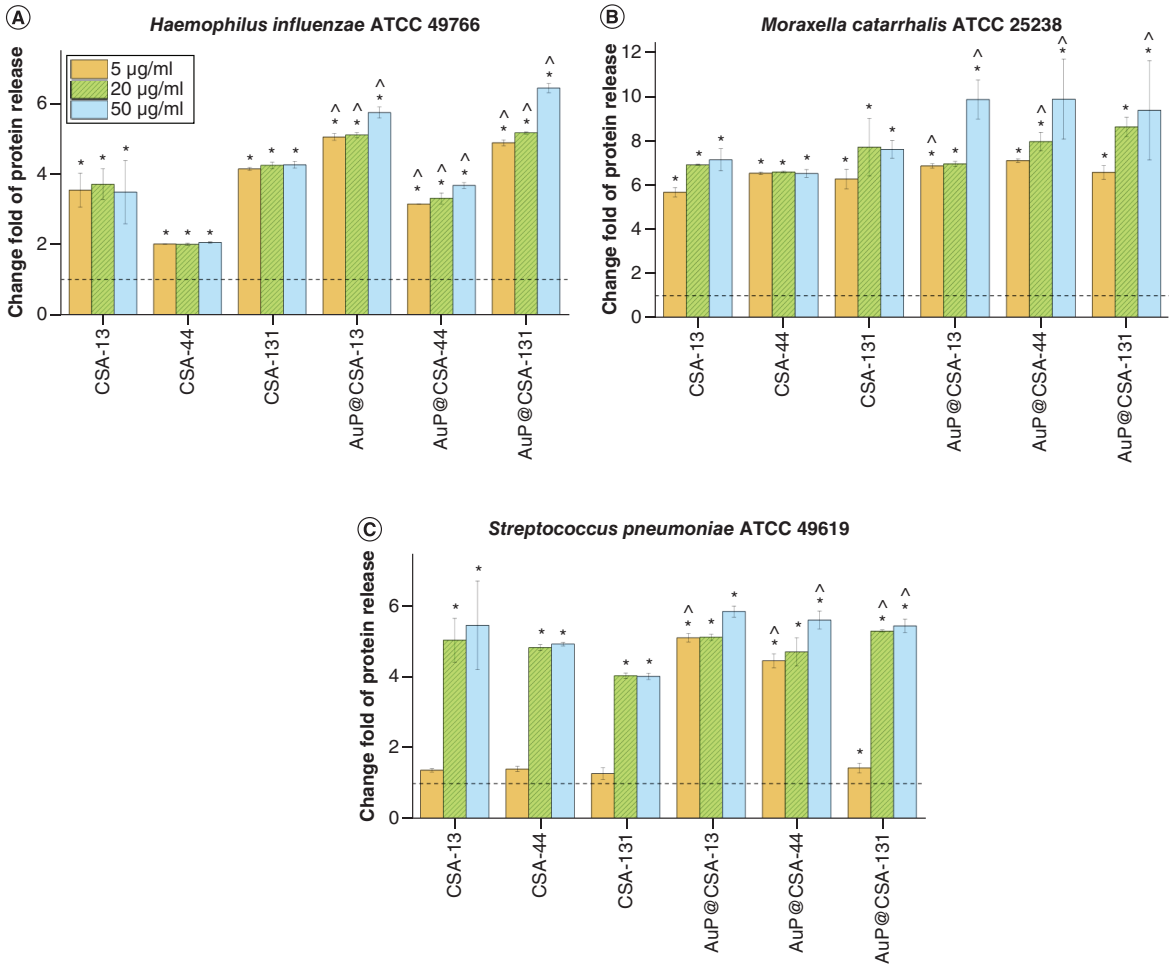

Figure 7. Release of intracellular content from cationic steroid antimicrobials and AuP@CSA-treated bacteria.
Release of proteins from **(A)** *Haemophillus influenzae* ATCC 49766, **(B)** *Moraxella cattarhalis* ATCC 25238 and **(C)** *Streptococcus pneumoniae* ATCC 49619 bacteria treated with ceragenins CSA-13, CSA-44, CSA-131 and CSA-based nanosystems (AuP@CSA-13, AuP@CSA-44, AuP@CSA-131) at concentrations of 5 (gold bars), 20 (green stripped bars) and 50 µg/ml (blue bars). Results are presented as mean ± standard deviation from three replicates.
* and ^ indicate statistical significance ($p < 0.05$) compared with untreated control and free ceragenins, respectively.
ATCC: American Type Culture Collection; AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial.

multiple studies confirm that attachment of conventional antibiotics to the surface of metallic NPs via either noncovalent interactions [64] or covalent bonds [65] might be used to combat MDR bacteria, including MRSA or *Acinetobacter baumannii* because both of these methods have been reported to enhance killing efficiency against pathogenic bacteria compared with free, unconjugated antibiotics [35,36]. Reasons for such phenomena include both improved internalization of antibiotics into the bacterial cells, as well synergistic effects between NPs and the attached antimicrobial without the disturbance of the inherent mechanism of action of the antibiotic [66]. Similarly, antimicrobial activity of ceragenins might also be modulated, as we demonstrated previously using iron oxide NPs as drug nanocarriers [67,68]. We verified that through NP-assisted, enhanced intracellular internalization of CSA and increased ability of compounds to generate ROS, a considerable membrane perturbation and leakage of intracellular content occurs [67,68]. Nevertheless, a majority of such developed nanosystems is based on spherical-shaped nanoparticles [35,36]. A compelling amount of data presented to date clearly point out the possibility of modifying the biological activity of Au NPs by controlling their size and shape, making them more therapeutically efficient and allowing them to attach more therapeutic compound on their surface [39–42]. In general, nonspherical Au NPs, including those with flower, star or rod morphology [40,69], are reported to exert enhanced bactericidal effects due to intensified ROS generation, which leads to excessive oxidative stress and enhanced bacterial membrane rupture [40,69]. Considering these promising reports, an attempt has been made to employ nonspherical nanomaterials

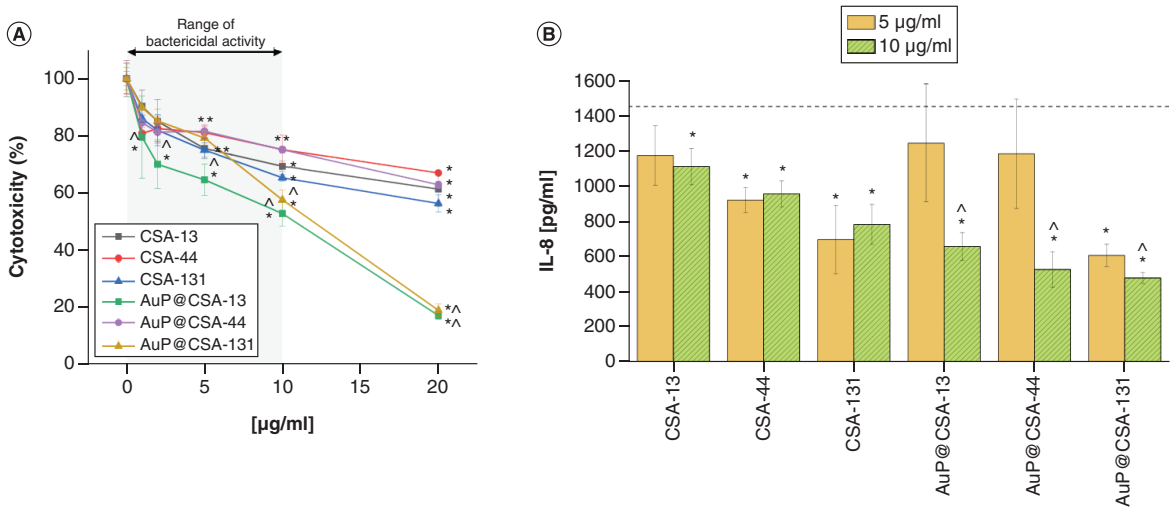

Figure 8. Decrease of *Haemophillus influenzae*-induced inflammation by ceragenins and ceragenin-containing nanosystems. (A) Cytotoxicity of ceragenins CSA-13, CSA-44, CSA-131 and CSA-containing nanosystems AuP@CSA-13, AuP@CSA-44 and AuP@CSA-131 in relations to human keratinocytes (HaCaT) treated with 24 h with tested agents at concentrations ranging from 1 to 20 µg/ml. **(B)** Decrease of IL-8 release from human keratinocytes stimulated with *H. influenzae* ATCC 49766 bacteria and treated with tested agents for 24 h. Results are presented as mean ± standard deviation from three replicates.
* and ^ indicate statistical significance ($p < 0.05$) compared with untreated control and free ceragenins, respectively. (Where there is more than one *, the values overlap).
AuP: Gold peanut-shaped nanoparticle; CSA: Cationic steroid antimicrobial.

