UNIVERSITAT DE BARCELONA

Super-Cationic peptides: insights on their antimicrobial action

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UNIVERSITAT DE BARCELONA

Faculty of Medicine and Health Sciences
PhD Thesis

Super-Cationic peptides: insights on their antimicrobial action.

Thesis submitted by **Isabel Pérez Guillén** in fulfilment of the requirements for the degree of PhD by the University of Barcelona

Supervised by Prof. Dr. Miguel Viñas Ciordia,
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Doctorate in Translational Medicine and Research

Department of Pathology and Experimental Therapeutics
Laboratory of Molecular Microbiology and Antimicrobials

Barcelona ~ 2022

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C E R T I F I C A N,

Que la tesis doctoral presentada por **Isabel Pérez Guillén**, y titulada ***"Super-Cationic peptides: insights on their antimicrobial action",*** ha sido realizada por la autora en el Laboratorio de Microbiología Molecular y Antimicrobianos del Departamento de Patología y Terapéutica Experimental del Campus de Bellvitge bajo nuestra dirección.

Que se autorizó su presentación a la Comisión del programa de doctorado *"Medicina i Recerca Translacional"* en fecha XX de XXX de 20XX.

Que la tesis cumple a nuestro juicio los requisitos necesarios formales y conceptuales para optar al título de doctor por la Universidad de Barcelona.

Y para que conste, firman el presente en l'Hospitalet de Llobregat a 11 de octubre de 2022.

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Fdo. Isabel Pérez Guillén

Title: **Super-Cationic peptides: insights on their antimicrobial action**

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Thesis submitted in fulfilment of the requirements for the degree of PhD by the University of Barcelona.

Signed: Isabel Pérez Guillén

L'Hospitalet de Llobregat, 11th October 2022

"It is our choices, Harry, that show what we truly are, far more than our abilities"

Harry Potter and the Chamber of Secrets (book) - J. K.Rowling

"Happiness can be found even in the darkest of times if one only remembers to turn on the light"

Harry Potter and the Prisoner of Azkaban (film) – Steve Kloves – J.K. Rowling

ACKNOWLEDGMENTS

Esta tesis se ha podido realizar gracias a un contrato de investigación realizado por la Fundación Josep Finestres en colaboración con el grupo liderado por el Prof. Dr. Miquel Viñas Ciordia (Julio 2020) en la unidad de microbiología del Departamento de Patología y Terapéutica Experimental de la Universidad de Barcelona.

La realización de la tesis está asociada al proyecto:
Fundació La Marató de TV3 (201C/2018): **B**reaking the borders of **A**ntimicrobial **R**esistance: Searching **N**ew **A**ntimicrobial compounds against multi-drug resistant bacteria: A study of **P**olicationic **A**MPs and lipid nanoparticles. (**BARNAPA**).

Además, agradecer a la Universidad de Barcelona por concederme la Beca de Retención de Talento al inicio de la actividad Investigadora. En el Campus de Bellvitge de Nov 2018 a Feb 2019.

El estar escribiendo esto supone el comienzo de un final, el final del doctorado, que se dice pronto, pero son muchos años de ciencia, esfuerzo, tiempo y anécdotas que se arremolinan en la cabeza cuando se intenta expresar aquí una pequeña parte. Es difícil cerrar una etapa y enfrentarse a los interrogantes que supone el comienzo de una nueva, pero en la vida igual que en la ciencia sin interrogantes no avanzamos.

Me gustaría aprovechar este espacio para recordar y agradecer todo el apoyo, ayuda, compañía, risas y ciencia que se me ha ofrecido durante este periodo y cuyo resultado está reflejado aquí, ya que, sin todas esas muchas personas, aportando su granito de arena, esta tesis no hubiese sido posible.

Al Prof. Dr. Miquel Viñas Ciordia por darme la oportunidad de entrar en su laboratorio en un momento personal muy complejo y confiar en mí en cada paso. He aprendido mucha ciencia, pero más importante he aprendido lecciones de vida, hemos trabajado, reído y a veces los péptidos nos han desesperado, pero el buen rollo que flota en el ambiente y que transmites engancha desde el primer momento. Muchas gracias por TODO.

Al Dr. Josep Maria Sierra codirector de esta tesis, por su gran ayuda y paciencia conmigo. Siempre dispuesto a ofrecer una nueva idea/enfoque o una solución a ese "gran" problema del momento.

A la Dra. Teresa Vinuesa por aportar esa energía y vitalidad en todo momento que son muy necesarios y acogerme en el laboratorio como una más desde el primer momento.

A la Dra. Ester Fusté por su infinita amabilidad y buen rollo, siempre ofreciendo ayuda sin dudarlo. A la Dra. Paula Espinal por aportar una visión nueva y ofrecer ayuda siempre con una gran sonrisa. A la Dra. Guadalupe Jiménez por esas charlas y risas de la tarde y por la ayuda ofrecida. A la Dra. Alexandra Merlos por su gran ayuda en el uso de la microscopia de fuerza atómica y sus bromas.

A la parte Sud-africana de esta tesis: Al Prof. Dr. Fernando Albericio y la Dra. Beatriz García de la Torre de la universidad de KwaZulu Natal (Sud-África) por sintetizar las moléculas que nos han permitido realizar esta tesis, por su paciencia y por sus ganas de ayudar en cualquier momento.

To the German group: To Prof. Dr. Roland Benz and Dr. Mathias Winterhalter for their kindness and their willingness to invite me abroad at Jacobs University (Bremen). It was an amazing experience to work and learn from you. To Dr. Claudio Piselli, Federica Linguardo and Eshita Paul, we made quite a group but it was a pleasure working alongside all of you and my Bremen experience would not have been the same without any of you.

Al Prof. Dr. Jordi Borrell, el Dr. Òscar Domènech, la Dra. Mª Teresa Montero y el Dr. Adrià Botet por acogerme y tratarme tan bien en su laboratorio de Fisicoquímica en la Facultad de Farmacia de la Universidad de Barcelona. He aprendido mucho, ha sido una experiencia muy gratificante y he disfrutado mucho trabajando a vuestro lado.

A nuestra técnica de laboratorio Sonia Suarez, por la ayuda y sobre todo por todas las risas y buenos momentos, no hemos reventado ninguna cabeza todavía pero nunca se sabe. A Eva Sánchez por su gran bondad y las conversaciones en el pasillo. A la Dra. Elena de Planell por las visitas con ese optimismo y alegría tan contagiosas. A los "nuevos" integrantes del laboratorio Eric, Felipe y Tanya que aportan frescura, vitalidad, nuevas risas y ganas de comerse el mundo.

A ese "*Lab Non-Stop*", mis amigos, cuyo camino hemos compartido en mayor o menor medida estos años, no sería lo mismo sin vosotros. A la Dra. Eulàlia Sans-Betancourt, la primera persona con la que empecé a trabajar en el laboratorio, tu bondad, tu alegría y tus ganas de enseñar fueron claves esos primeros días para pasar la vergüenza en ese sitio nuevo. Al Dr. Pablo Sans-Betancourt porque he aprendido de tu acento, vocabulario y costumbres tan divertidas, por las risas y los buenos momentos pasados juntos. A Anna Mur por tu calma, amabilidad y las risas compartidas.

A Elena (a.k.a Montxi), Idoia y Estela por esos momentos compartidos en el laboratorio, pero sobre todo esas escapadas, cenas, comidas, etc. A la Dra. Eva Armengol por tu energía y tu locura, has sido un fuerte apoyo todos estos años y eres de quien tomar ejemplo de resiliencia y ganas. A la Dra. Marta Jorba por tu capacidad de aportar tranquilidad y racionalidad en los momentos de caos, tienes un humor genial y he disfrutado mucho trabajando a tu lado todos estos años, además eres la única que puede decir que me has dado un puñetazo. A la Dra. Rocio Herráez, por ser tú, he disfrutado y reído muchísimo contigo, el mejor recuerdo siempre será ese día de verano cambiando el agua. Y sin duda al Dr. Héctor Rudilla, ese "chico" que me daba tanto respeto por su seriedad inicial y esa barba imponente, pero que has sido la persona que me ha acompañado y guiado durante mi trabajo de carrera y de máster, enseñándome a trabajar en el laboratorio y a manejar la tecnología un poco más, muchísimas gracias por guiarme, ayudarme en lo que he podido necesitar y tener paciencia con mis manías, no podía haberme tocado mejor mentor o amigo.

A mi familia, mis padres M.ª Antonia y Ricardo que siempre han estado apoyándome incondicionalmente y cuya ayuda ha sido vital e indispensable, no solo para la tesis sino para la vida, os quiero.

A mi tía Paqui por su ayuda en los retoques finales de la tesis (esos números romanos y espacios) y a mi Pablo, ambos han estado apoyándome sin parar pese a no saber muy bien qué hago y seguramente intentaran leer esto.

Por último, a mis amigos, mi grupo, a la Dra. Paula Ortiz, al Dr. Jan Huertas, a Anna Molinet (a.k.a Molly), a la Dra. Marta Jarque, a la Dra. María Bargues, el Dr. Alberto Meseguer y a Guillem Pina no hay mejores amigos posibles con los que compartir la vida.

Y a Carmen Vidal por creer siempre en mí.

CONTENTS

ACRONYMS AND ABBREVIATIONS

A

ABC: **A**TP-**B**inding **C**assette

AFM: **A**tomic **F**orce **M**icroscopy

AMS: **A**nti**M**icrobial **S**tewardship

AMPs: **A**nti**M**icrobial **P**eptides

ANS: **A**nilino-8-**N**aphthalene **S**ulfonate

C

CAP: **C**ovalently **A**ttached **P**rotein

D

DPH: 1,6-**D**i**P**henyl-1,3,5-**H**exatriene

E

*E. coli: **E**scherichia **coli***

ESBL: **E**xtended-**S**pectrum **B**eta-**L**actamase

F

FICi: **F**ractional **I**nhibitory **C**oncentration **I**ndex

G

GC: **G**rowth **C**urves

I

IM: **I**nner **M**embrane

IMP: **I**ntegral **M**embrane **P**rotein

L

LP: **L**ipo**P**rotein

LPS: **L**ipo**P**oly**S**accharide

LTA: **L**ipo**T**eichoic **A**cid

M

MATE: **M**ultidrug **A**nd **T**oxic **C**ompound **E**xtrusion

MBC: **M**inimum **B**actericidal **C**oncentration

MDR: **M**ulti**D**rug **R**esistant

MFS: **M**ajor **F**acilitator **S**uper

family

MIC: **M**inimum **I**nhibitory **C**oncentration

N

NAG: **N**-**A**cetyl**G**lucosamine

NAM: **N**-**A**cetyl**M**uramic acid

NPs: **N**ano**P**articles

O

OM: **O**uter **M**embrane

OMP: **O**uter **M**embrane **P**rotein

P

PG: **P**eptido**G**lycan

POPE: 1-**P**almitoyl-2-**O**leoyl-sn-glycero-3-**P**hospho**E**thanol-amine

POPG: 1-**P**almitoyl-2-**O**leoyl-sn-glycero-3-[**P**hospho-rac-(1- **G**lycerol)]

P. aeruginosa: ***P****seudomonas* ***aeruginosa***

R

RND: **R**esistance-**N**odulation-**D**ivision

S

SCPDS: **S**uper-**C**ationic **P**eptide **D**endrimer**S**

SLB: **S**upported **L**ipid **B**ilayer

SMR: **S**mall **M**ultidrug **R**esistance

S. aureus: ***S****taphylococcus* ***aureus***

T

TA: **T**eichoic **A**cids

TI: **T**herapeutic **I**ndex

TMA-DPH: 1-(4-
Tri**M**ethyl**A**mmoniumphen
yl)-6-**P**henyl1,3,5-
Hexatrienep-
toluenesulfonate

TKC: **T**ime-**K**ill **C**urves

W

WTA: **W**all **T**eichoic **A**cid

WHO: **W**orld **H**ealth
Organization

SCIENTIFIC PRODUCTION

The four year period employed in this PhD thesis has allowed the participation in research projects which production is presented in the following list:

Publications in international peer-reviewed journals:

- Ramchuran EJ*****, **Pérez-Guillén I***, Bester LA, Khan R, Albericio F, Viñas M, de la Torre BG. Super-Cationic Peptide Dendrimers Synthesis and Evaluation as Antimicrobial Agents. *Antibiotics*. 2021;10(6):695. The **Impact factor** according to 2021 Journal Citation Reports in Pharmacology & Pharmacy is **5.222** (**Q1**), and in Infectious Diseases (**Q2**).

- **Pérez-Guillén I**[$], Botet-Carreras A[$], Domènech Ò, Merlos A, Sierra JM, de la Torre BG, Albericio F, Viñas M, Borrell JH. Studying Lipid Membrane Interactions of a Super-Cationic Peptide in Model Membranes and Living Bacteria. *Pharmaceutics*. 2022;14(10):2191. The **Impact factor** according to 2021 Journal Citation Reports in Pharmacology & Pharmacy is **6.525** (**Q1**).

Publications in international peer-reviewed journals from complementary experimental collaborations:

- Rudilla H, **Pérez-Guillén I**, Rabanal F, Sierra JM, Vinuesa T, Viñas M. Novel synthetic polymyxins kill Gram-positive bacteria. *J Antimicrob Chemother*. 2018; 73: 3385–3390. The **Impact factor** according to 2018 Journal Citation Reports in Infectious Diseases and Microbiology is **5.113** (**Q1**).

- Armengol E, Domenech O, Fustè E, **Pérez-Guillén I**, Borell JH, Sierra JM, Viñas M. Efficacy of combinations of colistin with other antimicrobials involves membrane fluidity and efflux machinery. *Infect.Drug.Resist*. 2019;12:2031-2038. The **Impact factor** according to 2019 Journal Citation Reports in Infectious Diseases and Pharmacology & Pharmacy is **2.984** (**Q2**).

- Pérez-Tomás R, **Pérez-Guillén I**. Lactate in the Tumor Microenvironment: An essential Molecule in Cancer Progression and Treatment. *Cancers*. 2020;12(11):3244. The **Impact factor** according to 2020 Journal Citation Reports in Oncology is **6.639** (**Q1**).

Congress participations:

- Armengol E, **Pérez-Guillén I**, Viñas M, Sierra JM. Congress participation (Oral Communication): Actividad sinérgica de la combinación colistina/linezolid sobre bacterias Gram-negativas MDR y su efecto en las bombas de flujo. XXIII Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. 23-25 May 2019. Madrid, Spain.

- Pereira Riveros TV, Aguilera Muñoz FR, Sierra JM, Jiménez -Galisteo G, **Pérez-Guillen I**, Vinuesa T. (Poster): Aislamiento de Tannerella Forsythia para el diagnóstico de periodontitis a partir de muestras microbiológicas. XXV Congreso de la Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica. 2-4 June 2022. Granada. Spain.

ABSTRACT

Super-Cationic peptides: insights on their antimicrobial action.

Introduction:

Bacteria have been to the human civilization a threat as well as an ally, since the beginning of time. The introduction of antibiotics into clinics to treat infections was one of the best medical achievements ever accomplished. However, the increase of antimicrobial resistance (AMR) as well as the failure to develop new antibiotics is a cause of much concern, and is essential to create new strategies of development in order to fight this global threat.