as ceragenins carriers [43]; nevertheless their effectiveness in the presence of body secretions has not been tested to date. In this study, we used newly-developed, non-spherical AuNPs in the shape of nanopeanuts as nanocarriers for ceragenins CSA-13, CSA-44 and CSA-131. Notably, we observed the augmentation of antibacterial efficiency of ceragenins after their conjugation with Au NPs; however, the magnitude of this effect appears to be strongly dependent on a type of ceragenin and/or bacterial species as well as methodology used for its estimation. For example, the MIC/MBC values for AuP@CSA-13 and AuP@CSA-44 nanoparticles are twofold and threefold lower in comparison to CSA-13 and CSA-44 in a free form, but only against *M. catarrhalis*, whereas their activity, along with AuP@CSA-131, against *S. pneumoniae* and *H. influenzae* is comparable to CSAs alone in MIC/MBC testing. On the other hand, Au NPs clearly enhance the bactericidal action of ceragenins against *S. pneumoniae* in killing assay experiments. Hypothetically, such phenomena could be achievable due to potent activity of AuP NPs themselves and synergistic interactions between ceragenins and gold nanomaterials. According to our previously published data, Au NPs in shape of peanuts, similarly as those rod- and star-shaped [41,42], exert nanogram killing efficiency against spectrum of bacterial and fungal pathogens. Nevertheless, based on calculation of gold content in developed nanoformulations we exclude the possibility of synergistic interactions between AuP NPs and CSAs. As stated above, our developed nanosystems consist of two compounds: 2 mg/ml ceragenin (CSA-13, CSA-44 or CSA-131) and 2.93 ng/ml of nonspherical AuNPs. On this basis, we estimated that 5 µg/ml of Au NPs@CSA-131 (the highest bactericidal dose of nanosystems recorded using colony-counting measurements) includes 0.007325 ng/ml (7.325 pg/ml) of Au NPs. To test whether such amount of AuP NPs might affect the viability of OM pathogens, we performed an additional colony counting-based measurement. As recorded, such small concentrations of AuNPs does not exert any killing efficiency (Supplementary Figure 1), which is why we state that more potent activity of nanosystem depends on local immobilization of ceragenin CSA-131 on the surface of Au NPs than on additive effects of gold and ceragenin.

In our study, all CSAs and their nanosystems demonstrate strong activity against bacteria in biofilms – structures that play crucial role in pathogenesis of AOM and recurrent otitis infections as well as factors impeding their successful treatment. For instance, *S. pneumoniae* and *M. catarrhalis* are able to create multispecies biofilms where they can protect each other from the activity of macrolide and beta-lactam antibiotics, respectively [70]. Because CSAs are characterized by multiple mode of action and a low risk of resistance development this form of bacterial cooperation is highly unlikely to succeed [27,28]. Importantly, ceragenin-containing nanosystems effectively

inhibited the formation of biofilms by OM pathogens at doses significantly lower than free ceragenins. Possibly, this observation is attributed to the increased local concentration of CSA on the surface of AuP NPs inducing stress on the bacterial membrane as well as the electrostatic interactions between the gold nanoparticle functionalized ceragenins and the pathogen [62].

One of the primary antibacterial mechanism of ceragenins is their selective association with bacterial membranes, especially with Gram-negative bacteria, altering the cell morphology and microbial survival [18,71]. Likewise, metal nanoparticles also easily induce membrane permeabilization [72]. It must be noted that multiple antibacterial mechanisms of Au NPs against Gram-positive and Gram-negative bacteria have been reported. Most commonly, depolarization of membrane potential, cell wall damage, DNA fragmentation, oxidation of cell membrane fatty acids and inhibition of protein synthesis [40,41,73]. Similarly, nonspherical Au NPs were noted to decrease the formation and viability of pathogenic biofilms, which results from Au-NP-mediated ability to decrease the adhesion of microbes to abiotic surfaces [41,42]. Those reports strongly justify the further research on usefulness of these nanomaterials in therapy of drug-resistant pathogens. Because induction of ROS is recognized as one of the mechanisms determining bacterial killing by CSAs [74], we quantified intracellular ROS generation in all otopathogens with a DCFH-DA fluorescent probe [67,75,76]. Interestingly, a dose-dependent increase in ROS generation was recorded only for AuP@CSA-13 and AuP@CSA-44 in *S. pneumoniae*, whereas no significant ROS levels were measured for other compounds. Normally, the cellular systems within the bacteria maintain a redox state of balance between ROS generation and detoxification through various antioxidant defense strategies. As reported by Zhao *et al.*, oxidative stress intermediates can act as bifunctional factors such that at low stress levels, they potentiate the release of protective enzymes, while cellular dysfunction and bacterial death follows excessive stress production [77]. Therefore, in the latter case, other mechanisms of CSA nanosystems action are likely involved. For instance, it is noteworthy, that in *S. pneumoniae* the antibacterial effects of CSAs may be at least partially correlated with its natural autolytic behavior, that is, production of LytA autolysin [20]. Likewise, the effect of AuNPs inhibited the viability of *S. pneumoniae* without modulating the release of ROS but rather through formation of inclusion bodies of Au NPs within the bacteria [78,79]. In contrast, Au NPs enhanced the intracellular release of ROS and inhibited the energy metabolism and transcription process as a mechanism of killing in *S. aureus* [80]. An accumulation of ROS in response to treatment with CSA compounds, in particular CSA-131, AuP@CSA-13, AuP@CSA-131, AuP@CSA-44, was also recorded in *H. influenzae*, but surprisingly not in *M. catarrhalis*. Notably, *M. catarrhalis* shows relatively high innate level of resistance to exogenous oxidative stress, i.e., killing by exogenous H_2O_2, compared with *H. influenzae* or *S. pneumoniae* [81]. Thus, a presence of robust mechanisms responsible for dealing with this form of stress was suggested for this bacterium [82]. Although the molecular background behind this phenomenon is unknown [82], it may be associated with the nasopharyngeal mucosa niche occupied by *M. catarrhalis* as well as the lungs of patients with chronic obstructive pulmonary disease, who exhale more H_2O_2, especially at the time of an exacerbation, than healthy controls [83]. Therefore, differences in activity between the CSAs, aside from the structural diversity of these agents, e.g., lengths of lipid side chains, at least partially may to be attributed to unique traits of some bacterial pathogens. However, it should be noted that ROS measurements using fluorescein based approaches such as DCFDA dye can have some limitations; for example, its lack of direct interaction with intracellular H_2O_2 is one among others reported [84]. Therefore, ROS assessments using more specific fluorescent probes and alternative detection of lipid peroxidation, SOD/GST enzyme activity can provide more understanding on the possibility of ROS-based killing of the tested compounds. Collectively, the underlying killing mechanism of the tested compounds are not the same for all bacterial species. It appears that the activity of Au NP-based nanosystems is not specific and can have different targets depending on the bacterial species. Therefore, their mechanisms of antimicrobial action remains to be elucidated.

As we presented previously, ceragenins and ceragenin based nanosystems have demonstrated strong antimicrobial activity in the presence of body fluids such as blood plasma, serum, urine, CSF, sputum, abdominal fluid and dental plaque [67,85,86]. Our previous research confirmed the activity of CSAs at the site of infection, and this time we focused on the material for which the influence on the killing abilities of ceragenins and the nanosystems containing them has not yet been determined (i.e., human cerumen). Although cerumen is not commonly associated with middle-ear infections, it should be noted that in the treatment of middle-ear infection, apart from oral antibiotic therapy, an adjuvant therapy in the form of ear drops has also clinical significance. We propose that ceragenins and CSA-based nanosystems possess the great potential to be used as therapeutic agents for local treatment of infections and for this reason, the efficacy of these compounds in the presence of ear secretions should be estimated. Favorably, the bactericidal activity of the ceragenins and their conjugated nanosystems is not only preserved but

even augmented in the presence of the human cerumen. This observation possibly may be explained by natural antimicrobial properties of this substance mediated via several AMPs, including hBD1-3, lactoferrin, LL-37, BPI, hSLPI and HNP1-3 [87]. Previous reports demonstrated that ceragenin CSA-13 displays synergistic effects with naturally occurring compounds such as LL-37 peptide, lysozyme and lactoferrin [88]. Most likely, similar effect occurs in our experimental settings, which additionally confirms the utility of ceragenins and CSA-containing nanosystems in the treatment of ear infections. Moreover, slightly acidic pH of cerumen (~5.4) provides optimal conditions for the synergistic and additive effect of AMPs. However, it is noteworthy that the pH may increase as the result of ear infection, hence acidifying agents such as boric acid are used to support AOM therapy [89].

To introduce novel nanoantibiotics into clinical practice, it is crucial to ensure the adequate biocompatibility against host cells [50]. Notably, AuNPs have emerged as promising candidates for therapeutic and diagnostic applications in oncology owing to their minimal toxic effects [90–92]. To explore whether tested nanoformulations are more selective to bacteria than mammalian cells, we performed cell culture-based experiments using human immortalized keratinocyte HaCaT cell line. HaCaT cells are widely used as a model of eukaryotic system to investigate the toxicity of nanoparticles, with the subsequent ability to release a spectrum of inflammatory cytokines, including IL-8, in the response to infectious factors exposure [50]. Importantly, those cells are also used in a number of *in vitro* research aiming to understand the tympanic membrane biology [93,94]. In our study, human keratinocytes were used to explore the ability of tested compounds to decrease the release of pro-inflammatory IL-8 in the response to *H. influenzae* infection. Using this system we demonstrated that an additional benefit from CSAs, in particular their nanosystems, as anti-infective agents is related to their anti-inflammatory activity. Accordingly, collected data present that CSAs on the surface of AuNPs statistically decreased their inflammatory response in comparison to ceragenins alone. This phenomenon might potentially limit the use of other anti-inflammatory drugs, including corticosteroids, which are recognized as an alternative therapy for AOM [95].

Despite these promising preliminary data, there are a few limitations in this research. First, our research was performed using only ATCC strains, and carrying out the experiments with nonsusceptible clinical isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* would have improved the clinical relevance of the study due to the occurring of genomic variation in antibiotic susceptibility and other virulence genes of these three otopathogens. Second, a biological membrane interaction study with the NPN test was performed only for Gram-negative bacteria. Although the available literature indicates that ceragenins and nanosystems target the membranes of Gram-positive and Gram-negative pathogens to a similar degree, the final confirmation of this issue requires additional experimentation. Finally, to successfully elucidate the antimicrobial mechanisms of CSA and CSA-based nanosystems against OM, *in vivo* study models or cell lines pertaining to middle-ear epithelial cells mimicking the site of infections should be incorporated. This acknowledges the need for future perspectives on undertaking i*n vivo* efficacy studies of CSA-based nanosystems in OM infection.

Conclusion

Altogether, these observations strongly encourage and justify the development of ceragenins and ceragenin-containing nanoformulations as agents used in treatment of OM. However, a better understanding of the molecular mechanisms implicated in the bactericidal activity of the tested compounds is warranted.

Summary points

- Due to a high prevalence of otitis media (OM) and the continuous emergence of drug-resistant OM-causing pathogens, new therapeutic options are required.
- Ceragenins exert potent bactericidal activity against bacterial pathogens causing ear infections.
- Immobilization of ceragenins on the surface of nonspherical gold nanoparticles intensifies their antibacterial effects, against both planktonic and biofilm form of bacteria.
- Activity of both ceragenins and cationic steroid antimicrobial (CSA)-based nanosystems is enhanced in the presence of human cerumen.
- Nonspherical, ceragenin-based nanosystems developed in the course of our research exert potent bactericidal activity at concentrations which are recognized as nontoxic to host cells.
- Killing properties of developed nanosystems include the induction of oxidative stress in bacterial cells resulting in permeabilization of bacterial membranes and leakage of intracellular content.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2021-0370

Author contributions

E Piktel and R Bucki designed and supervised the research and preparation of the manuscript. SV Prasad, E Piktel, L Suprewicz, T Daniluk and U Wnorowska conducted the experiments and prepared the figures. J Depciuch, A Maximenko, P Zielinski and M Parlińska-Wojtan carried out the synthesis of the tested nanosystems, performed physicochemical analysis of the tested compounds and analyzed the collected data. J Spałek and S Okła collected the human cerumen from hospital patients. SV Prasad, E Piktel, J Depciuch, A Maximenko, T Daniluk and K Fiedoruk collected and analyzed the data. J Spałek, S Okła, K Fiedoruk and R Bucki made recommendations on the experiments. PB Savage carried out the synthesis of ceragenins. SV Prasad, E Piktel, J Depciuch, A Maximenko, L Suprewicz, U Wnorowska, M Parlińska-Wojtan, PB Savage, K Fiedoruk and R Bucki participated in the writing of the manuscript. All authors reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Cerumen samples were collected from adult healthy volunteers under approval of the Bioethics Committee at the Jan Kochanowski University in Kielce, Faculty of Medicine and Health Sciences (no. 20/2019). Informed written consent has been obtained from the participants involved.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Rettig E, Tunkel DE. Contemporary concepts in management of acute otitis media in children. *Otolaryngol. Clin. N. Am.* 47(5), 651–672 (2014).
2. Paul CR, Moreno MA. Acute otitis media. *JAMA Pediatr.* 174(3), 308 (2020).
3. Pichichero ME. Immunologic dysfunction contributes to the otitis prone condition. *J. Infect.* 80(6), 614–622 (2020).
4. Frost HM, Becker LF, Knepper BC *et al.* Antibiotic prescribing patterns for acute otitis media for children 2 years and older. *J. Pediatr.* 220, 109–115.e1 (2020).
5. Williams CJ, Jacobs AM. The impact of otitis media on cognitive and educational outcomes. *Med. J. Aust.* 191(S9), S69–S72 (2009).
6. Schilder AG, Chonmaitree T, Cripps AW *et al.* Otitis media. *Nat. Rev. Dis. Primers.* 2, 16063 (2016).
7. Ngo CC, Massa HM, Thornton RB, Cripps AW. Predominant bacteria detected from the middle ear fluid of children experiencing otitis media: a systematic review. *PLoS ONE* 11(3), e0150949 (2016).
8. Bair KL, Campagnari AA. *Moraxella catarrhalis* promotes stable polymicrobial biofilms with the major otopathogens. *Front. Microbiol.* 10, 3006 (2019).
9. Silva MD, Sillankorva S. Otitis media pathogens – a life entrapped in biofilm communities. *Crit. Rev. Microbiol.* 45(5-6), 595–612 (2019).