Bacterial cell membranes are a key factor in the emergence and evolution of resistant bacteria, due to their capacity to interact with other organisms, the environment or both. Therefore, the study of the physical and chemical properties of biological membranes is essential to understand their role in resistance.

One path to develop new strategies and create new antimicrobial agents to address the global threat of resistant bacteria is the synthesis of new compounds as an alternative therapy, like synthetic antimicrobial peptides (AMPs). In this context, the active exploration of peptides from the new AMP family, named the Super-Cationic peptide dendrimers (SCPDs) was carried out.

Hypothesis:

The main hypothesis of this thesis is that synthetic peptides may have antimicrobial properties against clinical bacterial strains, and may provide weapons to treat infections caused by multidrug-resistant strains. A deep research of the mechanisms underlying antimicrobial action should be carried out.

Objectives:

The main objective of this thesis is to determine the antimicrobial properties of synthetic peptides from a new AMP family, SCPDs, specifically the peptide G1OLO-L_2OL_2.

The secondary objectives of this thesis are divided in three parts:
First, finding the eventual target and interactions those peptides may have with the bacteria, particularly its possible interaction with the bacterial cell membrane.
Second, to determine the cytotoxicity these molecules may have over eukaryotic cells.
And third, to observe the effect these molecules may have on the bacteria, the cell membranes and on artificial membranes by means of atomic force microscopy (AFM).

Methodology:

The antimicrobial activity of the synthetic peptides was explored against planktonic bacteria of several microorganisms and the cytotoxicity of all the compounds was also tested.

Furthermore, in order to decipher the possible effects caused by the integration of G1OLO-L_2OL_2 into the bilayer, observation of the effects generated by AFM along with measurements, as a function of temperature of the steady-state fluorescence anisotropy (*r*) of Laurdan, DPH and TMA-DPH incorporated into liposomes were done. On top of that, this peptide may have an effect over the electrostatic surface membrane potential ($\Delta\Psi$) which was assessed using ANS probe, in order to establish a possible relationship between the electrical properties of the phospholipid bilayer surface and the peptide activity.

Results:

The screening of SCPDs revealed that almost all compounds showed antibacterial activity and seem to be antimicrobials of broad-spectrum.

The study of time-kill curves (TKC) and growth curves (GC) allowed to see differences in action of the peptides, specifically over *Escherichia coli* where the tested compounds affected significantly the bacterial growth. These results prompted to evaluate the cytotoxicity of these molecules in eukaryotic cell lines where, G1KLK-L_2KL_2 and G1OLO-L_2OL_2 were considered as the best candidates as new antimicrobial agents against *E. coli*.

No significant modification of the liposome size was observed when the effect of G1OLO-L_2OL_2 loading was determined. However, loading increase the zeta potential value of blank liposomes in nearly 5 mV, likewise it was stablished that the presence of G1OLO-L_2OL_2 modifies the effective surface charge of the liposome increasing in + 5.06 mV the surface potential of the liposomes.

Moreover, the evaluation of the variation of DPH and TMA-DPH anisotropy values (*r*) as a function of temperature with (0.005 μM) and without peptide was also studied. With TMA-DPH significant changes towards higher temperatures were observed in the transition temperature (Tm). Furthermore, the Laurdan study to analyze the induction of lipid microdomains caused by presence of the peptide in liposomes clearly indicates a transition towards a more fluidic phase without the existence of lipid domains of different composition.

AFM allowed us to observe how the addition of 0.0005 μM peptide results in the fluidification of the lower lipid layer in an initial stage after the peptide addition. Up to 35 minutes after the peptide addition it was feasible to observe a degradation of the lipid domains disorganizing from the center of the domain to the margins.

Moreover, bacterial AFM showed remarkable differences between the control strains and the treated ones where a total

destruction of the integrity of the planktonic bacteria occurs. The cells seem to shorten and granulate its external membrane while losing some of their intracellular content.

Conclusions:

In conclusion, we have designed a set of branched peptides to obtain constructs that we have called super-cationic peptide dendrimers (SCPDs) and tested them as potential antimicrobial agents, which appear to be broad-spectrum antibacterial compounds. TKC kinetics revealed higher activity against Gram-negative bacteria and growth curves reinforced this conclusion. Moreover, the cytotoxicity study showed that the higher the toxicity the higher branched are the peptides. Furthermore, the biophysical experiments demonstrate a surface effect of G1OLO-L_2OL_2 on the model lipid membranes.

RESUMEN

Super-Cationic peptides: insights on their antimicrobial action.

Introducción:

Las bacterias han sido desde el principio de los tiempos una amenaza y un aliado para la civilización humana.

La introducción de los antibióticos en la clínica para tratar infecciones fue uno de los mejores logros médicos jamás logrados. No obstante, el aumento de las resistencias a los antimicrobianos, así como la falta de desarrollo de nuevos antibióticos es motivo de gran preocupación, y por ello es fundamental la creación de nuevas estrategias de desarrollo para combatir esta amenaza global.

Las membranas celulares bacterianas son uno de los factores clave en la evolución de bacterias resistentes, debido a su capacidad para interactuar con otros organismos, el medio ambiente o ambos. Por lo tanto, el estudio de las propiedades físicas y químicas de las membranas biológicas es fundamental para comprender su papel en las resistencias.

Un camino para desarrollar nuevas estrategias y crear nuevos agentes antimicrobianos para abordar la amenaza global de la resistencia bacteriana es la síntesis de nuevos compuestos como terapias alternativas, tal y como serían los péptidos antimicrobianos sintéticos (AMPs).

En este contexto, se llevó a cabo la exploración de la actividad antimicrobiana de los péptidos de la nueva familia AMP, denominados Super-Cationic peptide dendrimers (SCPDs).

Hipótesis:

La principal hipótesis de esta tesis es que los péptidos sintéticos pueden tener propiedades antimicrobianas contra cepas bacterianas clínicas y pueden proporcionar armas para tratar infecciones causadas por cepas multirresistentes. Se debe realizar una investigación profunda de los mecanismos que subyacen a la acción antimicrobiana.

Objetivos:

El principal objetivo de esta tesis es determinar las propiedades antimicrobianas de los péptidos sintéticos de una nueva familia de AMP, conocida como SCPDs, concretamente del péptido G1OLO-L_2OL_2.

El objetivo secundario de esta tesis se divide en tres partes:

Primero, encontrar la posible diana y las interacciones que estos péptidos pueden tener con la bacteria, particularmente su posible interacción con la membrana celular bacteriana.

Segundo, determinar el efecto citotóxico que estas moléculas pueden generar sobre células eucariotas.

Y, en tercer lugar, observar el efecto que estas moléculas pueden tener sobre las bacterias, la membrana celular y las membranas artificiales mediante microscopía de fuerza atómica (AFM).

Metodología:

Se exploró la actividad antimicrobiana de los péptidos sintéticos frente a bacterias planctónicas de varios microorganismos y se ensayó la citotoxicidad de todos los compuestos. Asimismo, con el fin de descifrar los posibles efectos causados por la integración de G1OLO-L_2OL_2 en la bicapa, se llevó a cabo la observación de los efectos generados por AFM junto con mediciones en función de la temperatura de la anisotropía de fluorescencia en estado estacionario (*r*) de Laurdan, DPH y TMA-DPH incorporados en liposomas.

A su vez, este péptido podría tener un efecto sobre el potencial de membrana ($\Delta\Psi$) evaluado mediante la sonda ANS con el fin de establecer una posible relación entre las propiedades eléctricas de la superficie de la bicapa lipídica y la actividad del péptido.

Resultados:

El estudio de los SCPDs reveló que casi todos los compuestos mostraron actividad antibacteriana indicando que los compuestos probados parecen ser antimicrobianos de amplio espectro.

El estudio de curvas de muerte (TKC) y curvas de crecimiento (GC) permitió ver diferencias en la acción de los péptidos, específicamente sobre *Escherichia coli* donde los compuestos probados afectaron significativamente el crecimiento bacteriano Estos resultados impulsaron a evaluar la citotoxicidad de estas moléculas en líneas celulares donde, G1KLK-L_2KL_2 y G1OLO-L_2OL_2 fueron considerados como los mejores candidatos como nuevos agentes antimicrobianos contra *E. coli*.

G1OLO-L_2OL_2 no parece modificar significativamente el tamaño de los liposomas, sin embargo, aumenta el valor del potencial zeta de estos en casi 5 mV. Asimismo, se estableció que la presencia de G1OLO-L_2OL_2 modifica la carga superficial efectiva del liposoma aumentando en + 5,06 mV el potencial superficial de los liposomas. Además, también se estudió de la variación de los valores de anisotropía (*r*) de DPH y TMA-DPH en función de la temperatura con (0,005 μM) y sin péptido.

Con TMA-DPH se observó un cambio significativo en la temperatura de transición (Tm) hacia temperaturas más altas.

Además, el estudio con Laurdan para analizar la inducción de microdominios lipídicos provocados por la presencia del péptido en los liposomas indica claramente una transición hacia una fase más fluida sin la existencia de dominios lipídicos de diferente composición.

La microscopía de fuerza atómica nos permitió observar cómo la adición de 0,0005 μM de péptido genera la fluidificación de la capa lipídica inferior en un estadio inicial, después de 35 minutos de la adición del péptido se observa también una degradación de los dominios lipídicos que parecen perder su estructura desorganizándose desde el centro del dominio hacia los márgenes.

Conjuntamente, en el estudio *in vivo* con bacterias se observan diferencias notables entre el control y las células tratadas, en las que se produce una destrucción total de la integridad de las bacterias planctónicas. Las células parecen acortarse y granular su membrana externa mientras pierden algo de su contenido intracelular.

Conclusiones:

En conclusión, hemos diseñado un conjunto de péptidos ramificados para obtener moléculas denominadas dendrímeros peptídicos supercatiónicos (SCPDs) y los hemos probado como posibles agentes antimicrobianos, que parecen ser compuestos antibacterianos de amplio espectro. La cinética de TKC reveló una mayor actividad contra bacterias Gram-negativas y las curvas de crecimiento reforzaron esta conclusión. Además, el estudio de citotoxicidad mostró que cuanto mayor es la toxicidad, más ramificados son los péptidos. Además, los experimentos

biofísicos demuestran un efecto superficial de G1OLO-L_2OL_2 en las membranas lipídicas modelo.

INTRODUCTION

Humankind has been, since the beginning of time, threatened by infectious diseases.

Microbial infections had caused injury, pain and in plenteous of cases death, leading to near extinction in some zones of the Earth due to epidemics like the plague, Spanish influenza, tuberculosis, leprosy or cholera.

Bacteria were the first living organisms on Earth, they vary in size and shape and without their discovery and description the outstanding role of antibiotics could not have been written (1–3).

1. The Bacterial Cell Envelope.

Cells are the fundamental element that form a living organism. They are encased by a biological membrane, which allows the interaction and communication with other cells, the environment or both (2).

Bacteria are prokaryotic organisms and their cell envelope is a dynamic intricate structure formed by multiple layers that protects them from their unreliable and usually hostile surroundings. Moreover, the bacterial envelope has a crucial role in the physiology and morphogenesis of the bacteria and is a key factor in the development of resistant bacteria against antimicrobial agents (4,5).

Most bacteria can be classified into two major groups, based on the Gram staining technique defined more than 100 years ago (1884) by a Danish bacteriologist, Hans Christian Gram. It allows discerning their cell wall structure between Gram-positive and Gram-negative bacteria applying differential staining using a crystal violet-iodine complex and a safranin counterstain (6).In essence, it can be said that the bacterial cell envelope is formed by the cytoplasmic membrane, the cell wall and, in Gram-negative bacteria the outer membrane (5).

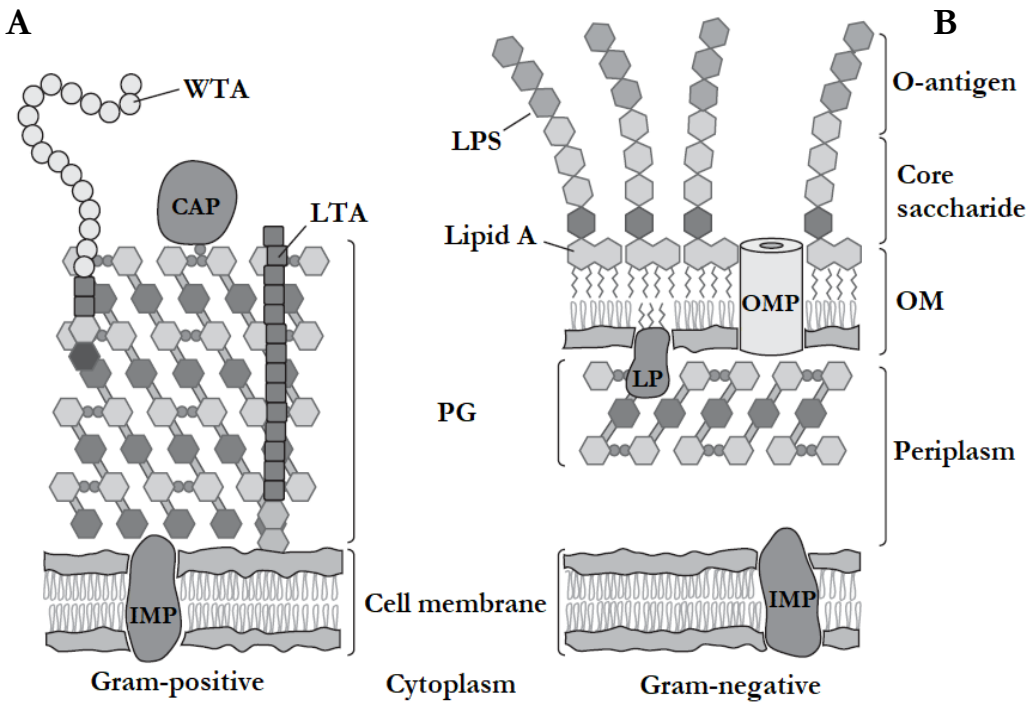

Figure 1: Structures of Gram-positive (A) and Gram-negative (B) bacteria cell envelopes. Image modified and obtained from (4). CAP: covalently attached protein, LP: lipoprotein, LPS: lipopolysaccharide, LTA: lipoteichoic acid, IMP: integral membrane protein, OM: outer membrane, OMP: outer membrane protein, PG: peptidoglycan, WTA: wall teichoic acid.

1.1 Gram-positive Bacteria.

The Gram-positive bacteria envelope consists of two substructures. The first and more internal is the lipid bilayer membrane that encases the bacterium cytoplasm (7).

The second, external and the main trait of the Gram-positive bacteria is the peptidoglycan (PG) that forms the outer surface of the bacterium (8) (Figure 1A).

Chemically, the PG is composed of alternating β-1,4-linked linear units of the disaccharide N-acetylglucosamine (GlcNAc, NAG) and N-acetylmuramic acid (MurNAc, NAM) forming a mesh-like framework. Since, unlike Gram-negative bacteria, Gram-positive bacteria are not surrounded by an outer membrane its thickness can reach a size between 30 to100 nm or even more (8,9).

Up to 60% of the cell wall mass is composed of teichoic acids (TA) that enclose two different bacterial cell wall polymers: lipoteichoic acids (LTAs) anchored via lipid to the lipid membrane and wall teichoic acids (WTAs) covalently attached among the PG layers. Both play a central role in numerous cellular processes and confer the cell highly negatively charged properties (4,7,8,10).