- **A review on the role of potential otopathogens in the formation of biofilms and appropriate treatment strategies to prevent recurrence.**

10. Vermee Q, Cohen R, Hays C *et al.* Biofilm production by *Haemophilus influenzae* and *Streptococcus pneumoniae* isolated from the nasopharynx of children with acute otitis media. *BMC Infect. Dis.* 19(1), 44 (2019).
11. Obolski U, Lourenço J, Thompson C *et al*. Vaccination can drive an increase in frequencies of antibiotic resistance among nonvaccine serotypes of. *Proc. Natl. Acad. Sci. USA* 115(12), 3102–3107 (2018).
12. Cleary D, Devine V, Morris D *et al.* Pneumococcal vaccine impacts on the population genomics of non-typeable *Haemophilus influenzae*. *Microb. Genom.* 4(9), (2018).
13. Kaur R, Pham M, Yu KOA, Pichichero ME. Rising pneumococcal antibiotic resistance in the post-13-valent pneumococcal conjugate vaccine era in pediatric isolates from a primary care setting. *Clin. Infect. Dis.* 72(5), 797–805 (2021).
14. Marom T, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions in acute otitis media. *Curr. Allergy Asthma Rep.* 12(6), 551–558 (2012).
15. Qureishi A, Lee Y, Belfield K *et al*. Update on otitis media – prevention and treatment. *Infect. Drug Resist.* 7, 15–24 (2014).
16. Yang R, Sabharwal V, Okonkwo OS *et al.* Treatment of otitis media by transtympanic delivery of antibiotics. *Sci. Transl. Med.* 8(356), 356ra120 (2016).
17. Surel U, Niemirowicz K, Marzec M *et al*. Ceragenins – a new weapon to fight multidrug resistant bacterial infections. *Med. Stud.* 30(3), 207–213 (2014).
18. Lai XZ, Feng Y, Pollard J *et al.* Ceragenins: cholic acid-based mimics of antimicrobial peptides. *Acc. Chem. Res.* 41(10), 1233–1240 (2008).
19. Leszczynska K, Namiot D, Byfield FJ *et al.* Antibacterial activity of the human host defence peptide LL-37 and selected synthetic cationic lipids against bacteria associated with oral and upper respiratory tract infections. *J. Antimicrob. Chemother.* 68(3), 610–618 (2013).
20. Moscoso M, Esteban-Torres M, Menendez M, Garcia E. *In vitro* bactericidal and bacteriolytic activity of ceragenin CSA-13 against planktonic cultures and biofilms of *Streptococcus pneumoniae* and other pathogenic streptococci. *PLoS ONE* 9(7), e101037 (2014).
21. Hacioglu M, Haciosmanoglu E, Birteksoz-Tan AS, *et al*. Effects of ceragenins and conventional antimicrobials on *Candida albicans* and *Staphylococcus aureus* mono and multispecies biofilms. *Diagn. Microbiol. Infect. Dis.* 95(3), 114863 (2019).
22. Howell MD, Streib JE, Kim BE *et al.* Ceragenins: a class of antiviral compounds to treat orthopox infections. *J. Invest. Dermatol.* 129(11), 2668–2675 (2009).
23. Piktel E, Pogoda K, Roman M *et al.* Sporicidal activity of ceragenin CSA-13 against *Bacillus subtilis*. *Sci. Rep.* 7, 44452 (2017).
24. Durnas B, Wnorowska U, Pogoda K *et al.* Candidacidal activity of selected ceragenins and human cathelicidin LL-37 in experimental settings mimicking infection sites. *PLoS ONE* 11(6), e0157242 (2016).
25. Bucki R, Namiot DB, Namiot Z *et al.* Salivary mucins inhibit antibacterial activity of the cathelicidin-derived LL-37 peptide but not the cationic steroid CSA-13. *J. Antimicrob. Chemother.* 62(2), 329–335 (2008).
26. Bucki R, Sostarecz AG, Byfield FJ *et al*. Resistance of the antibacterial agent ceragenin CSA-13 to inactivation by DNA or F-actin and its activity in cystic fibrosis sputum. *J. Antimicrob. Chemother.* 60(3), 535–545 (2007).
27. Hashemi MM, Rovig J, Weber S *et al*. Susceptibility of colistin-resistant, gram-negative bacteria to antimicrobial peptides and ceragenins. *Antimicrob. Agents Chemother.* 61(8), e00292 (2017).
28. Pollard JE, Snarr J, Chaudhary V *et al. In vitro* evaluation of the potential for resistance development to ceragenin CSA-13. *J. Antimicrob. Chemother.* 67(11), 2665–2672 (2012).
29. Wnorowska U, Fiedoruk K, Piktel E *et al.* Nanoantibiotics containing membrane-active human cathelicidin LL-37 or synthetic ceragenins attached to the surface of magnetic nanoparticles as novel and innovative therapeutic tools: current status and potential future applications. *J. Nanobiotechnol.* 18(1), 3 (2020).
30. Alkilany AM, Murphy CJ. Toxicity and cellular uptake of gold nanoparticles: what we have learned so far? *J. Nanopart. Res.* 12(7), 2313–2333 (2010).
31. Shamaila S, Zafar N, Riaz S *et al*. Gold nanoparticles: an efficient antimicrobial agent against enteric bacterial human pathogen. *Nanomaterials (Basel)* 6(4), 71 (2016).
32. Mohamed MM, Fouad SA, Elshoky HA *et al.* Antibacterial effect of gold nanoparticles against *Corynebacterium pseudotuberculosis*. *Int. J. Vet. Sci. Med.* 5(1), 23–29 (2017).
33. Khan MA, Khan MJ. Nano-gold displayed anti-inflammatory property via NF-κB pathways by suppressing COX-2 activity. *Artif. Cells Nanomed. Biotechnol.* 46(Suppl 1.), 1149–1158 (2018).
34. Lee B, Lee DG. Synergistic antibacterial activity of gold nanoparticles caused by apoptosis-like death. *J. Appl. Microbiol.* 127(3), 701–712 (2019).
35. Rad MR, Kazemian H, Yazdani F *et al.* Antibacterial activity of gold nanoparticles conjugated by aminoglycosides against *A. baumannii* isolates from burn patients. *Recent Pat. Antiinfect. Drug Discov.* 13(3), 256–264 (2018).

36. Kalita S, Kandimalla R, Sharma KK *et al.* Amoxicillin functionalized gold nanoparticles reverts MRSA resistance. *Mater. Sci. Eng. C Mater. Biol. Appl.* 61, 720–727 (2016).
37. Díez-Martínez R, García-Fernández E, Manzano M *et al.* Auranofin-loaded nanoparticles as a new therapeutic tool to fight streptococcal infections. *Sci. Rep.* 6, 19525 (2016).
38. Slomberg DL, Lu Y, Broadnax AD *et al.* Role of size and shape on biofilm eradication for nitric oxide-releasing silica nanoparticles. *ACS Appl. Mater. Interfaces,* 5(19), 9322–9329 (2013).
39. Piktel E, Ościłowska I, Suprewicz Ł *et al.* ROS-mediated apoptosis and autophagy in ovarian cancer cells treated with peanut-shaped gold nanoparticles. *Int. J. Nanomedicine* 16, 1993–2011 (2021).
40. Penders J, Stolzoff M, Hickey DJ *et al.* Shape-dependent antibacterial effects of non-cytotoxic gold nanoparticles. *Int. J. Nanomedicine* 12, 2457–2468 (2017).
- **A study on the antibacterial activity of various shapes of gold nanoparticles (AuNPs) against the Gram-positive *Staphylococcus aureus*.**
41. Piktel E, Suprewicz Ł, Depciuch J *et al.* Varied-shaped gold nanoparticles with nanogram killing efficiency as potential antimicrobial surface coatings for the medical devices. *Sci. Rep.* 11(1), 12546 (2021).
- **Describes the evidence for the *in vitro* effects of varied Au NPs in shapes of rods, peanut, stars and spherical against bacteria and fungi. It addresses the antibiofilm properties and potential use of gold nanoparticles as antimicrobial surface coatings for medical devices.**
42. Piktel E, Suprewicz Ł, Depciuch J *et al.* Rod-shaped gold nanoparticles exert potent candidacidal activity and decrease the adhesion of fungal cells. *Nanomedicine (Lond.)* 15(28), 2733–2752 (2020).
43. Chmielewska SJ, Skłodowski K, Depciuch J *et al.* Bactericidal properties of rod-, peanut-, and star-shaped gold nanoparticles coated with ceragenin CSA-131 against multidrug-resistant bacterial strains. *Pharmaceutics* 13(3), 425 (2021).
- **Describes the evidence for the antimicrobial effects of rods, peanut and star shaped Au NPs functionalized with CSA-131 as potential nanoantibiotics against multidrug resistant Gram-negative and Gram-positive bacteria.**
44. Ding B, Guan Q, Walsh JP *et al.* Correlation of the antibacterial activities of cationic peptide antibiotics and cationic steroid antibiotics. *J. Med. Chem.* 45(3), 663–669 (2002).
45. Rodriguez-Carvajal J. FULLPROF: a program for rietveld refinement and pattern matching analysis. In: *Abstracts of the Satellite Meeting on Powder Diffraction of the XV Congress of the IUCr.* Toulouse, France, 127 (1990).
46. Jorgensen JH, Maher LA, Howell AW. Use of Haemophilus test medium for broth microdilution antimicrobial susceptibility testing of *Streptococcus pneumoniae*. *J. Clin. Microbiol.* 28(3), 430–434 (1990).
47. Durnas B, Piktel E, Watek M *et al.* Anaerobic bacteria growth in the presence of cathelicidin LL-37 and selected ceragenins delivered as magnetic nanoparticles cargo. *BMC Microbiol.* 17(1), 167 (2017).
48. Chai TJ, Chai TC. Bactericidal activity of cerumen. *Antimicrob. Agents Chemother.* 18(4), 638–641 (1980).
49. Wnorowska U, Piktel E, Durnaś B *et al.* Use of ceragenins as a potential treatment for urinary tract infections. *BMC Infect Dis.* 19(1), 369 (2019).
50. Piktel E, Wnorowska U, Cieśluk M *et al.* Inhibition of inflammatory response in human keratinocytes by magnetic nanoparticles functionalized with PBP10 peptide derived from the PIP2-binding site of human plasma gelsolin. *J. Nanobiotechnol.* 17(1), 22 (2019).
51. Suh I, Ohta H, Waseda Y. High-temperature thermal expansion of six metallic elements measured by dilatation method and x-ray diffraction. *J. Mater. Sci.* 23, 757–760 (1988).
52. Sneba K, Sathishkumar M, Sok K, YeoungSang Y. Counter ions and temperature incorporated tailoring of biogenic gold nanoparticles. *Process Biochem.* 45(9), 1450–1458 (2010).
53. Levin CS, Janesko BG, Bardhan R *et al.* Chain-length-dependent vibrational resonances in alkanethiol self-assembled monolayers observed on plasmonic nanoparticle substrates. *Nano Lett.* 6(11), 2617–2621 (2006).
54. Bazylewski P, Divigalpitiyab R, Fanchiniac G. *In situ* Raman spectroscopy distinguishes between reversible and irreversible thiol modifications in l-cysteine. *RCS Adv.* 7, 2964–2970 (2017).
55. Deręgowska A, Depciuch J, Wojnarowska R *et al.* Study of optical properties of a glutathione capped gold nanoparticles using linker (MHDA) by Fourier transform infra red spectroscopy and surface enhanced Raman scattering. *Int. J. Biol. Biomol. Agricult. Food Biotechnol. Eng.* 7(1), 80–83 (2013).
56. Charlé K, Frank F, Schulze W. The optical properties of silver microcrystallites in dependence on size and the influence of the matrix environment. *Berichte Bunsengesellschaft Phys. Chem.* 88(4), 350–354 (1984).
57. Chandekar A, Sengupta SK, Whitten JE. Thermal stability of thiol and silane monolayers: A comparative study. *Appl. Surf. Sci.* 256(9), 2742–2749 (2010).
58. Frost HM, Gerber JS, Hersh AL. Antibiotic recommendations for acute otitis media and acute bacterial sinusitis. *Pediatr. Infect. Dis. J.* 38(2), 217 (2019).

59. Gerber JS, Ross RK, Bryan M *et al.* Association of broad- vs narrow-spectrum antibiotics with treatment failure, adverse events, and quality of life in children with acute respiratory tract infections. *JAMA* 318(23), 2325–2336 (2017).

60. Leso V, Fontana L, Ercolano ML, Romano R, Iavicoli I. Opportunities and challenging issues of nanomaterials in otological fields: an occupational health perspective. *Nanomedicine(Lond.)* 14(19), 2613–2629 (2019).

• **A review on the use of nanotechnology for efficient and targeted treatment of inner ear diseases.**

61. Ziąbka M, Dziadek M, Menaszek E *et al*. Middle ear prosthesis with bactericidal efficacy-*in vitro* investigation. *Molecules* 22(10), 1681 (2017).

62. Yu Q, Li J, Zhang Y, Wang Y *et al*. Inhibition of gold nanoparticles (AuNPs) on pathogenic biofilm formation and invasion to host cells. *Sci. Rep.* 6, 26667 (2016).

• **Review on the wide-spectrum antimicrobial activity of gold nanoclusters against Gram-positive and Gram-negative bacteria.**

63. Kayyali MN, Brake L, Ramsey AJ *et al*. A novel nano-approach for targeted inner ear imaging. *J. Nanomed. Nanotechnol.* 8(4), 2017).

64. Ahangari A, Salouti M, Heidari Z *et al*. Development of gentamicin-gold nanospheres for antimicrobial drug delivery to Staphylococcal infected foci. *Drug Deliv.* 20(1), 34–39 (2013).

65. Rai A, Prabhuneb A, Perry C. Antibiotic mediated synthesis of gold nanoparticles with potent antimicrobial activity and their application in antimicrobial coatings. *J. Mater. Chem.* 20, 6789–6798 (2010).

66. Brown AN, Smith K, Samuels TA *et al*. Nanoparticles functionalized with ampicillin destroy multiple-antibiotic-resistant isolates of *Pseudomonas aeruginosa* and *Enterobacter aerogenes* and methicillin-resistant *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 78(8), 2768–2774 (2012).

67. Niemirowicz K, Durnaś B, Tokajuk G *et al.* Formulation and candidacidal activity of magnetic nanoparticles coated with cathelicidin LL-37 and ceragenin CSA-13. *Sci. Rep.* 7(1), 4610 (2017).

68. Piktel E, Prokop I, Wnorowska U *et al.* Ceragenin CSA-13 as free molecules and attached to magnetic nanoparticle surfaces induce caspase-dependent apoptosis in human breast cancer cells via disruption of cell oxidative balance. *Oncotarget* 9(31), 21904–21920 (2018).

69. Chatterjee T, Chatterjee BK, Chakrabarti P. Modelling of growth kinetics of *Vibrio cholerae* in presence of gold nanoparticles: effect of size and morphology. *Sci. Rep.* 7(1), 9671 (2017).

70. Perez AC, Pang B, King LB *et al.* Residence of *Streptococcus pneumoniae* and *Moraxella catarrhalis* within polymicrobial biofilm promotes antibiotic resistance and bacterial persistence *in vivo*. *Pathog. Dis.* 70(3), 280–288 (2014).

71. Epand RF, Pollard JE, Wright JO *et al*. Depolarization, bacterial membrane composition, and the antimicrobial action of ceragenins. *Antimicrob. Agents Chemother.* 54(9), 3708–3713 (2010).

•• **A study on the bactericidal efficiency and mechanism of action of various ceragenins against *Escherichia coli*.**

72. Slavin YN, Asnis J, Hafeli UO, Bach H. Metal nanoparticles: understanding the mechanisms behind antibacterial activity. *J. Nanobiotechnol.* 15(1), 65 (2017).

73. Lee H, Lee DG. Gold nanoparticles induce a reactive oxygen species-independent apoptotic pathway in *Escherichia coli*. *Colloids Surf. B Biointerfaces* 167, 1–7 (2018).

74. Rowe-Magnus DA, Kao AY, Prieto AC *et al*. Cathelicidin peptides restrict bacterial growth via membrane perturbation and induction of reactive oxygen species. *mBio* 10(5), e02021 (2019).