Surface proteins attach through noncovalent ionic interactions to the PG or TA, but many are bonded covalently to stem peptides within the peptidoglycan layers (4).

1.2 Gram-negative Bacteria.

The Gram-negative bacterial cell envelope is more challenging and consists of three substructures. Starting from the external layer the first encountered is the outer membrane (OM), which is the main trait that differentiates Gram- negative bacteria.

The OM is a highly asymmetric bilayer composed by phospholipids and predominantly glycolipids, being the lipopolysaccharide (LPS) the principal one (4,11).

LPS is a macromolecule with three main structural components: The Lipid A, the core oligosaccharide (inner and outer), and the O-antigen. This molecule is a definite indicator of infection and that is why the human innate immune system is sensitized to it (12).

In addition, the OM contains bounded proteins that can be divided into two different classes, lipoproteins and □-barrel proteins (considered to be almost all of the transmembrane proteins) called outer membrane proteins (OMPs).

The PG whose thickness is only a few nanometers in contrast to the Gram-positive bacteria is enclosed in the periplasm, an

aqueous and viscous cellular compartment vastly populated by proteins delimited by the OM and the inner membrane (IM) (Figure 1B).

The IM is formed by a fluid phospholipid membrane that encases the bacterium cytoplasm and due to the lack of organelles all of the functions occur in (or on) it (4,11).

2. A brief history of Antibiotics.

The usage of microbes capable to produce antibiotic to prevent or avoid infectious diseases extends back millennia, long before the onset of modern medicine. Using moldy bread in order to treat open wounds or burns was common more than 2000 years ago in ancient civilizations like the Greeks and Egyptians. The existence of the Eber's papyrus (from 1550bc) or and Anglo-Saxon recipe (from 1000 years ago) are the oldest saved medical documents that include these natural treatments in its lists of remedies.

It was in 1546 that particles (contagious and indistinguishable) were described as the causal agent of certain diseases. It took several centuries to demonstrate bacteria as one example of said particles.

Two contributions proved to be transformative in understanding the underlying causes of infectious disease and propelled bacteriology towards the modern era. The germ theory of disease by Louis Pasteur and the four Koch postulates by Robert Koch, established the causal relation between infection, disease and microbes (13,14).

The word "antibiotic" can be tracked to describe the antagonistic action between different microorganisms (15). Later on, it would be used to describe the natural action of secondary metabolites

produced by bacteria or fungi with the capability to either inhibit growth or kill others. Today, its meaning is broader, including partially or totally synthesized molecules (14) although is being slowly ,but constantly, replaced by the term "antimicrobial agents".

During the first decade of the XX century Paul Ehrlich laboratories synthesized the first man-made antibacterial agent, arsphenamine (Salvarsan), approved in 1910 as a treatment for syphilis (caused by the bacterium *Treponema pallidum*) (16).

Nonetheless, on September 1928 one of the most impactful discoveries in the history of microbiology was made. The bacteriologist Alexander Fleming found an intriguing pattern on a *Staphylococcus aureus* Petri dish contaminated with a fungus (*Penicillium notatum*). Near the fungus the colonies had endured lysis whereas the colonies farther from the fungus appear normal. Fleming grew the fungus pure and tried it on several pathogenic bacterial strains, obtaining similar effects as previously observed. He concluded that the fungus must have produce a substance that killed the bacteria, and he called it penicillin (14,17).

Isolation and purification of penicillin was not successful initially, until in 1940 when Howard Walter Florey, Ernst Boris Chain and Norman Heatley worked on it and managed to get the isolated compound.

Due to necessity and World War II a project to produce and distribute penicillin for the need of the forces fighting was launched. It was in 1945 that penicilin became available to the public (14).

Meanwhile during the late 1920s and early 1930s the German chemical industry underwent a dramatic increase in synthesized molecules (specifically dye compounds), and among them one stood out: sulphonamidochrysoidine. The discovery of the sulfonamide drugs marked the next advance in antibiotic history (13,18).

The amount of discoveries of antimicrobial compounds produced by microorganism led Selman Waksman to start the study of microbes as producers of antimicrobial compounds, in particular he focused on the spore-forming bacteria grouped under the term Actinomycetes.

The Waksman program led to the golden era of antibiotic discovery range between the 1940s and 1960s (Figure 2) where more than 20 classes of antibiotic were discovered from dozens of bacterial species and fungi (16,19,20).

In addition to discovering antimicrobial agents from natural sources, the interest of synthetic designed agents raised extremely and it actually proved to be equally if not more efficient than their natural compounds.

Therefore, is logical to say that the introduction of antibiotics into clinical use was one of the greatest medical achievements ever accomplished.

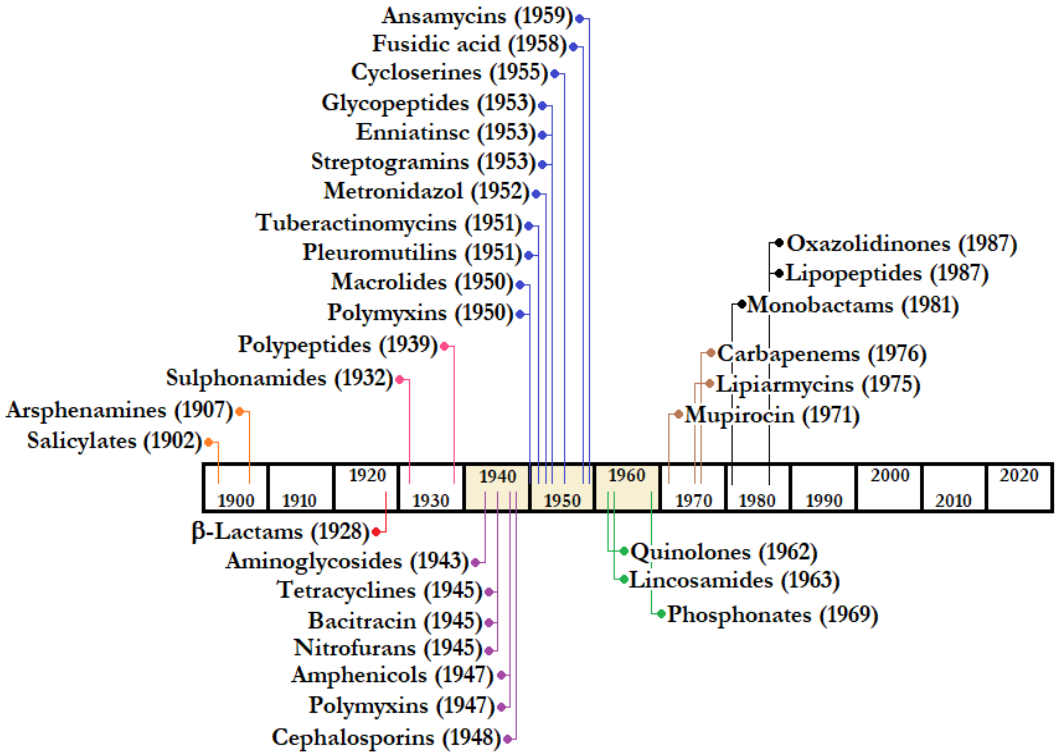

Figure 2: Timeline showing the new classes of antibiotic first report in literature.

Nevertheless, it seems that the more there is to know about antibiotics, the fewer they are discovered. In the last 45 years only two new class of antibiotics have been discovered (lipopeptides and oxazolidinones). Actually even though scarcely some molecule makes it into the clinical practice they usually are new generations of the already existing antibiotic classes or combinations between them (1,21).

3. Antibiotics mechanism of action.

The antibiotic effect over bacteria usually is directed at some feature of the bacterial structure or its metabolic processes. There are five most common targets for antibiotics (Figure 3) (16,19,22–24).

3.1 Inhibition of cell wall synthesis.

As a result of the cytoplasmic inhibition of the synthesis of the precursors of the PG, the inhibition of the transport phase of the precursors of PG or the inhibition of the PG formation, making unavailable the formation of new PG layers therefore leading to the bacterium death (25–27). Good examples are β-lactams, bacterial enzymes able to destroy the penicillin molecule, Bacitracin, able to inhibit the central role bactoprenol has in the transport of wall subunits through the membrane, and Fosfomycin that inhibits the first metabolic biochemical pathways of wall components.

3.2 Disruption of the cell membrane structure or function.

Hugely related to the types of lipid in the cell membrane of the bacteria, and the antibiotic class. Producing alterations on the permeability of the membrane (25–27). As examples, Daptomycin that inhibits cell envelope synthesis by interfering with fluid membrane microdomains or Polymyxins causing a complete alteration of the membrane structure and roles.

3.3 Inhibition of the nucleic acids synthesis.

The antibiotic effect occurs by blocking DNA replication or stopping DNA transcription (affecting the RNA synthesis and consequently RNA translation) (26,27). Good examples are Fluoroquinolones able to inhibit enzymes directly involved in DNA replication, and Ansamycins able to inhibit DNA-dependent RNA polymerase, thus inhibiting DNA transcription.

3.4 Inhibition of protein synthesis.

The disruption of the protein synthesis in the bacterium would lead eventually to the incapacitation of the bacterial cell, inhibiting its growth or even killing it completely. The target of these antibiotics can be divided in two: the inhibition of the 50S

ribosomal subunit (*e.g.* oxazolidinones and macrolides) or the inhibition of the 30S ribosomal subunit (*e.g.* tetracyclines and aminoglycosides) (26,27).

3.5 Blockage of key metabolic pathways.

Some antibiotics mimic a substrate essential for the correct metabolism of the bacteria, causing the enzymes to attach themselves to it rather than the correct substrates (26,27).

Since folate synthetic pathway is needed for bacterial survival because its alteration leads to a deficiency in Thymine, one of the main affected pathways is the folate synthesis metabolism. The best-known examples are Sulphonamides (inhibiting dihydropteroate synthetase) and Trimethoprim that inhibits dihydrofolate reductase, both key enzymes in the synthesis of pyrimidins.

Antibiotics mechanism of action can result in a bactericidal effect which will cause the bacterial cell death or a bacteriostatic effect leading to the inhibition of cell growth (28).

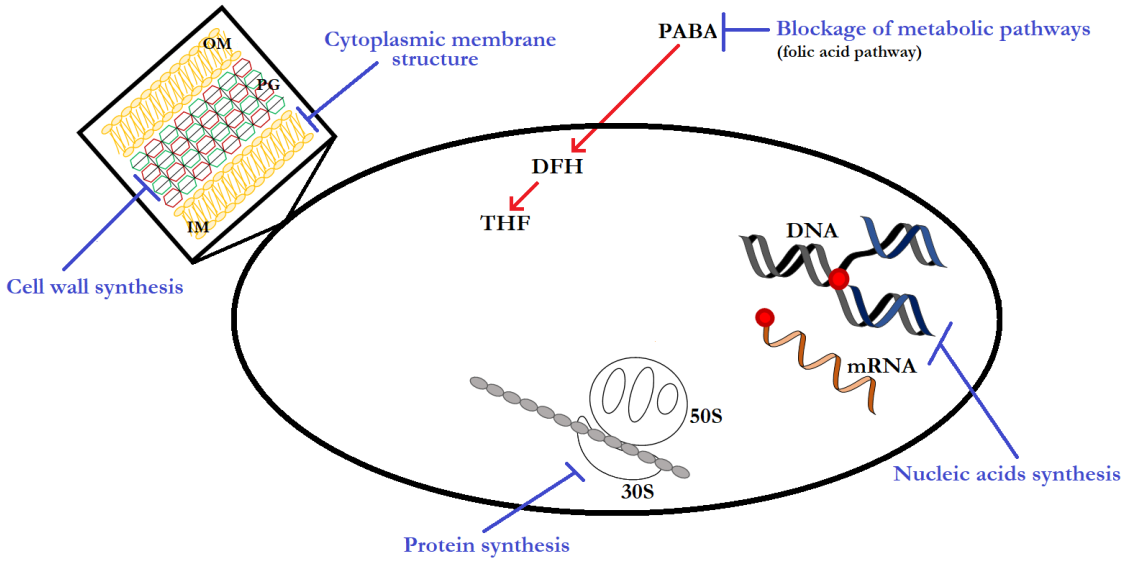

Figure 3: Schematics of the five most common targets for antibiotics.

4. Multidrug resistant bacteria.

Multidrug resistant (MDR) bacteria have an increasing role threating the mortality rates caused by infectious diseases worldwide (29).

The antibiotic era in which the use of antibiotics was usually successful may be ending. Bacterial infections that once were treatable are, due to the growing antimicrobial resistance (AMR) and reducing antibiotic storage untreatable, making people die. The post antibiotic era is emerging, even though envisioning a world without antibiotics is hard (30,31).

Microbes have always had the tools to drive resistance. The usage of large quantities of antibiotics to treat and control infections in human, animal and agriculture has created the unprecedented conditions of selective pressure on bacteria and a stimulus of selection of new forms created through the mobilization of resistance elements (19).

Pathogenic bacterial strains resistant to most, or all, available antibiotics are now isolated routinely, and every year a total of 700.000 people die from infections caused by drug resistant strains (25.000 deaths per year in the EU alone). Therefore, unless action is taken it has been estimated that the number of death from AMR infections could increase up to 10 million

deaths per year by 2050 creating a particularly worrisome scenario(Figure 4) (32–34).

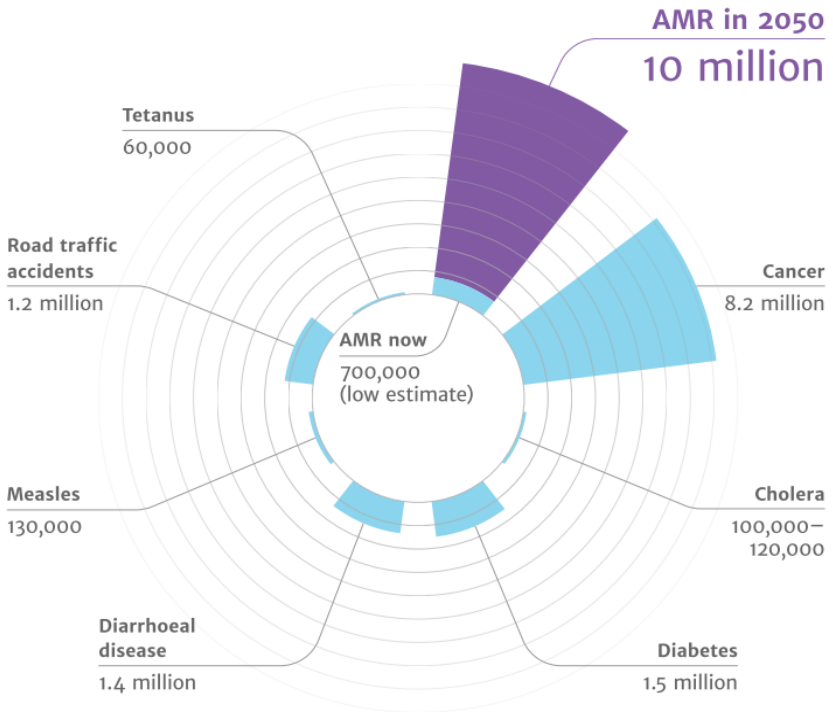

Figure 4: Deaths attributable to AMR every year. Image obtained from (33).