75. Eruslanov E, Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods Mol. Biol.* 594, 57–72 (2010).

76. Dwyer DJ, Belenky PA, Yang JH *et al.* Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. USA* 111(20), E2100–E2109 (2014).

77. Zhao X, Drlica K. Reactive oxygen species and the bacterial response to lethal stress. *Curr. Opin. Microbiol.* 21, 1–6 (2014).

78. Ortiz-Benitez EA, Carrillo-Morales M, Velázquez-Guadarrama N *et al*. Inclusion bodies and pH lowering: as an effect of gold nanoparticles in *Streptococcus pneumoniae*. *Metallomics* 7(7), 1173–1179 (2015).

79. Ortiz-Benítez EA, Velázquez-Guadarrama N, Durán Figueroa NV *et al*. Antibacterial mechanism of gold nanoparticles on *Streptococcus pneumoniae*. *Metallomics* 11(7), 1265–1276 (2019).

80. Zheng K, Setyawati MI, Leong DT, Xie J. Antimicrobial gold nanoclusters. *ACS Nano* 11(7), 6904–6910 (2017).

81. Pericone CD, Overweg K, Hermans PW, Weiser JN. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infect. Immun.* 68(7), 3990–3997 (2000).

82. Hoopman TC, Liu W, Joslin SN *et al*. Identification of gene products involved in the oxidative stress response of *Moraxella catarrhalis*. *Infect. Immun.* 79(2), 745–755 (2011).

83. Borrill ZL, Roy K, Singh D. Exhaled breath condensate biomarkers in COPD. *Eur. Respir. J.* 32(2), 472–486 (2008).

84. Kalyanaraman B, Darley-Usmar V, Davies KJ *et al.* Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* 52(1), 1–6 (2012).

85. Niemirowicz K, Surel U, Wilczewska AZ *et al.* Bactericidal activity and biocompatibility of ceragenin-coated magnetic nanoparticles. *J. Nanobiotechnology* 13(1), 32 (2015).

86. Durnaś B, Wnorowska U, Pogoda K *et al.* Candidacidal activity of selected ceragenins and human cathelicidin LL-37 in experimental settings mimicking infection sites. *PLoS ONE* 11(6), e0157242 (2016).
87. Schwaab M, Gurr A, Neumann A *et al.* Human antimicrobial proteins in ear wax. *Eur. J. Clin. Microbiol. Infect. Dis.* 30(8), 997–1004 (2011).
88. Leszczyńska K, Namiot A, Cruz K *et al.* Potential of ceragenin CSA-13 and its mixture with pluronic F-127 as treatment of topical bacterial infections. *J. Appl. Microbiol.* 110(1), 229–238 (2011).
89. Kim JK, Cho JH. Change of external auditory canal pH in acute otitis externa. *Ann. Otol. Rhinol. Laryngol.* 118(11), 769–772 (2009).
90. Sulaiman GM, Waheeb HM, Jabir MS *et al.* Hesperidin loaded on gold nanoparticles as a drug delivery system for a successful biocompatible, anti-cancer, anti-inflammatory and phagocytosis inducer model. *Sci. Rep.* 10(1), 9362 (2020).
91. Farooq MU, Novosad V, Rozhkova EA *et al.* Gold nanoparticles-enabled efficient dual delivery of anticancer therapeutics to HeLa cells. *Sci. Rep.* 8(1), 2907 (2018).
92. Cheheltani R, Ezzibdeh RM, Chhour P *et al.* Tunable, biodegradable gold nanoparticles as contrast agents for computed tomography and photoacoustic imaging. *Biomaterials* 102, 87–97 (2016).
93. Raynov AM, Choung YH, Park HY *et al.* Establishment and characterization of an *In vitro* model for cholesteatoma. *Clin. Exp. Otorhinolaryngol.* 1(2), 86–91 (2008).
94. von Witzleben M, Stoppe T, Ahlfeld T *et al.* Biomimetic tympanic membrane replacement made by melt electrowriting. *Adv. Healthc. Mater.* 10(10), e2002089 (2021).
95. Ranakusuma RW, McCullough AR, Safitri ED *et al.* Oral prednisolone for acute otitis media in children: a pilot, pragmatic, randomised, open-label, controlled study (OPAL study). *Pilot Feasibility Stud.* 4, 146 (2020).

7. ABSTRACT

Antimicrobial resistance (AMR) is a major concern in clinical settings, that has been related to the misuse and overuse use of antibiotics, as well as crisis in the development of novel antimicrobial agents. Ceragenins (CSAs) are promising candidates for the founding of novel antibiotics. These non-peptide mimics of endogenous antimicrobial peptides (AMPs) are bactericidal and broad-spectrum antimicrobials with immunomodulatory properties. In addition, antibacterial activity and biocompatibility of ceragenins may be improved by their attachment on the surface of nanomaterials, such as gold nanoparticles (AuNPs).

The objective of this research was to study the antibacterial efficacy of ceragenins alone and in combination with peanut-shaped gold nanoparticles (AuP NP@CSA) against the leading etiological agents of middle ear infection – *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis.* Enhanced antibacterial activity, reduction of the proinflammatory cytokine IL-8 secretion, and improved biocompatibility with host cells characterized the tested ceragenin-based nanosystems. Remarkably, the antibacterial activity of ceragenins was not affected by human cerumen (earwax).

In vitro experiments with induction of resistance to ceragenin CSA-13 and colistin in an emerging hospital pathogen – *Enterobacter hormaechei* subsp. *steigerwaltii* (ST89) were used to investigate the potential mechanisms of resistance to these compounds. Notably, despite the common cationic nature of both agents, the prolonged exposure of *E. hormaechei* to CSA-13 induced only moderate level resistance to this ceragenin, compared to the high level of resistance to colistin observed in *E. hormaechei* upon exposure to this antibiotic. Furthermore, the whole-genome (WGS) and transcriptome (RNA-seq) analyses revealed that molecular mechanisms responsible for the high level of resistance to colistin developed by the studied *E. hormaechei* strain have no impact on its susceptibility to ceragenin CSA-13. Additionally, several novel mechanisms that directly or indirectly may contribute to the development of resistance to ceragenin CSA-13 and/or colistin in *E. hormaechei* have been identified.

8. ABSTRACT IN POLISH

Antybiotykooporność (*ang. antimicrobial resistance; AMR*) stanowi jeden z głównych problemów klinicznych, wynikający z nadmiernego stosowania oraz nadużywania antybiotyków, jak również braku badań and rozwojem nowych leków. Cerageniny (CSAs) są obiecującymi kandydatami do opracowania nowych generacji związków przeciwdrobnoustrojowych. Te steroidowe analogi naturalnych peptydów przeciwdrobnoustrojowych (*ang. antimicrobial peptides,* AMPs) cechuje bakteriobójcze i szerokie spektrum działania oraz właściwości immunomodulujące. Ponadto aktywność przeciwbakteryjna i biokompatybilność ceragenin może być modyfikowana poprzez ich przyłączenie do powierzchni nanomateriałów, np. nanocząstek złota (AuNPs, *ang. Au nanoparticles*).

Celem badań była ocena skuteczności przeciwbakteryjnej ceragenin oraz zawierających je nanosystemów w formie nanocząstek złota w kształcie orzeszków ziemnych (AuP NP, *ang. Au peanut-shaped nanoparticles*) – AuP NP@CSA wobec głównych czynników etiologicznych zapalenia ucha środkowego – *Streptococcus pneumoniae, Haemophilus influenzae* i *Moraxella catarrhalis*. Wykazano zwiększoną aktywność przeciwbakteryjną, zmniejszenie produkcji prozapalnej cytokiny IL-8 oraz lepszą biokompatybilność z komórkami gospodarza nanosystemów AuP NP@CSA w porównaniu do działania samych ceragenin. Ponadto, nie zaobserwowano wpływu ludzkiej woskowiny na aktywność przeciwbakteryjną ceragenin.