One of the main contributing factors of the current need of new antibiotics is the emergence and spreading of resistance mechanisms among bacteria faster than the antibiotic discovery and development. Additionally, antibiotics lack of commercially attractiveness market for drug developers due to the cost associated to bring one antimicrobial successfully to market, the clinical trials and the periods of treatment.

Moreover, a critical fact is the prospect that the new molecule may become ineffective in the short term. A spent money that developers may not earn back latter due to lack of patented time and competing with cheaper generics.

Price pressure exists and furthermore, authorities will restrict the usage of these products to slow down the development of resistance and strict price controls (34,35).

To help prioritize the research and development of new and effective antimicrobials, the world health organization (WHO) published in 2017 a list of different families of bacteria that are the greatest threat to human health. The list is organized with three priority tiers: critical, high and medium, based on the urgency of need to develop new antibiotics (36).

Priority 1: CRITICAL

- *Acinetobacter baumannii*, carbapenem-resistant.
- *Pseudomonas aeruginosa*, carbapenem-resistant.
- *Enterobacteriaceae*, carbapenem-resistant, extended-spectrum beta-lactamase (ESBL)-producing.

Priority 2: HIGH

- *Enterococcus faecium*, vancomycin-resistant.
- *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant.
- *Helicobacter pylori*, clarithromycin-resistant.
- *Campylobacter* spp., fluoroquinolone-resistant.
- *Salmonellae*, fluoroquinolone-resistant.
- *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant.

Priority 3: MEDIUM

- *Streptococcus pneumoniae*, penicillin-non-susceptible.
- *Haemophilus influenzae*, ampicillin-resistant.
- *Shigella* spp., fluoroquinolone-resistant.

5. Mechanisms of antimicrobial resistance.

Resistance is a natural process observed since the first antibiotics were discovered.

Some bacterial strains are naturally resistant to certain types of antibiotics independently to its exposure to them, and is called intrinsic resistance. In other cases, exposure to antimicrobials has led to the selection of resistant individuals of a heterogenic population (heteroresistance) or individuals that have acquired genes conferring resistance, or are carrying mutations that encode resistance.

That is to say antibiotics do not induce resistance but select the increase of resistant bacteria proportion.

Likewise, acquired resistance is also a possibility and can occur through two distinct process: via mutation of the DNA during the replication or DNA transfer or through the horizontal gene transfer processes that occur in bacteria (transformation, transduction and conjugation).

As a matter of fact, the main antibiotic resistance mechanism that bacteria adopt can be divided in four main categories, and in view of the structural differences between Gram-positive and

Gram-negative bacteria there is a difference between the mechanisms used (24,27,37–39).

5.1 Decreased or limited drug uptake.

As previously mentioned a natural difference exists in the capability of bacteria to limit the uptake of antimicrobial agents.

In Gram-positive bacteria due to the none-existent OM restricting drug access is not as common in comparison with Gram-negative bacteria that are intrinsically less permeable to many antibiotics because their OM creates a permeability barrier. Mycolata are an exception to this general rule.

The bacteria goal in Gram-negative bacteria is to reduce the OM permeability and restrain penetration of the antibiotic and other exogenous compounds inside the bacteria. Therefore, the downregulation of porins leading to a decrease in the number of porins in the OM or the replacement of porins for more selective channels and even mutations that change the selectivity of the porin allow the bacteria to accomplish its "aim" (24,37–40).

5.2 Modification of the drug target.

The bacterial structure has a considerable diversity of components to be antibiotic targets. Consequently, there are as many "targets" that can be modified by the bacteria to gain

resistance to those drugs. The changes may prevent the antibiotic binding but enable the bacteria to carry out its regular function (24,37–40).

For example, the mutation of gene encoding for PBPs, results in the expression of unique PBPs whit low affinity for all antibiotics whose target is PBPs, thus enabling the survival of the bacteria.

5.3 Drug alteration or inactivation.

Bacteria can produce enzymes that will alter irreversibly and inactivate the antibiotics. This antibiotic alteration is a major mechanism of antibiotic resistance.

Thousands of enzymes have since been identified to be able to alter and deteriorate antibiotics of different classes, preventing their binding to its target (24,37–40).

For example, one of the well-characterized enzymes is the β-lactamase enzyme which effect is the hydrolysis of the β-lactam ring present in all β-lactams, thus, are essential to their activity.

5.4 Drug efflux pumps activation.

Efflux pumps are membrane proteins that function as exporters whose primary objective is to get rid of all the toxic substances

from the bacterial cell. Numerous pumps will be able to transport a large assortment of compounds.

Bacteria have chromosomally encoded genes for efflux pumps which could be in some cases expressed constitutively, and in others induced or overexpressed under certain stimuli.

In order to rise the antibiotic removal from the intracellular compartment bacteria will overexpress the efflux pumps and this would lead to intracellular drug concentrations never sufficiently high to prompt an antibiotic effect. This mechanism confers resistance to a wide range of antibiotics, and helps to other mechanisms by making stoichiometry antibiotic/target unfavorable for antibiotic action, thus contributing to MDR.

Up to date, five super families of efflux pumps have been described: the ATP-binding cassette (ABC) family, the small multidrug resistance (SMR) family, the major facilitator super (MFS) family, the resistance-nodulation-division (RND) family, and the multidrug and toxic compound extrusion family (MATE) (24,37–40).

Even though, bacteria carry multiple genes encoding MDR efflux pumps, some have been mobilized onto plasmids, a worrying development as it shows that this resistance mechanism is transmissible and could be rapidly disseminated to other clinically relevant pathogens.

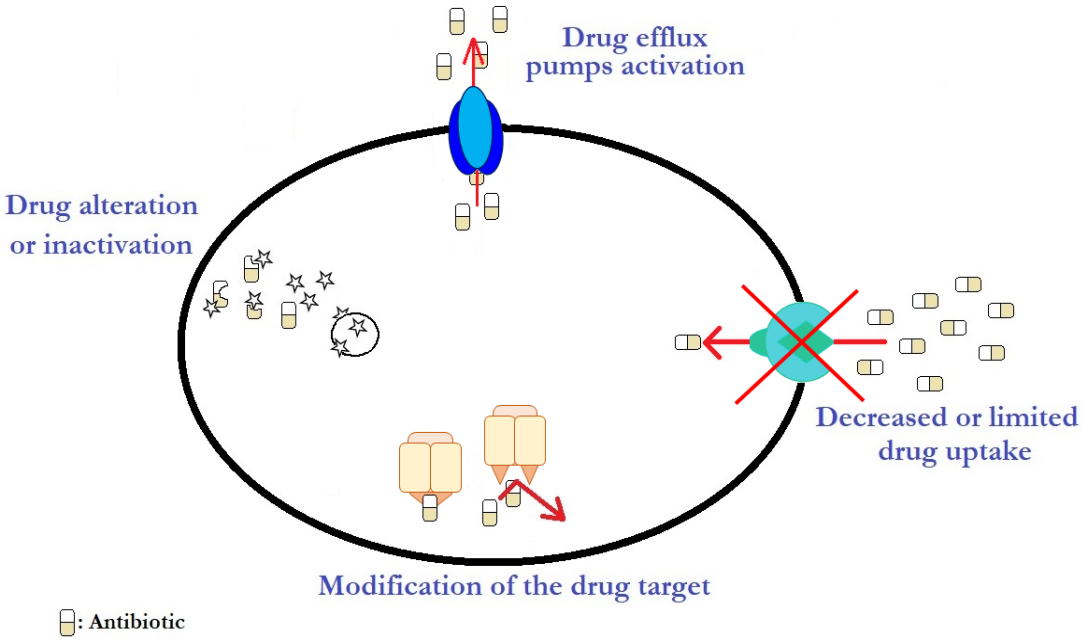

Figure 5: Schematics of the four main antibiotic resistance mechanisms.

6. *Present and future perspectives of antibiotics.*

As previously mentioned, treatment of bacterial infections through the usage of cheap, safe and copious antibiotics can no longer be taken for granted in the face of increasing resistance. We are entering a post-antibiotic era where controlling infections has not been encountered for more than three-quarters of a century, and in which many common infections would no longer be treatable.

Years of unsuccessful research have led pharmaceutical firms interest in antibiotic research and development, to pull the plug in antibiotic discovery, either by leaving the field, or by downsizing their effort. Moreover, this pull out is strongly associated with the economic expenses derived from the process of discovery of a new drug, from its initial research to final commercialization and the added administrative restrictions applied by the governments.

The rise of resistant bacteria combined with the failure to provide new antibiotics is a cause of much distress. Therefore, encouraging antibiotic research and development is essential in order to develop new strategies to address the global threat of MDR bacteria (19,21,41).

7. Strategies to fight MDR bacteria.

Taking into account the limited and shrinking supply of antibiotics, the governments together with the medical organizations have stablished the antibiotic stewardship (AMS) whose approach is to reserve the newest antibiotics for extreme cases and treat whenever possible with the most common and older compounds (42).

The increase in MDR bacteria is generating a growing awareness for the need of new strategies in order to fight resistance and create new antibacterial agents (43).

7.1 Discovery of natural compounds.

Antibiotic discovery history leads to believe that natural products produced or not by microorganism exhibit the chemical properties for the discovery of new antibiotics.

Identifying the correct types of producers from screening environmental isolates is key in the success of finding new classes of antibiotics. One of the most promising sources of secondary metabolites based on metagenomics analyses are uncultured bacteria, which nowadays cannot grow under laboratory conditions.

This potential source of natural compounds opens up the possibility of reviving the Waksman platform of antibiotic discovery by several groups in the academia and the industry, combining the new-found compounds with new tools for genome mining.

This capability is already leading to a surprising discovery where many bacteria and fungi already "known" seem to encode many more natural products than observed in the laboratory (16,19,44).

7.2 Discovery of synthetic compounds.

The first man-made fully synthetic compound began with the development of the arsphenamines (1907), and despite the continuous synthetization of more than 10 million new molecules, only a few active molecules have reached the market.

The source of countless medicines essential for human health are in the synthetic libraries that are the backbone of most pharmaceutical companies.

Nowadays, it is not clear why, despite the rising amount of compounds in the synthetic libraries along with the advances in chemistry, in general have not led for more than three decades to the discovery of new and safer antibiotics with increased potencies and extended spectra of activity (17,19,45).

7.3 Combination of antibiotics.

Aiming to find a possible synergistic effect the combination of antibiotics as treatment has been tested and demonstrated to have successful results in the clinic.

The combination of two drugs seems to generate much less resistance on the pathogen and has an increased spectrum of coverage and antibacterial activity gaining approval as a new design strategy for novel antibiotics (46).

As a matter of fact, combinations are the pillar of treatment for tenacious infections such as tuberculosis (44,47,48).

7.4 Old backbones, new molecules.

Improvement of already known molecules is another strategy that can yield benefits.

One strategy as another approach to generate new antibiotics is to synthetize based on the scaffold of already known antibiotics new synthetic molecules.

These modifications can be done in order to optimize drugs with an initial thought of a promising future such as polymyxins that with further investigation have severe side effects, therefore have been subsequently abandoned because of it (49,50).

The main objective is to create new molecules with enhanced antibiotic properties improving for example their pharmacokinetic and pharmacodynamics characteristics or reducing its cytotoxic effects (17,45) or both.

7.5 Alternative therapies.

Furthermore, alternative therapies exist and are currently in practice or under clinical trial.

The usage of bacteriophage therapy (on the rise in the last years), photodynamic therapy, antibacterial adjuvants, phytochemicals and nanoparticles (NPs) as antibacterial agents, and antimicrobial peptides (AMPs) is seen as a feasible option to reduce resistance.

Although it's important to take into account they may have one or several limitations (47,51).

8. Antimicrobial Peptides (AMPs).

As previously mentioned AMPs have been considered as alternative for, or an addition to the already used antibiotics, as one of the many options to overcome resistance (47,52,53).

AMPs are as well-known as host defense oligopeptides found in most living organisms, both prokaryotes and eukaryotes, and act as natural defenders. Probably, they are one of the most conserved molecules in nature in order to control pathogenic microorganisms.

Natural AMPs are mainly short (between 5-50 amino acids), and 2-9KDa in size and almost everyone has a positive net charge ranging from +2 to +11, due to arginine and lysine amino acids on their structure. AMPs are versatile molecules with a broad diversity of structural and physiochemical properties (54,55).

These peptides are folded in a way that allows them to be amphipathic. They vary in size, length, secondary structure and sequence.

Their classification is often related to the structure they adopt in hydrophilic/hydrophobic interfaces.

Although the main mechanism of action is membrane permeabilization due to their hydrophobic and aliphatic residues on their sequence, other mechanisms have been described like

membrane destabilization, macromolecular synthesis inhibition, intracellular translocation of the peptide and inhibition of DNA/RNA/protein synthesis (54,55).

It seems that the development rate of resistance in comparison with conventional antibiotics is much less frequent.

The advances achieved in chemical and biological synthesis and the progress in structural understanding have allowed to improve the antimicrobial spectrum and tissue compatibility (56). Nevertheless, despite the potential displayed it has been a challenge to translate the result into clinical approval.

During the development of this thesis, we have focused on the exploration of the antimicrobial activity of synthetic AMPs.

HYPOTHESIS AND OBJECTIVES

Hypothesis.

The main hypothesis of this thesis is that **synthetic peptides may have antimicrobial properties against clinical bacterial strain**s, and may **provide weapons** to treat infections caused by multidrug-resistant strains. A deep research of the **mechanisms underlying antimicrobial action** should be carried out.

Justification of the study and Objectives.

Nowadays the increase of antimicrobial resistance (AMR) as well as the failure to develop new antibiotics is a cause of much concern worldwide. It has been estimated that 10 million deaths per year would occur due to AMR infections by 2050 unless action is taken.

One path to develop new strategies and create new antimicrobial agents to address the global threat of resistant bacteria is the synthesis of new compounds as an alternative therapy, like synthetic antimicrobial peptides (AMPs).

In this context, the activity exploration of peptides from the new AMP family, named the Super-Cationic peptide dendrimers (SCPDs) was carried out.

Objectives:

The main objective of this thesis is to determine the antimicrobial properties of synthetic peptides from a new AMP family, SCPDs, specifically peptide G1OLO-L_2OL_2.

Secondary objectives (divided in three parts):

1) Study of the antimicrobial activity of synthetic peptides from the new AMP family, SCPDs.

1.1) Determination of their **antimicrobial effect on planktonic** bacteria (determining Minimum Inhibitory Concentrations (MIC), the Minimum Bactericidal Concentrations (MBC), Fractional Inhibitory Concentration Index (FICi), drawing growth curves (GC) and Time-Kill curves (TKC)).

2) Determination of the **cytotoxicity** of the new molecules

3) Finding the eventual target and interactions those peptides may have with the bacteria, specifically its possible interaction with the bacterial cell membrane.

- Using synthetic liposomes to evaluate the effect of the peptides have on particle size and Z potential along with measurements as a function of temperature, of the steady-state fluorescence anisotropy (*r*) and the electrostatic surface membrane potential.

3.1) Observe the effect these molecules may have on the bacteria, the cell membrane and artificial membranes by means of atomic force microscopy (AFM).

RESULTS

ARTICLE 1

ARTICLE 1: Super-Cationic Peptide Dendrimers—Synthesis and Evaluation as Antimicrobial Agents.

Estelle J. Ramchuran†, **Isabel Pérez-Guillén†**, Linda A. Bester, René Khan, Fernando Albericio, Miguel Viñas, Beatriz G. de la Torre.

† These two authors contribute equally to the paper.

Antibiotics 2021

Addressed objectives in this article:

1) Study of the antimicrobial activity of synthetic peptides from the new AMP family, $SCPD_S$.

1.1) Determination of their antimicrobial effect on planktonic bacteria (determining Minimum Inhibitory Concentration (MIC), the Minimum Bactericidal Concentration (MBC), Fractional Inhibitory Concentration Index (FICi), growth curves (GC) and Time-Kill curves (TKC).

2) Determination of the **cytotoxicity** of the molecules.

antibiotics

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Article

Super-Cationic Peptide Dendrimers—Synthesis and Evaluation as Antimicrobial Agents

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Citation: Ramchuran, E.J.; Pérez-Guillén, I.; Bester, L.A.; Khan, R.; Albericio, F.; Viñas, M.; de la Torre, B.G. Super-Cationic Peptide Dendrimers—Synthesis and Evaluation as Antimicrobial Agents. *Antibiotics* **2021**, *10*, 695. https://doi.org/10.3390/antibiotics10060695

Academic Editors: Jean-Marc Sabatier and Zhijian Cao

Received: 18 May 2021
Accepted: 8 June 2021
Published: 10 June 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Microbial infections are a major public health concern. Antimicrobial peptides (AMPs) have been demonstrated to be a plausible alternative to the current arsenal of drugs that has become inefficient due to multidrug resistance. Herein we describe a new AMP family, namely the super-cationic peptide dendrimers (SCPDs). Although all members of the series exert some antibacterial activity, we propose that special attention should be given to $(KLK)_2KLLKLL\text{-}NH_2$ (G1KLK-L2KL2), which shows selectivity for Gram-negative bacteria and virtually no cytotoxicity in HepG2 and HEK293. These results reinforce the validity of the SCPD family as a valuable class of AMP and support G1KLK-L2KL2 as a strong lead candidate for the future development of an antibacterial agent against Gram-negative bacteria.

Keywords: solid-phase peptide synthesis; antimicrobial peptides; Gram-positive; Gram-negative; therapeutic index

1. Introduction

The emergence and spread of multidrug-resistant (MDR) bacteria have become a major challenge for antimicrobial therapy [1]. Indeed, multidrug resistance is a major public health issue [2]. The scenario has become increasingly more alarming given the large gap between the development of novel antibiotics and the burden of infections due to MDR bacteria [3]. Thus, research into new antimicrobial products and strategies is urgent [4].

Antimicrobial peptides (AMPs), either natural products produced by microorganisms, animals, plants and bacteria, or synthetic ones, are one of the main options to overcome bacterial drug resistance [5]. In principle, this is based on the fact that they are potent antimicrobials and they do not share mechanisms of action with known traditional antibiotics. Moreover, some peptides, particularly those similar to animal defense AMPs, have immunomodulatory properties [6]. Widespread resistance, including extremely resistant microorganisms, together with the lack of new antimicrobial agents, has led to the revival of abandoned antimicrobials, such as colistin, a natural AMP, and has driven research

into the synthesis of similar molecules [7], as well as the search for new ones [8,9]. In general, AMPs have a positive net charge that allows them to interact selectively with bacterial membranes and also with other negatively charged structures, including DNA, lipopolysaccharide (LPS) and lipoteichoic acids (LTAs). Although membrane permeabilization seems to be the main mechanism of action of AMPs, a few additional mechanisms have also been reported, such as inhibition of the biosynthesis of macromolecules, inhibition of nucleic acid synthesis and alterations in intracellular translocation and in metabolism. Transcriptomics studies have revealed that AMPs commonly lead to a dramatic alteration of bacterial gene expression. Surprisingly, the toxicity of AMPs on eukaryotic cells in vitro is generally low.

Numerous AMPs, including linear alpha helical amphipathic peptides [10] and also peptidomimetics such as peptoids [11], foldamers [12] and other amide-containing oligomers [13], act by disrupting microbial membranes. Peptides based on a dendrimer structure are an interesting case and could be considered multiple ramified peptides through a branched unit, which is usually a Lys residue. This concept has been explored by Reymond et al. using mostly units based on Leu-Lys [14–16]. In the study herein, we examined the antimicrobial activity of different generations of super-cationic peptide dendrimers based on the triad XLX, where X are residues of Lys, Orn or Arg.

2. Results and Discussion

2.1. Design and Synthesis of Super-Cationic Peptide Dendrimers (SCPDs)

Molecule design was based on the following three main physical driving forces behind the antibacterial activity of cationic AMPs: (i) highly positive charge to enhance interaction with the anionic lipids of the cell wall in bacteria, (ii) hydrophobic content to facilitate membrane insertion and (iii) flexibility to allow conformational changes when interacting with membranes [17,18]. With this in mind, two "mirror sequences" were used: LLKLL for G0, which has a high hydrophobic content, and XLX for the branches of the following generations, where X is a basic residue, and hence this sequence contributes two positive charges to each branch. The branching is afforded by the incorporation of a Lys residue, which, after reaction on the α- and ε- amino groups, can be considered as contributing to the hydrophobicity of the molecule. Moreover, we included a series of compounds with N-terminals acylated with different lengths of the acylating moiety (acetyl, hexyl and dodecyl), which directly affects the hydrophobicity of the resulting molecule (Table 1).

Table 1. SPCDs and their charges.

	Peptide Dendrimer Sequence	Short Name	Residues (Basic/Hydrophobic)	Positive Charges
1	$(KLK)_8(\mathbf{K}KLK)_4(\mathbf{K}KLK)_2\mathbf{K}LLKLL\text{-}NH_2$	G3KLK-L_2KL_2	54 (29/25)	37
2	$(KLK)_4(\mathbf{K}KLK)_2\mathbf{K}LLKLL\text{-}NH_2$	G2KLK-L_2KL_2	26 (13/13)	17
3	$(Ac\text{-}KLK)_4(\mathbf{K}KLK)_2\mathbf{K}LLKLL\text{-}NH_2$ [a]	Ac-G2KLK-L_2KL_2	30 [a] (13/17)	13
4	$(Hx\text{-}KLK)_4(\mathbf{K}KLK)_2\mathbf{K}LLKLL\text{-}NH_2$	Hx-G2KLK-L_2KL_2	30 [a] (13/17)	13
5	$(Dd\text{-}KLK)_4(\mathbf{K}KLK)_2\mathbf{K}LLKLL\text{-}NH_2$	Dd-G2KLK-L_2KL_2	30 [a] (13/17)	13
6	$(RLR)_4(\mathbf{K}RLR)_2\mathbf{K}LLRLL\text{-}NH_2$	G2RLR-L_2RL_2	26 (13/13)	17

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Table 1. *Cont.*

	Peptide Dendrimer Sequence	Short Name	Residues (Basic/Hydrophobic)	Positive Charges
7	$(OLO)_4(KOLO)_2KLLOLL\text{-}NH_2$	G2OLO-L_2OL_2	26 (13/13)	17
8	$(KLK)_2KLLKLL\text{-}NH_2$	G1KLK-L_2KL_2	12 (5/7)	7
9	$(RLR)_2KLLRLL\text{-}NH_2$	G1RLR-L_2RL_2	12 (5/7)	7
10	$(OLO)_2KLLOLL\text{-}NH_2$	G1OLO-L_2OL_2	12 (5/7)	7

In blue are the basic residues, positively charged at physiological pH. [a] The acyl moieties have been considered as hydrophobic residues.

All the constructs were synthesized by standard solid-phase peptide synthesis (SPPS) following the Fmoc/tBu methodology. They were then purified to >95% homogeneity by reverse-phase HPLC and characterized by LCMS. The schematic representation of the 10 compounds is given in Figure 1A, and a 2-D representation of G3KLK-L_2KL_2 is shown in Figure 1B.

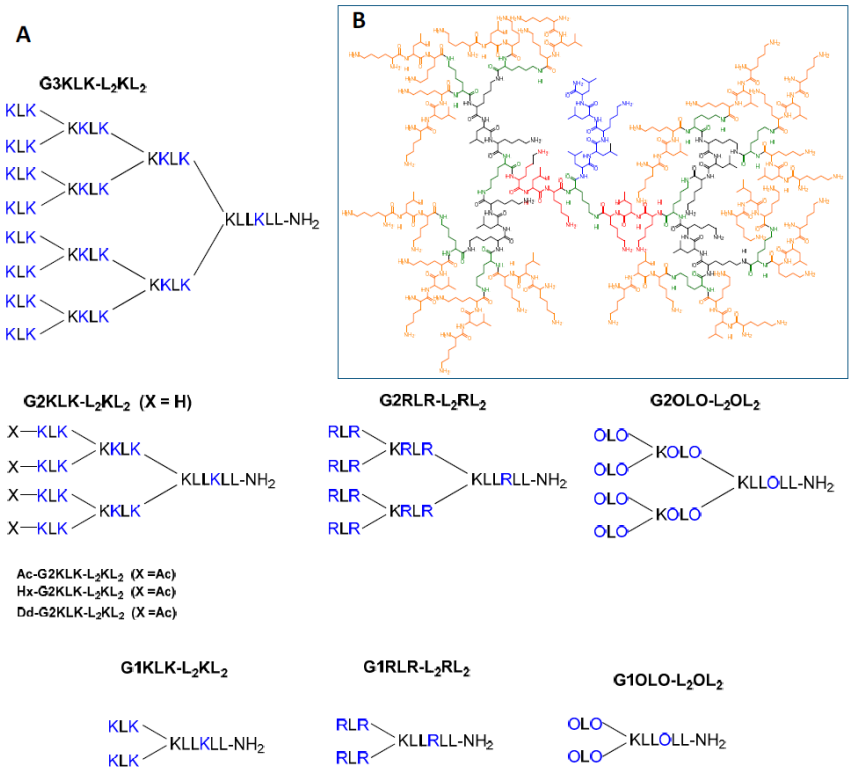

Figure 1. (**A**) Schematic representation of the peptide dendrimers synthesized. (**B**) 2-D representation of G3KLK-L_2KL_2; the green residues are the branching Lys, then blue, red, black and orange indicate G0, G1, G2 and G3, respectively.

2.2. Antimicrobial Action (MIC and MBC)

The antimicrobial activity of the 10 peptide dendrimers described before was first assayed against a panel of antibiotic-susceptible reference bacterial strains (three Gram-positive and two Gram-negative). The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values ranged from 8 to 256 μg/mL, as can be seen in Table 2.

It is acknowledged that there is a poor understanding of why the bacteria in vitro susceptibility against an antibacterial agent results in pitfalls when tested in clinical infections. The MIC determination only concerns two factors, bacteria and drug while in clinical infection, but there is an extra factor to be considered: the patient. To simulate an environment more realistic, the MICs are determined by adding 50% of human serum to the testing media. In fact, MIC values, as well as time–kill studies, of some compounds can present significant modifications in their antimicrobial capability when tested in the presence of human serum. This is the case of Mu1140, a peptide produced by *Lactococcus lactis*; its MIC increased up to fourfold when tested against *S. pneumoniae* in the presence of human serum, likely because the antibiotic binding to serum proteins results in a lower effective concentration. In contrast, the opposite outcome was seen against *S. aureus*—MIC decreases to one-fourth [17]. In our study, the addition of 50% human serum had no effect on MIC values in any of the bacterial strains tested.

Table 2. MIC and MBC (μg/mL) values of the SCPDs tested on susceptible bacteria.

Amtimicrobial Agents	Gram-Positive									Gram-Negative					
	S. aureus ATCC 29213			*B. subtilis* ATCC 6051			*E. faecium* ATCC 35667			*E. coli* ATCC 25822			*P. aeruginosa* ATCC 27853		
	MIC	50% HS *	MBC	MIC	50% HS *	MBC	MIC	50% HS *	MBC	MIC	50% HS *	MBC	MIC	50% HS	MBC
G3KLK-L_2KL_2	16	16	16	4–8	8	8	64	64	64	32	32	32	16	16	16
G2KLK-L_2KL_2	32	32	32	8	8	8	32	64	32	16	32	32	8–16	16	16
Ac-G2KLK-L_2KL_2	64	64	128	8–16	16	16	64	128	128	8–16	16	16	8–16	16	16
Hx-G2KLK-L_2KL_2	128	256	128	64	64	128	32	32	64	64	64	64	64	64	64
Dd-G2KLK-L_2KL_2	>128	ND	ND	>128	ND	ND	>128	ND	ND	>128	ND	ND	>128	ND	ND
G2RLR-L_2RL_2	32	32	32	16	32	16	16	32	16	64	64	64	64	64	64
G2OLO-L_2OL_2	32	32	32	32	32	32	8	16	8	32	32	32	32	32	32
G1KLK-L_2KL_2	128	>128	128	64	128	64	>128	ND	ND	32	32	32	32	32	32
G1RLR-L_2RL_2	16	32	16	32	32	32	4	8	4	8	16	8	16	32	16
G1OLO-L_2OL_2	64	64	64	32	32	32	16	16	16	8	8	8	16	16	16
Meropenem	0.25	ND	ND	0.062	ND	ND	<0.25	ND	ND	0.125	ND	ND	1	ND	ND

* MIC values in the presence of 50% human serum.

The MIC values appear to indicate that the tested compounds are broad-spectrum antimicrobials since they exerted activity against both Gram-positive and -negative strains. Only compound G1KLK-L_2KL_2 showed a certain degree of selectivity against Gram-negative strains. In spite of the fragility of the information provided by MIC values, a comparison of the compounds indicated that (i) the different levels of branching does not significantly affect antimicrobial activity against the bacterial strains tested, and (ii) the acylation of the N-termini does not contribute to improving this activity. In contrast, a longer acyl chain was revealed as being detrimental, giving higher MIC values. In this regard, the peptide dendrimer Dd-G2KLK-L_2KL_2 was discarded for further experiments.

Comparison of the MBC and MIC values revealed that they were very close, in most cases differing in one level of dilution, which is insignificant. This finding would suggest that this family of peptide dendrimers exert bactericidal activity. To confirm this hypothesis, time–kill kinetics and growth curve studies were conducted, and they are discussed in a further section.

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Next, the antimicrobial action of the compounds was tested on resistant strains: MRSA (methicillin-resistant *S. aureus*, Table 3), VRE (vancomycin-resistant *Enterococcus*, Table 4) and CRE (carbapenem-resistant Enterobacteriaceae, Table 5). As expected, antimicrobial activity against resistant isolates did not differ significantly from the values measured in susceptible strains. This observation suggests that although bacterial resistance to SCPDs cannot be ruled out, the mechanisms of such resistance would be different to those determining resistance to conventional antibiotics.

Table 3. MIC (μg/mL) values of the SCPDs tested on MRSA vancomycin susceptible isolates.

Isolates	G3KLK -L_2KL_2	G2KLK -L_2KL_2	Ac-G2KLK -L_2KL_2	Hx-G2KLK -L_2KL_2	G2RLR- L_2RL_2	G2OLO -L_2OL_2	G1RLR- L_2RL_2	G1OLO- L_2OL_2	Van	Amp
B11970	32	64	128	64	32	64	16	64	1	>512
P10781	16	64	128	64	64	64	32	64	1	>512
P10747	16	64	128	64	64	64	32	64	1	>512
S37938	32	64	128	64	32	64	32	64	1	>512
S18155	32	64	128	64	64	64	16	64	1	>512
B13178	32	64	128	64	32	64	16	64	1	>512
440260	32	64	128	64	32	64	16	64	1	>512
S18970	32	64	128	64	32	64	16	64	1	>512
P11520	16	64	128	64	32	64	16	64	1	512
T5683	32	64	128	64	64	64	16	64	1	>512

Table 4. MIC (μg/mL) values of the SCPDs tested on VRE, *E. faecium* isolates.