Potencjalne mechanizmy oporności na cerageniny zbadano w oparciu o przeprowadzone w warunkach *in vitro* eksperymenty mające na celu indukcję oporności na cerageninę CSA-13 i kolistynę w nowym patogenie szpitalnym, tzw. *emerging pathogen,* jakim jest gram-ujemna pałeczka – *Enterobacter hormaechei* subsp. *steigerwaltii* (ST89). Przeprowadzone badania wykazały, że pomimo wspólnej kationowej natury obu związków, długotrwała ekspozycja *E. hormaechei* na CSA-13 indukowała jedynie umiarkowany poziom oporności na tę cerageninę, w porównaniu do wysokiego stopnia oporności zaobserwowanego po ekspozycji na kolistynę. Co więcej, analizy oparte na sekwencjonowaniu nowej generacji genomu (*ang. whole genome sequencing, WGS*) i transkryptomu (RNA-seq) badanych szczepów wykazały, że mechanizmy molekularne odpowiedzialne za wysoki poziom oporności na kolistynę

wykształcone przez badany szczep *E. hormaechei* podczas długotrwałej ekspozycji na ten antybiotyk nie mają wpływu na jego wrażliwość na cerageninę CSA-13. Dodatkowo zidentyfikowano nowe mechanizmy, które bezpośrednio lub pośrednio mogą przyczyniać się do rozwoju oporności na cerageninę CSA-13 i/lub kolistynę u *E. hormaechei*.

9. FUNDING AND CONSENT FROM THE BIOETHICS COMMITTEE

9.1. Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 754432 and the Polish Ministry of Science and Higher Education, from financial resources for science in 2018-2023 granted for the implementation of an international co-financed project.

9.2. Consent from the Bioethics Committee

KOMISJA BIOETYCZNA
WYDZIAŁ LEKARSKI I NAUK O ZDROWIU
UNIWERSYTET
Jana Kochanowskiego w Kielcach

Uchwała Komisji Bioetycznej

Nr 20/2019

Na podstawie art. 29 ustawy z dnia 05 grudnia 1996 r. o zawodach lekarza i lekarza dentysty (t.j. Dz.U. z 2015 r. poz. 464) oraz Rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz. U. z 1999 r. Nr 47 poz. 480) oraz działając zgodnie z zasadami GCP (Good Clinical Practice):

Komisja Bioetyczna przy Wydziale Lekarskim i Nauk o Zdrowiu Uniwersytetu Jana Kochanowskiego w Kielcach na posiedzeniu w dniu **04.03.2019 r.** zapoznała się z projektem badania pt.: **„Ocena możliwość wykorzystania peptydów przeciwdrobnoustrojowych i ich syntetycznych analogów (ceragenin) w terapii zakażeń narządu słuchu "**

Przedstawionym przez: **Lek. Jakub Spałek**

Do Komisji wpłynęły następujące dokumenty:

1. Wniosek do Komisji Bioetycznej
2. Oświadczenia badacza
3. Informacja dla pacjenta

Po zapoznaniu się z całością dokumentacji Komisja Bioetyczna stwierdza, że jest ona kompletna i zawiera wszelkie ustawowo wymagane dokumenty. Zawarte w dokumentach informacje dotyczące badanego problemu są jasne i wyczerpujące.

Celem projektu jest ocena potencjału terapeutycznego naturalnych peptydów p/drobnoustrojowych i syntetycznych pochodnych ceragenin. Planowane jest badanie aktywności p/drobnoustrojowej tych substancji w stosunku do najczęstszych czynników etiologicznych tych zakażeń w obecności emulsyfikowanej woskowiny.

Metody badawcze zastosowane w pracy mają charakter nieinwazyjny.

Na podstawie przedstawionej dokumentacji i jej analizy Komisja Bioetyki wyraża pozytywną opinię na temat prezentowanej pracy i zgodę na jej przeprowadzenie.

Wydana uchwała dotyczy tylko rozpatrywanego wniosku z uwzględnieniem przedstawionego projektu; każda zmiana i modyfikacja wymaga uzyskania odrębnej uchwały. Wnioskodawca zobowiązany jest do informowania o wszelkich poprawkach, które mogłyby mieć wpływ na opinię Komisji, o ciężkich lub niespodziewanych zdarzeniach niepożądanych i nieprzewidzianych okolicznościach; o zakończeniu badania, o jego wynikach i istotnych decyzjach innych komisji bioetycznych.

Wyrażona przez komisję bioetyczną opinia może zawierać uzupełniające warunki dopuszczające przeprowadzenie opiniowanego projektu.

Komisja bioetyczna może wyrazić stanowisko w sprawie uzupełnienia projektu opiniowanego eksperymentu medycznego o dodatkowe warunki dopuszczające jego przeprowadzenie. *[1]

POUCZENIE

1. *Odwołanie od uchwały komisji bioetycznej wyrażającej opinię może wnieść:*
   1) podmiot zamierzający przeprowadzić eksperyment medyczny,
   2) kierownik zakładu opieki zdrowotnej, w którym eksperyment medyczny ma być przeprowadzony,
   3)komisja bioetyczna właściwa dla ośrodka, który ma uczestniczyć w wieloośrodkowym eksperymencie medycznym.
2. *Odwołanie, o którym mowa w ust. 1, wnosi się za pośrednictwem komisji bioetycznej, która podjęła uchwałę, do Odwoławczej Komisji Bioetycznej, o której mowa w art. 29 ust. 5 ustawy o zawodach lekarza i lekarza dentysty, w terminie 14 dni od dnia otrzymania uchwały wyrażającej opinię.*
3. *Odwołanie powinno być rozpatrzone nie później niż w ciągu dwóch miesięcy od dnia jego wniesienia.*
4. *Do postępowania odwoławczego stosuje się odpowiednio przepisy z Rozporządzenia Ministra Zdrowia i Opieki Społecznej z dnia 11 maja 1999 r. w sprawie szczegółowych zasad powoływania i finansowania oraz trybu działania komisji bioetycznych (Dz. U. Nr 47, poz. 480) § 6 ust. 3-5, 7, 8 i 10.*

Kielce, 5.03.2019 r.

PRZEWODNICZĄCA
KOMISJI BIOETYCZNEJ
WYDZIAŁU LEKARSKIEGO I NAUK O ZDROWIU

dr n. med. Agata Horecka-Lewitowicz

Zał.

- podpisy członków Komisji

* jeśli dotyczy

10. AUTHOR AND CO-AUTHOR CONTRIBUTION TO DISSERTATION PUBLICATIONS

Review article

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. *Expression and Function of Host Defense Peptides at Inflammation Sites.* Int J Mol Sci. 2019 Dec 22;21(1):104. doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121.

IF: 5.924; MEiN: 140

Information on the nature of the co-authors' participation in publications, including with an estimate of the percentage contribution (review work)

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. **Expression and Function of Host Defense Peptides at Inflammation Sites**. Int J Mol Sci. 2019 Dec 22;21(1):104. Doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121.

Author's name and surname	Nature of participation	Contribution in %
Doctoral candidate – Suhanya Prasad, MSc	Conceptualization, writing, and original draft preparation	60%
dr hab. n. med. Krzysztof Fiedoruk	Conceptualization, writing, figure preparation, review, and editing,	15%
dr n. med. Tamara Daniluk	Writing and figure preparation	10%
dr n. med. Ewelina Piktel	Conceptualization and writing	5%
Prof. dr hab. Robert Bucki	Conceptualization, review, and editing	10%

I hereby declare that all co-authors agreed to use these articles in the dissertation.

Signature

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STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. Expression and Function of Host Defense Peptides at Inflammation Sites. Int J Mol Sci. 2019 Dec 22;21(1):104. Doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121., being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 15 % and contributed to conceptualization, writing, figure preparation, review, and editing in the above review.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences.

Sincerely,

.....

ADIUNKT
Zakładu Mikrobiologii Lekarskiej
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STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. Expression and Function of Host Defense Peptides at Inflammation Sites. Int J Mol Sci. 2019 Dec 22;21(1):104. Doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121., being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 10% and contributed to writing and figure preparation in the above review.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences.

Sincerely,

.....Daniluk Tamara.....

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STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. Expression and Function of Host Defense Peptides at Inflammation Sites. Int J Mol Sci. 2019 Dec 22;21(1):104. Doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121., being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 5 % and contributed to conceptualization and writing in the above review.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences.

Sincerely,

Ewelina Piktel

.....

9/14/2022

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STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Fiedoruk K, Daniluk T, Piktel E, Bucki R. Expression and Function of Host Defense Peptides at Inflammation Sites. Int J Mol Sci. 2019 Dec 22;21(1):104. Doi: 10.3390/ijms21010104. PMID: 31877866; PMCID: PMC6982121., being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 10 % and contributed to conceptualization, review, and editing of the mentioned publication.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences and health sciences in the discipline of medical sciences.

Sincerely,

KIEROWNIK
Zakładu Mikrobiologii Lekarskiej
Inżynierii Nanobiomedycznej

.....
Prof. dr hab. Robert Bucki

Original article

Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. *Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins*. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374.

IF: 6.096; MEiN: 100

Information on the nature of the co-authors' participation in publications, including with an estimate of the percentage contribution (original work)

Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. **Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins**. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374.

Author's name and surname	Nature of participation	Contribution in %
Doctoral candidate – Suhanya Prasad, MSc	Conducted the experiments, prepared the figures, analyzed the data, and writing of the manuscript	55%
dr n. med. Ewelina Piktel	Designed and supervised the research, collected and analyzed the data, preparation of the manuscript and figures	5%
dr inż. Joanna Depciuch	Synthesis, and physicochemical data analysis of the tested nanosystems	4%
dr. Alexey Maximenko	Synthesis, and physicochemical data analysis of the tested nanosystems	4%
mgr Łukasz Suprewicz	Conducted the experiments, and prepared the figures	3%
dr n. med. Tamara Daniluk	Conducted the experiments and prepared the figures	3%
dr n. med. Jakub Spałek	Collected the human cerumen from hospital patients	2%
dr n. med. Urszula Wnorowska	Conducted the experiments and prepared the figures	5%
dr hab. Piotr Zieliński prof.UwB	Synthesis, and physicochemical analysis of the tested nanosystems	2%
Prof. dr hab. inż. Magdalena Parlinska-Wojtan	Synthesis, and physicochemical analysis of the tested nanosystems	2%
Prof. Paul B Savage	Synthesis of ceragenins	3%

Continued on next page

Continued

dr n. med. Sławomir Okła	Collected the human cerumen from hospital patients	2%
dr hab. n. med. Krzysztof Fiedoruk	Participated in writing of the manuscript, analyzed the data, and made recommendations on the experiments	5%
Prof. dr hab. Robert Bucki	Designed and supervised the research, preparation of the manuscript, and made recommendations on the experiments	5%

I hereby declare that all co-authors agreed to use these articles in the dissertation.

Signature

dr n. med. Ewelina Piktel
Department of Medical Microbiology and Nanobiomedical Engineering
Medical University of Bialystok
ul. Mickiewicza 2C
15-222 Bialystok

STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. **Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins**. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374, being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 5 % and designed, supervised the research, collected and analyzed the data, preparation of the manuscript and figures in the above research.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences.

Sincerely,

.....

dr inż. Joanna Depciuch
Institute of Nuclear Physics Polish Academy of Sciences
PL-31342, Krakow, Poland

STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. **Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins**. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374, being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 4% and consisted of synthesis and physicochemical data analysis of the tested nanosystems used in the above research.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences and health sciences in the discipline of medical sciences.

Sincerely,

.....Joanna Depciuch.....

JAGIELLONIAN UNIVERSITY
31-007 Kraków, Gołębia 24
NIP 675-000-22-36
National Synchrotron Radiation
Centre SOLARIS
30-392 Kraków, Czerwone Maki 98

dr. Alexey Maximenko

SOLARIS National Synchrotron Radiation Centre,

Jagiellonian University, PL-30392, Krakow, Poland

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Sincerely,

Alexey Maximenko

mgr Łukasz Suprewicz
Department of Medical Microbiology and Nanobiomedical Engineering
Medical University of Bialystok
ul. Mickiewicza 2C
15-222 Bialystok

STATEMENT

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I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences.

Sincerely,

Łukasz Suprewicz 14.09.2022 r.

dr n. med. Tamara Daniluk
Department of Medical Microbiology and Nanobiomedical Engineering
Medical University of Bialystok
ul. Mickiewicza 2C
15-222 Bialystok

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Sincerely,

.....Daniluk Tamara.....

lek med. Jakub Spałek
Department of Pathology, Collegium Medicum
Jan Kochanowski University in Kielce, PL-25317, Kielce, Poland

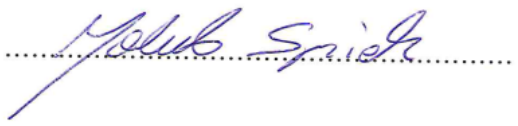
STATEMENT

I declare and confirm my participation in the publication:

Prasad SV, Piktel E, Depciuch J, Maximenko A, Suprewicz Ł, Daniluk T, Spałek J, Wnorowska U, M Zielinski P, Parlinska-Wojtan M, B Savage P, Okła S, Fiedoruk K, Bucki R. **Targeting bacteria causing otitis media using nanosystems containing nonspherical gold nanoparticles and ceragenins**. Nanomedicine (Lond). 2021 Dec;16(30):2657-2678. doi: 10.2217/nnm-2021-0370. Epub 2021 Nov 26. PMID: 34823374, being a part of the doctoral dissertation of Suhanya Prasad, MSc, was 2% and consisted of collecting the cerumen samples from hospital patients used in the above research.

I agree that Suhanya Prasad, MSc, may use the publication described above in the proceedings for granting the degree of doctor of medical sciences and health sciences in the discipline of medical sciences.

Sincerely,



dr n. med. Urszula Wnorowska
Department of Medical Microbiology and Nanobiomedical Engineering
Medical University of Bialystok
ul. Mickiewicza 2C
15-222 Bialystok

STATEMENT

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Sincerely,

Urszula Wnorowska

Professor Paul B. Savage
Department of Chemistry and Biochemistry
Brigham Young University,
C100 BNSN
Provo, UT 84602
Phone: (801) 422-4020/ Fax: (801) 422-0153
Email: paul_savage@byu.edu

9/13/22

STATEMENT

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Sincerely,

.....

Paul B. Savage

Reed M. Izatt Professor of Chemistry and Biochemistry

dr n. med. Sławomir Okła
Department of Otolaryngology, Head & Neck Surgery
Holy Cross Cancer Center in Kielce, PL-25734, Kielce, Poland

STATEMENT

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Sincerely,

KIEROWNIK KLINIKI
Otolaryngologii, Chirurgii Głowy i Szyi
(2)
Dr n. med. Sławomir Okła

dr hab. Piotr Zieliński
Institute of Nuclear Physics Polish Academy of Sciences
PL-31342, Krakow, Poland

STATEMENT

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Sincerely,

.....

prof. dr hab. inż. Magdalena Parlińska-Wojtan
Institute of Nuclear Physics Polish Academy of Sciences
PL-31342, Krakow, Poland

STATEMENT

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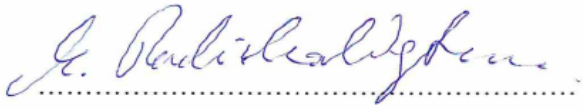
Sincerely,



dr hab. n. med. Krzysztof Fiedoruk
Department of Medical Microbiology and Nanobiomedical Engineering
Medical University of Bialystok
ul. Mickiewicza 2C
15-222 Bialystok

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Sincerely,

ADIUNKT
Zakładu Mikrobiologii Lekarskiej
i Inżynierii Nanobiomedycznej

dr hab. n. med. Krzysztof Fiedoruk

9/14/2022

Prof. dr hab. Robert Bucki
Department of Medical Microbiology and Nanobiomedical Engineering
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