Isolates	G3KLK -L_2KL_2	G2KLK -L_2KL_2	Ac-G2KLK -L_2KL_2	Hx-G2KLK -L_2KL_2	G2RLR -L_2RL_2	G2OLO -L_2OL_2	G1RLR- L_2RL_2	G1OLO- L_2OL_2	Van
951245262	16	64	64	128	16	32	64	16	>128
951234856	32	32	64	64	32	64	32	16	>128
951208931	16	32	128	64	16	32	32	32	>128
938636470	32	32	64	128	16	64	64	16	>128
938666613	16	32	128	64	16	32	32	32	>128
938600912	16	32	64	64	16	32	32	16	>128
938072607	32	32	128	64	32	64	32	32	>128
944414000	32	32	128	64	16	64	32	32	>128
945530665	32	32	128	64	16	32	32	32	>128
U43821	32	32	128	64	16	32	32	32	>128

Table 5. MIC (μg/mL) values of the SCPDs tested on CRE, *E. coli* isolates.

Isolates	G3KLK -L_2KL_2	G2KLK -L_2KL_2	Ac-G2KLK -L_2KL_2	Hx-G2KLK -L_2KL_2	G2RLR- L_2RL_2	G2OLO -L_2OL_2	G1RLR- L_2RL_2	G1OLO- L_2OL_2	Merop
VIM-1 BM-12	>256	64	64	128	64	64	8	8	>32
NDM4-FEK	256	32	64	64	32	32	8	8	>32
KPC L2l	32	32	64	64	64	32	8	8	16
OXA-48 501	16	32	32	128	64	16	16	16	2
IMP JAP	64	32	64	>256	64	8	8	8	8

As reflected by the MIC values, the SCPDs synthesized apparently work as broad-spectrum antimicrobials since they show activity against both Gram-positive and -negative bacterial strains. The observation that the MIC values are very close for the two types of bacteria would suggest no differences in the mode of action exerted by the compounds. Thus, this is an indication that permeability is not an obstacle for SCPD action. In principle,

the outer membrane of Gram-negative bacteria is a permeability barrier and a first obstacle preventing antimicrobials from exerting their action.

Furthermore, it is relevant to bear in mind that vancomycin is the last resort to treat MRSA infections as it is nephrotoxic, inducing interstitial nephritis and tubular cell damage. Furthermore, vancomycin resistance is spreading. In this context, there is an urgent need to explore new, less toxic compounds with activity against MRSA as alternatives to vancomycin. In this regard, SCPDs emerge as promising molecules since they also covered vancomycin-resistant infections.

2.3. Cytotoxicity

To assess the toxicity of the synthesized peptide dendrimers on eukaryotic cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in two cell lines: HepG2 (human liver cancer cell line) and HEK293 (line derived from human embryonic kidney cells). The most branched peptide, G3KLK-L_2KL_2, showed the highest toxicity, with an IC_{50} of 25.7 and 53.9 µg/mL for HepG2 and HEK293, respectively (Figure 2). Compared with the MIC for this compound, these viability values make it unsuitable as a therapeutic application. On the other hand, the least branched peptide consisting of Lys as basic residues (G1KLK-L_2KL_2) did not show toxicity at the range of concentrations tested.

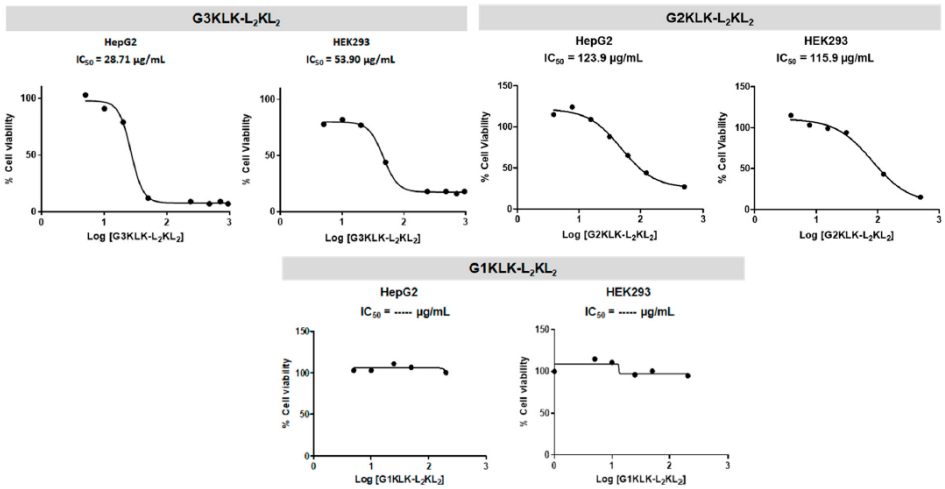

Figure 2. Cytotoxicity of G3KLK-L_2KL_2, G3KLK-L_2KL_2 and G3KLK-L_2KL_2 in HepG2 and HEK293 cell lines.

The compounds included in the G2 group (G2KLK-L_2KL_2, G2RLR-L_2RL_2, G2OLO-L_2OL_2) showed less toxicity than the G3 one. All the IC_{50} values for the G2 group were 3 to 4 times higher than the corresponding MIC. However, of note, the lower cell viability was exhibited by the compounds consisting of Arg as basic residues, especially in the case of HEK293 cells (Figure 3). The improvement of cell viability testing the G1 group, with one less branching level, was significant. Moreover, a similar behavior was observed in this group, the Arg-containing compounds once again being the most toxic.

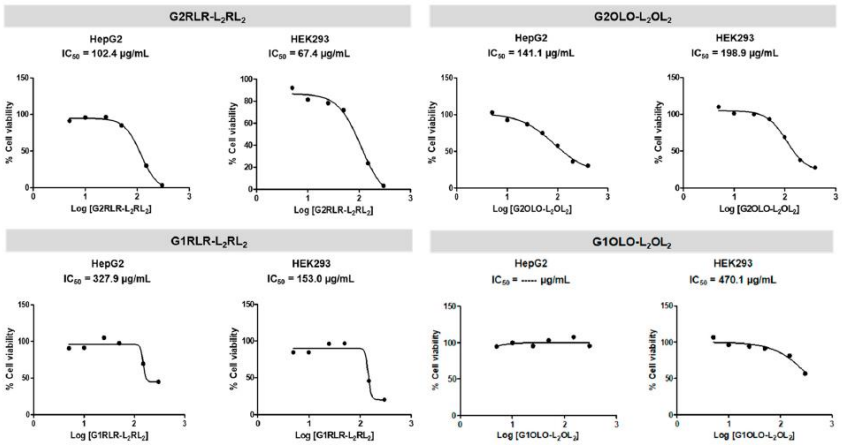

Figure 3. Cytotoxicity of G2RLR-L_2RL_2, G2OLO-L_2OL_2, G1RLR-L_2RL_2 and G1OLO-L_2OL_2 in HepG2 and HEK293 cell lines.

2.4. Time–Kill Curves

Another approach to evaluate the antibacterial effect of one molecule is to consider the killing kinetics or time–kill curves on standardized cultures of bacteria. We did this against a Gram-positive strain, *S. aureus*, for compounds G2RLR-L_2RL_2, G2OLO-L_2OL_2 and G1OLO-L_2OL_2 as representative of those with the lowest MIC and highest IC_{50} values and thus with a potentially better therapeutic index. We also tested these compounds against two Gram-negative strains (*E. coli* and *P. aeruginosa*). In this case, we also included G1KLK-L_2KL_2, which showed some selectivity against Gram-negative bacteria and low toxicity in eukaryotic cells.

The time–kill curves for *S. aureus* (Figure 4) revealed that the three compounds had no effect on bacterial growth at MIC, 1/2 MIC and 1/4 MIC, showing approximately the same behavior as the positive control at all the concentrations. These results seem to not be in agreement with the MIC values obtained, where these compounds showed an antibacterial effect at concentrations between 32 and 64 µg/mL. Nevertheless, the differences between quantitative results between MIC determinations and growth curves in the presence of antibacterials should be regarded as a result of the methods themselves. The ability of bacterial growth in a shaken 10 mL container (the conditions in which the growth curves are obtained in mini-fermenters) is much more favorable for the bacterium than microplate wells. Thus, it is reasonable that some differences in the concentrations to inhibit growth may differ between both culture conditions. On the other hand, the time–kill curves have been run at below MIC concentrations.

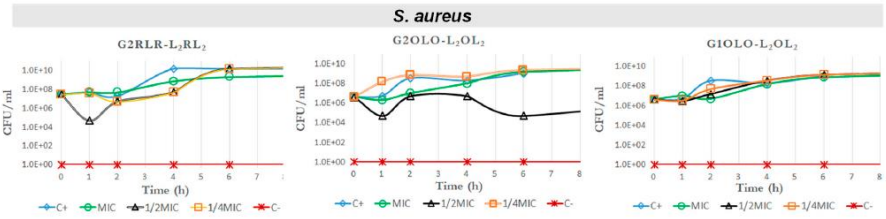

Figure 4. Time–kill curves against *S. aureus* for G2RLR-L_2RL_2, G2OLO-L_2OL_2 and G1OLO-L_2OL_2.

In contrast, the curves for *E.coli* (Figure 5) showed that all the tested compounds killed the bacteria in less than 1 h at MIC, 1/2 MIC and 1/4 MIC, and no regrowth was seen after 24 h (Figure S1, SI).

This devastating action of all four compounds even at 1/4 MIC prompted us to examine the potential therapeutic index (TI) for each one, considering this concentration and toxicity (IC_{50}) in HepG2 and Hek293 cell lines. In this regard, we found that G2RLR-L_2RL_2 would have a TI of approximately 6 and 4, respectively, which are not acceptable values for therapeutic applications. Compound G2OLO-L_2OL_2 showed better results, with a TI of 70 and 100 for HepG2 and Hek293 cells, respectively. These values could allow this compound to be studied further. Finally, the compounds G1KLK-L_2KL_2 and G1OLO-L_2OL_2 did not show toxicity for the two cell lines. The TI could be determined only for G1OLO-L_2OL_2, which had an index of 235 for the kidney cell line. On the basis of our results, G1KLK-L_2KL_2 and G1OLO-L_2OL_2 emerge as the best candidates as new antimicrobial agents against *E. coli*.

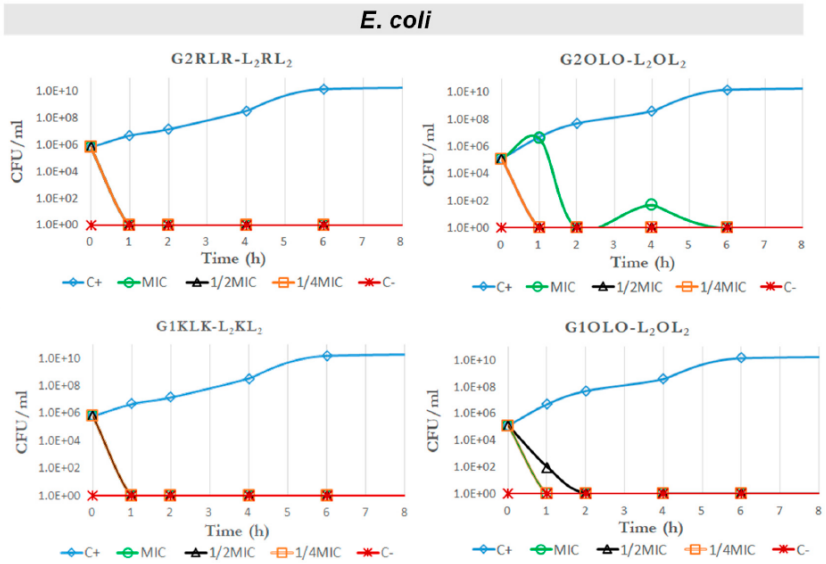

Figure 5. Time–kill curves against *E. coli* for G2RLR-L_2RL_2, G2OLO-L_2OL_2, G1KLK-L_2KL_2 and G1OLO-L_2OL_2.

In another Gram-negative bacteria, *P. aeruginosa* (Figure 6), a bacterial species characterized by intrinsic resistance to many antimicrobials, the action of the tested compounds was less dramatic that in previous case. Nevertheless, at MIC and 1/2 MIC, compound G2RLR-L_2RL_2 reduced the bacterial population to almost zero and no regrowth was appreciated after 24 h. However, at 1/4 MIC, the bacterial population remained partially inhibited at the beginning of the exposure. At the MIC, G2OLO-L_2OL_2 killed all bacteria within the first hour of exposure and regrowth was observed after the second hour. A similar pattern was seen when this compound was tested at 1/2 MIC and 1/4 MIC, although the bacterial population was not totally eliminated in these cases. After the second hour onwards, the bacterial population started to grow, but at a slower rate than in the control. This observation may suggest that, at low concentrations, G2OLO-L_2OL_2 acts as a bacteriostatic agent. A delay in antimicrobial action was also observed at 1/2 MIC in comparison with 1/4 MIC. This phenomenon has been referred to as a paradoxical effect (or Eagle effect) [18]. In the case of G1KLK-L_2KL_2, all bacteria were eliminated after the first hour of exposure at the MIC and no regrowth was detected in the following 24 h. When 1/2 MIC was used,

a reduction in bacteria of four orders of magnitude was observed after 1 h, but bacteria showed regrowth later on. The bacteria showed the same behavior in response to 1/4 MIC, but the reduction was only two magnitudes of order. Finally, G1OLO-L_2OL_2 had no effect at any concentration tested.

Regarding the TIs, again as in the case of *E. coli*, G2RLR-L_2RL_2 gave small values and were lower (<2) in the case of *P. aeruginosa*, meaning this compound will have very poor therapeutic applicability. G2OLO-L_2OL_2 showed TI values (TI = 35 for HepG2 and TI = 50 for Hek293) that could be considered for therapeutic use, although the killing effect was observed only for a limited time. G1KLK-L_2KL_2 is the compound of choice since it showed the lowest cytotoxicity.

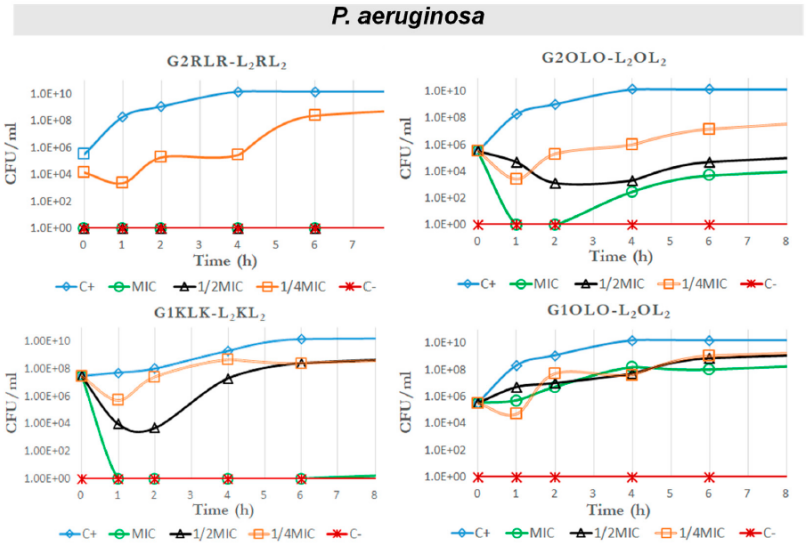

Figure 6. Time–kill curves against *P. aeruginosa* for G2RLR-L_2RL_2, G2OLO-L_2OL_2, G1KLK-L_2KL_2 and G1OLO-L_2OL_2.

2.5. Effects on Growth Kinetics

The two compounds based on Orn as basic residues, G2OLO-L_2OL_2 and G1OLO-L_2OL_2, and G1KLK-L_2KL_2 as the most promising, were assayed for their effect on the growth curve of the same bacterial strains used in the time–killing study. The effect of these compounds on *S. aureus* was negligible (Figure 7, upper panel) at MIC, 1/2 MIC and 1/4 MIC. This lack of effect confirmed the results of the time–kill kinetics experiments shown above and are in disagreement with the values shown in MIC determinations. The assay in *E. coli* revealed that the three compounds abolished bacterial growth (Figure 7, central panel), irrespective of the concentration used (MIC, 1/2 MIC and 1/4 MIC). These results also are in agreement with the observations made in the time–killing study. In *P. aeruginosa* (Figure 7, lower panel), G2OLO-L_2OL_2 abolished growth only at the MIC, and caused a 10 h delay in growth at 1/2 MIC and about a 2 h delay at 1/4 MIC. These findings thus reveal a concentration-dependent effect. A similar effect was observed for G2KLK-L_2KL_2, but this compound showed less capacity to delay growth when the bacteria were exposed to 1/2 MIC and 1/4 MIC. When the bacteria were treated with G1OLO-L_2OL_2 at the MIC, a delay of approximately 6 h in growth was observed, while at 1/2 MIC or 1/4 MIC, there was no effect.

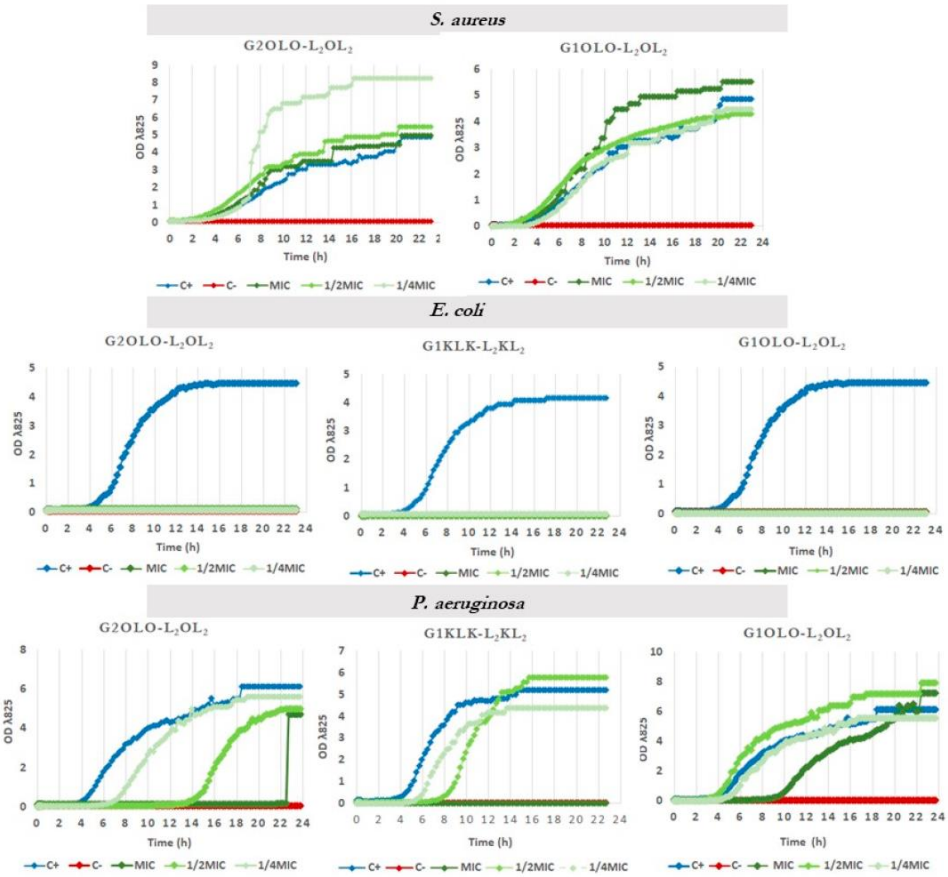

Figure 7. Growth curves of *S. aureus*, *E. coli* and *P aeruginosa* in the presence of MIC, 1/2 MIC and 1/4 MIC of G2OLO-L_2OL_2 and G1OLO-L_2OL_2.

3. Conclusions

The search for new antimicrobial agents to fight bacterial resistance is a challenge. Here, we designed a set of branched peptides to obtain constructs that we have called super-cationic peptide dendrimers (SCPDs) and tested them as potential antimicrobial agents. The first screening determined their MIC and MBC values, revealing that almost all the compounds showed some antibacterial activity, with no significant differences between Gram-positive and Gram-negative microorganisms. Thus, these SCPDs appear to be broad-spectrum antibacterial compounds, except G1KLK-L_2KL_2, which was Gram-negative selective. Nevertheless, further testing, i.e., time–kill kinetics and growth curves, revealed considerable differences in the action, showing higher activity against Gram-negative bacteria, especially *E. coli*. On the other hand, the cytotoxicity study in HepG2 and HEK293 cell lines showed higher toxicity for the higher branched peptides. Although some of the compounds showed good antibacterial activity, the calculated TI make them unsuitable as therapeutic agents. However, this was not the case for compound G1KLK-L2KL2, whose TI makes it a strong lead candidate for the future development of an antibacterial agent against Gram-negative bacteria.

4. Materials and Methods

4.1. Chemical Synthesis

The SCPDs were synthesized manually by solid phase peptide synthesis methodology using Fmoc/*t*Bu strategy [19]. Briefly, Fmoc-Rink-amide-resin (0.76 mmol/g) was used in combination with Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Orn(Boc)-OH and Fmoc-Lys(Boc)-OH for the units and Fmoc-Lys(Fmoc)-OH for the branching (5 equiv. for each coupling). *N*,*N*′-diisopropylcarbodiimide (DIC) and OxymaPure (5 equiv. each) were used as coupling reagents and *N*,*N*-dimethylformamide (DMF) as a solvent for a 30 min reaction. Fmoc was removed by two treatments with piperidine-DMF (2:8) for 1 and 5 min. At the end of the synthesis, the dendrimers were detached from the resin by treatment with trifluoroacetic acid (TFA)-triisopropylsilane (TIS)-H_2O (95:2.5:2.5) for 1 h. The filtrates were collected in cold diethyl ether (DEE) and the precipitated was isolated by centrifugation and decantation. The solid was washed three times with DEE, dissolved in 10% acetic acid and lyophilized. All peptides were purified using C18 reverse phase high-performance liquid chromatography (HPLC) and characterized by liquid chromatography-mass spectrometry (LC-MS) (see SI).

4.2. Bacterial Strains, Media and Antimicrobial Substances

Nunclon Delta Surface sterile microtiter plates (including the Edge 2.0 plate) and fetal calf serum were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human serum from male AB plasma (sterile and filtered), antibiotics, antimycotic solution, methylthiazol terazolium salt and all other reagents were obtained from Sigma (St. Louis, MO, USA). All cell culture media and plasticware were procured from Whitehead Scientific (Cape Town, South Africa).

Clinical isolates of CRE, MRSA and VRE were obtained from Lancet Laboratories, Durban, South Africa, with approval BE394/15 from the Biomedical Research Ethical Committee of the University of KwaZulu-Natal. Five reference strains of bacteria, namely *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecium* ATCC 35667, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 6051 were supplied by the American Type Culture Collection (ATCC).

4.3. Determination of Minimum Inhibitory Concentrations

MIC values were determined by the broth microdilution method and the significance of the values obtained was interpreted following the guidelines of the Clinical and Laboratory Standards for Antimicrobial Susceptibility Testing. [20]. The effect of human serum on the antimicrobial effect of the molecules tested was determined in a similar way to the MIC method used above in a solution of 50% human serum plus Mueller–Hinton broth.

4.4. Cytotoxicity

Cell viability was evaluated using the colorimetric MTT assay [21]. Human hepatocarcinoma (HepG2) cells and human embryonic kidney 293 (HEK-293) cells used in the drug toxicity assays were obtained from Cellonex (Johannesburg, South Africa). Viable HepG2 and HEK293 cells (20,000 cells/well) were seeded into a 96-well microtiter plate (200 µL) and allowed to attach overnight. The cells were exposed to varying concentrations of the peptides for 24 h. After removal of the treatment medium, 20 µL of MTT salt solution (5 mg/mL in 0.1M phosphate buffer saline (PBS)) and 100 µL of complete cell culture medium (CCM) were added to each well. After a 4 h (37 °C) incubation, the MTT salt solution was discarded and 100 µL of dimethyl sulphoxide (DMSO) were added to each well (1 h, 37 °C). The optical density of the solubilized formazan product was measured at 570 nm (reference λ of 690 nm) using a Bio-tek µQuant spectrophotometer (Winooski, VT, USA). The results were reported as percentage of cell viability $\left(\left(\frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ control\ cells}\right) \times 100\right)$ versus log concentration of the extract. GraphPad Prism (V5) (La Jolla, CA, USA) was used to obtain the half maximum inhibitory concentration (IC_{50}) for each compound.

4.5. Time–Kill Curves

Killing curve assays were performed with a starting inoculum of 6×10^6 CFU/mL. Strains were tested against the peptides at concentrations below the MICs. Antimicrobials were added to 5 mL of bacteria in the exponential phase of growth and incubated at 37 °C with shaking. Samples were obtained aseptically at 0, 1, 2, 4, 6 and 24 h, diluted in Ringer 1/4 and plated on TSA for colony counting. The response of microbial strains to a single antimicrobial was determined by lowering logarithms of viable bacteria. In accordance with Lora-Tamayo et al. [22], an antimicrobial was considered active when a reduction of $\geq$1 log10 relative to the initial inoculum was observed.

4.6. Effect on Bacterial Growth Curves

The study of the growth dynamic of the *S. aureus*, *E. coli* and *P.aeruginosa* strains against CAMPDs were assayed with a starting inoculum of 10^6–10^8 CFU/mL in a 10 mL specific falcon tube. The experiment was performed by a real-time reverse-spin bioreactor RTS-1 (Biosan SIA, Riga, Latvia) that applies non-invasive, mechanically driven rotation; thus, the cell suspension is mixed efficiently, mixing and oxygening the culture at 37 °C. Cell growth kinetics were recorded non-invasively in real time from data obtained from a near-infrared optical system, every 15 min.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10060695/s1. **Figure S1**. 24 h Time-kill curves against *E. coli* for G2RLR-L_2RL_2, G2OLO-L_2OL_2, G1KLK-L_2KL_2, and G1OLO-L_2OL_2. **Figure S2**. HPLC chromatogras and Mass Spectra of all compounds synthesized.

Author Contributions: The strategy was designed by B.G.d.l.T., M.V. and F.A., who supervised the whole study. The experiment was carried out by E.J.R. and I.P.-G. The biological studies at UKZN were supervised by L.A.B. and R.K. The first draft of the manuscript was written primarily by E.J.R. and I.P.-G. All authors have read and agreed to the published version of the manuscript.

Funding: The work was funded in part by the Marató TV3 Foundation 2018 (#20183530) and the National Research Foundation (NRF) (Blue Sky's Research Programme # 120386).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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ARTICLE 2

ARTICLE 2: Studying Lipid Membrane Interactions of a Super-Cationic 2 Peptide in Model Membranes and Living Bacteria.

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Pharmaceutics 2022

Addressed objectives in this article:

3) Finding the eventual target and interactions those peptides may have with the bacteria, specifically its possible interaction with the bacterial cell membrane.

- Using synthetic liposomes to evaluate the effect of the peptides have on particle size and Z potential along with measurements as a function of temperature, of the steady-state fluorescence anisotropy (*r*) and the electrostatic surface membrane potential.

3.1) Observe the effect these molecules may have on the bacteria, the cell membrane and artificial membranes by means of atomic force microscopy (AFM).

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MDPI

Article

Studying Lipid Membrane Interactions of a Super-Cationic Peptide in Model Membranes and Living Bacteria

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Abstract: The super-cationic peptide dendrimers (SCPD) family is a valuable class of antimicrobial peptide candidates for the future development of antibacterial agents against multidrug-resistant gram-negative bacteria. The deep knowledge of their mechanism of action is a major challenge in research, since it may be the basis for future modifications/optimizations. In this work we have explored the interaction between SCPD and membranes through biophysical and microbiological approaches in the case of the G1OLO-L_2OL_2 peptide. Results support the idea that the peptide is not only adsorbed or close to the surface of the membrane but associated/absorbed to some extent to the hydrophobic-hydrophilic region of the phospholipids. The presence of low concentrations of the peptide at the surface level is concomitant with destabilization of the cell integrity and this may contribute to osmotic stress, although other mechanisms of action cannot be ruled out.

Keywords: super-cationic peptides; atomic force microscopy; anisotropy; antimicrobials interactions

Citation: Pérez-Guillén, I.; Domènech, Ò.; Botet-Carreras, A.; Merlos, A.; Sierra, J.M.; Albericio, F.; de la Torre, B.G.; Montero, M.T.; Viñas, M.; Borrell, J.H. Studying Lipid Membrane Interactions of a Super-Cationic Peptide in Model Membranes and Living Bacteria. *Pharmaceutics* **2022**, *14*, 2191. https://doi.org/10.3390/pharmaceutics14102191

Received: 9 September 2022
Accepted: 10 October 2022
Published: 14 October 2022

1. Introduction

Bacteria are essential for human survival, but they can also cause severe and potentially fatal diseases [1]. One of the greatest achievements in medical history was the discovery of antibiotics and their clinical introduction to treat infections [2]. Nowadays, however, infections that were once treatable are no longer responsive to many antibiotics, due to the increase in antimicrobial resistance (AMR). Indeed, roughly 700,000 people die every year due to drug-resistant infections, with predictions of up to 10 million deaths per year by 2050 [3]. The rise in antimicrobial resistant bacteria, especially multidrug-resistant (MDR) bacteria, together with the failure to develop new antibiotics has motivated researcher aimed at the development of novel therapeutic strategies, including those based on new antimicrobial agents [4–7], such as antimicrobial peptides (AMPs) [8,9]. A number of AMPs are currently in use or under study in clinical trials as a viable option to overcome resistance, either in combination with or as an alternative to existing antibiotics [10]. AMPs are produced by most living organisms as natural defensive molecules that protect against pathogenic microorganisms [11]. Despite their small size (2–9 kDa), AMPs are versatile, due to their structural and physicochemical properties. Moreover, the sequence of AMPs facilitates their amphipathic nature [12,13]. Nearly all AMPs have a positive net charge

that allows them to interact selectively with bacterial membranes or other negatively charged structures. In order to obtain new antibacterial molecules, we have investigated branched peptides which has led us to the synthesis of what we call super-cationic peptide dendrimers (SCPDs) that appear to be broad-spectrum antibacterial compounds acting on Gram-positive and Gram-negative bacteria. Nevertheless, time–kill kinetics and growth curves, revealed considerable differences in their action, showing higher activity against Gram-negative bacteria. Among a long series of molecules G1OLO-L_2OL_2 displayed excellent microbiological results. The most prominent characteristic of SCPDs is their number of positive charges.

The bacterial membrane mediates interactions with both other organisms and the environment and is a key factor in the development of drug resistance [14]. The physical and chemical properties of biological membranes are directly linked to their functions [15]. In this work, we investigated the membrane interactions of the branched AMP rich in Ornitine $(OLO)_2$KLLOLL-NH_2 (G1OLO-L_2OL_2), from the recently described super-cationic peptide dendrimers (SCPDS) family [16]. To do so, we monitored the steady-state fluorescence of the polarity-sensitive probe Laurdan, and the fluorescence anisotropy of liposome-incorporated fluorescent molecules as a function of temperature. Membrane-peptide interactions were followed using atomic force microscopy (AFM).

2. Materials and Methods

2.1. Materials

Lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and dissolved in a chloroform:methanol (3:1,*v*/*v*) solution to a final concentration of 1 mg/mL. G1OLO-L_2OL_2 was synthesized as previously described [16]. Figure 1 shows the chemical structure of the G1OLO-L_2OL_2 peptide. The buffer used throughout the experiments was 10 mM Tris-HCl (pH 7.40) supplemented with 150 mM NaCl, prepared in ultrapure water (Milli-Q reverse osmosis system, 18.2 mΩ·cm resistivity). 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl1,3,5-hexatriene p-toluenesulfonate (TMA-DPH), 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), and 1-anilinonaphthalene-8-sulfonic acid (ANS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). G1OLO-L_2OL_2 lyophilized peptide was fully dissolved in ultrapure water supplemented with 5% acetic acid to a final concentration of 5 mM. Volumes from this stock solution were used for all the experiments. Concentration in experiments was 0.005 µM but for AFM of SLB (0.0005 µM).

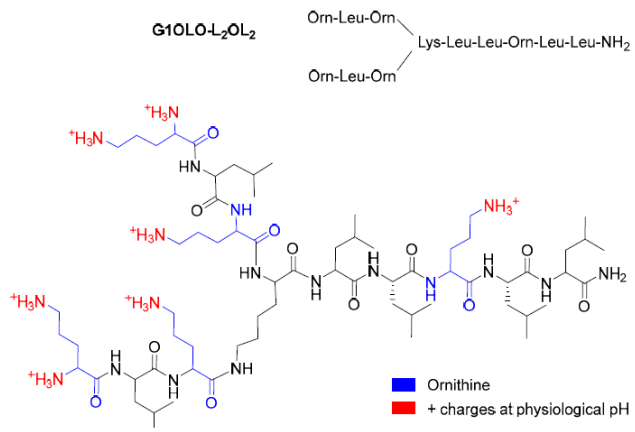

Figure 1. Chemical structure of the G1OLO-L_2OL_2 peptide.

2.2. Bacterial Strains

Two imipenem-resistant *Pseudomonas aeruginosa* isolates (846VH, 536SJD) and, as a quality control, one collection strain of *P. aeruginosa* (ATCC 27853) were studied. For the AFM bacterial studies, *Escherichia coli* strain ATCC 25922 was also used. All isolates were stored as tryptic soy broth (TSB)-glycerol (15%) stocks at −80 °C and subcultured for use in the experiments.

2.3. Liposome Preparation

Liposomes were prepared as follows: the corresponding volume of each phospholipid was prepared in a conical glass tube as described above. The solvent was then evaporated under a stream of oxygen-free N_2 during constant rotation of the tube. The tube was kept under a vacuum overnight and protected from light. The dry lipid film was then resuspended in buffer to a final concentration of 500 μM. Multilamellar vesicles (MLVs) were formed after several freeze–thaw cycles below and above the transition temperature of the lipids (22 °C). The MLVs were then extruded through an Avanti® Mini-extruder (Avanti Polar Lipids, Inc.), using polycarbonate membranes with a pore size of 100 nm.

The mean particle size and polydispersity of the liposomes were measured by dynamic light scattering, using a Nanosizer Nano S (Malvern Instruments, Malvern, UK). Electrophoretic mobility, indicating the effective surface electrical charge (potential), was determined using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Each sample was measured in triplicate.

2.4. Electrostatic Surface Membrane Potential (ΔΨ) Measurements

The interaction of liposomes with macromolecules, including peptides, will depend on the balance of electrostatic repulsion vs. attraction forces and thus on the surface charge of the structures involved. In this study, the surface charge of the liposomes in the presence or absence of G1OLO-L_2OL$_2$, was determined using ANS, a negatively charged fluorescent probe with a low fluorescence yield in polar environments. Liposomes (500 μM) in the presence or absence of G1OLO-L_2OL$_2$ (incubated under the same conditions as in the DPH experiments) were titrated with 5 mM ANS in methanol and fluorescence was monitored at λ_{ex} and λ_{em} of 380 and 480 nm, respectively, using a lipid:probe ratio of 300/1 (mol/mol). The concentration of bound ANS ($[ANS]_B$) vs. free ANS ($[ANS]_{free}$) was adjusted using a modified Langmuir isotherm (Equation (1)):

$$[ANS]_B = C_{max} \frac{\left(k \cdot [ANS]_{free}\right)^b}{1 + \left(k \cdot [ANS]_{free}\right)^b} \quad (1)$$

where C_{max} is the maximum concentration of ANS bound to the liposomes, k is the binding constant, and b is a parameter value related to the cooperativity of the process.

$[ANS]_B$ values can be calculated as shown in Equation (2):

$$[ANS]_B = \frac{F_b - F_0}{A_b - A_0} \quad (2)$$

where F_b and F_0 are the fluorescence intensities, and A_b and A_0 are the emission coefficients of ANS in the presence or absence of lipid, respectively. Emission coefficient A_b can be evaluated as the slope of a high-lipid-concentration sample (2 mM) titrated with a diluted ANS solution (0.1–1 μM), and A_0 from the same slope in the absence of lipid.

The change in the electrostatic surface potential (ΔΨ) of the liposomes due to incorporation of the peptide can be calculated as shown in Equation (3):

$$\Delta\Psi = \frac{RT}{F} ln \left(\frac{k_{liposomes+peptide}}{k_{liposomes}} \right) \quad (3)$$

where $k_{peptide}$ and $k_{liposomes}$ are the ANS binding constants for liposomes in the presence or absence of the peptide, respectively, R is the universal gas constant, T is the absolute temperature, and F is Faraday's constant.

All fluorescent determinations were carried out using an SLM-Aminco 8100 spectrofluorimeter equipped with a jacketed cuvette holder. The temperature was controlled (±0.1 °C) using a circulating water bath (Haake, Thermo Scientific, Waltham, MA, USA). The excitation and emission slits were 8/8 and 4/4, respectively.

2.5. Fluorescence Measurements

DPH tends to embed in the phospholipid bilayer, whereas TMA-DPH tends to anchor at its aqueous interface [17]. This difference was used to investigate the liposome phase behavior of the hydrocarbon domain of the bilayer. The liposomes were incubated with 0.005 μM of G1OLO-L_2OL$_2$ at 37 °C overnight. DPH or TMA-DPH was then added to the sample to a final lipid-to-probe ratio of 300/1 (mol:mol), followed by incubation for 30 min at 37 °C to allow the probe to interact with the liposomes. The anisotropy (r) of the samples over a temperature range of 3–45 °C was recorded at excitation and emission wavelengths of 381 nm and 526 nm, respectively. Vertically and horizontally polarized emission intensities were corrected for background scattering by subtracting the corresponding polarized intensities of a blank containing the unlabeled suspension (liposomes in buffer without the probe). The r values were calculated as shown in Equation (4):

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad (4)$$

where I_{ij} is the fluorescence intensity when the excitation (i) and emission (j) polarizers are fixed in the vertical (V) or horizontal (H) position, and G is the instrument sensitivity ratio of the detection system for vertically and horizontally polarized light.

The values of r as a function of temperature were adjusted using a modified Boltzmann equation (Equation (5)):

$$r = r_2 \frac{r_1 - r_2}{1 + 10^{-b(\frac{T}{T_m} - 1)}} \quad (5)$$

where r_1 and r_2 are the maximum and minimum values of r, T_m is the L_β-to-L_α phase transition temperature of the sample, and b is a parameter that provides information on the cooperativity of the transition process.

Laurdan is a polarity-sensitive probe with an affinity for the glycerol backbone of the bilayer; its lauric acid tail anchors to the phospholipid acyl chain region [18]. In this study, Laurdan was used to monitor the bilayer fluidity related to a fluorescence shift, by taking advantage of its dipolar relaxation characteristics. Laurdan excitation was measured over a range of 320–420 nm, using emission wavelengths of 440 nm and 490 nm. The lipid concentration in the liposome suspension was adjusted to 250 μM, with Laurdan added to obtain a lipid:probe ratio of 300:1. The generalized polarization (GP_{ex}) for the emission spectra was calculated as shown in Equation (6)

$$GP_{ex} = \frac{I_{440} - I_{490}}{I_{440} + I_{490}} \quad (6)$$

where I_{440} and I_{490} are the fluorescence intensities at emission wavelengths of 440 nm (gel phase, L_β) and 490 nm (liquid crystalline phase L_α), respectively. GP_{ex} values depend on the excitation wavelength (λ_{ex}). In lipid mixtures and at constant temperature, positive slope values of GP_{ex} vs. λ_{ex} indicate the coexistence of domains of different composition, and negative slope values a thermal transition towards a more fluid phase.

2.6. Atomic Force Microscopy Imaging

2.6.1. Bacteria

The in vivo effects of G1OLO-L_2OL_2 onto the surfaces of *Pseudomonas* strain 27,853 and *E. coli* strain ATCC 25,922 after 4 h of exposure to the peptide at concentrations of 0.02 µM (2 × MIC) and 0.04 µM (4 × MIC) and 0.01 µM (2 × MIC) and 0.02 µM (4 × MIC), respectively, were assessed using AFM. Both strains were grown on Muller-Hinton broth cation-adjusted (MHBCA) medium to a concentration of 10^6 colony-forming units (CFU)/mL. After incubation of the bacteria in fresh medium at 37 °C for 24 h to obtain cultures in the exponential growth phase, G1OLO-L_2OL_2 was added to the cultures for 4 h at an incubation temperature of 37 °C. The cells were then harvested by centrifugation, resuspended in 2% glutaraldehyde in 0.2 M PBS overnight, and washed three times with distilled water to remove cell debris. The pellets were then suspended in 1.5 mL of distilled water. A 10 µL drop of the suspension was placed on a Thermanox® coverslip and glued to a mica disc for AFM imaging.

The samples were imaged in air using an atomic force microscope XE-70 (Park Systems, Suwon, Korea). Images were obtained in non-contact mode using pyramidal-shaped silicon cantilevers with a spring constant of ±40 N/m and a resonance frequency of ±300 kHz; the upper sides were coated with aluminum to enhance the reflectivity of the laser beam. AFM images were acquired with a scan size of 5 μm^2 at a scan rate of 0.3–0.6 Hz. Data acquired during surface scanning were converted into images of topography and amplitude and analyzed using XEP and XEI software (Park Systems, Korea). The topography images were then used to observe the shape, structure, and surface of the planktonic bacteria. In addition, they were used to determine the average surface nano-roughness (R_a) of the treated and untreated planktonic bacteria, with (R_a) calculated as the average distance from the roughness profile to the center plane of the profile.

2.6.2. Liposomes

AFM was carried out on a commercial multimode atomic force microscope controlled by Nanoscope V electronics (Bruker AXS Corp., Madison, WI, USA). Freshly cleaved mica discs (1 cm^2) mounted on round Teflon discs were glued to steel discs. Liposome suspensions were incubated overnight on the mica discs at 37 °C. To prevent sample evaporation, the steel discs containing the mica discs and the sample were enclosed in a small Petri dish placed inside a larger Petri dish with a small amount of water at the bottom as a reservoir. The large Petri dish was then sealed with Teflon ribbon and placed inside an oven (Termaks AS, Bergen, Norway) for 20 min with a temperature control of ±0.2 °C. Non-adsorbed liposomes were removed by gently rinsing the samples with buffer, covering the mica surface with 60 µL of buffer. The samples were then directly mounted on the AFM scanner ("E" scanner, 10 µm) and allowed to stabilize. Images were acquired in liquid using MSNL-10 sharpened silicon nitride tips (Bruker AXS Probes, Camarillo, CA, USA) with a mean spring constant of 30 pN nm^{-1}, in contact mode at a 0° scan angle, and with a scan rate of 1.5 Hz. To minimize the applied force on the sample, the set point was continuously adjusted during imaging. Peptide was injected to a final concentration of 5 nM. All images were processed using NanoScope analysis software (Bruker AXS Corp., Santa Barbara, CA, USA).

2.7. Synergy Study

Checkerboard testing was used to assess the susceptibility of the planktonic, imipenem-resistant bacteria (*P. aeruginosa* strains 846VH and 536SJD) to G1OLO-L_2OL_2, added in combination with imipenem. Bacteria at a concentration of 10^6 CFU/mL were added together with G1OLO-L_2OL_2 in imipenem-containing MHB (pH of 7.3 ± 0.2) to the wells of a 96-well round-bottom microtiter plate. Concentrations assayed were between 0.5 µg/mL to 16 µg/mL (Imipenem) and between 0.125 µg/mL to 64 µg/mL of the peptide. All experiments were performed in triplicate. The interactions of the bacteria with the peptide were quantitatively evaluated by calculating the fractional inhibitory concentration index

(FICi) according to the following formula: FICi = ([MIC drug X in combination)/(MIC of drug X alone])+([MIC of drug Y in combination)/(MIC of drug Y alone]). An FICi < 0.5 was considered to indicate a synergistic interaction, an FICi > 4 an antagonistic interaction, and an FICi $\geq$ 0.5 and $\leq$ 4 an indifferent interaction [19].

3. Results

3.1. Particle Size and Z Potential

The size, polydispersity index (PDI), and zeta potential of the liposome and peptide are summarized in Table 1. The peptide was positively charged at the pH studied and, when incubated with the liposomes, did not significantly modify their size. Interestingly, the peptide did increase the zeta potential of blank liposomes by nearly 5 mV, from −31 mV to −26 mV.

Table 1. Liposome and peptide characteristics: size, PDI, and zeta potential. Measures were made by triplicate.

	Diameter (nm)	PDI	Zeta Potential ± SE (mV)
G1OLO-L_2OL_2	-	-	+5.6 ± 0.4
Blank liposomes	62.09	0.061	−30.8 ± 0.8
Liposomes + G1OLO-L_2OL_2	70.07	0.108	−26 ± 2

3.2. Fluorescence Experiments

Changes in fluidity due to the presence of the peptide in the lipid membrane were analyzed based on the fluorescence anisotropy of the membranes when incubated with the probes DPH and TMA-DPH. Figure 2 shows the changes in DPH and TMA-DPH anisotropy (r) as a function of temperature, both for blank liposomes (Figure 2A,C) and liposomes incubated with 5 nM G1OLO-L_2OL_2 (Figure 2B,D).

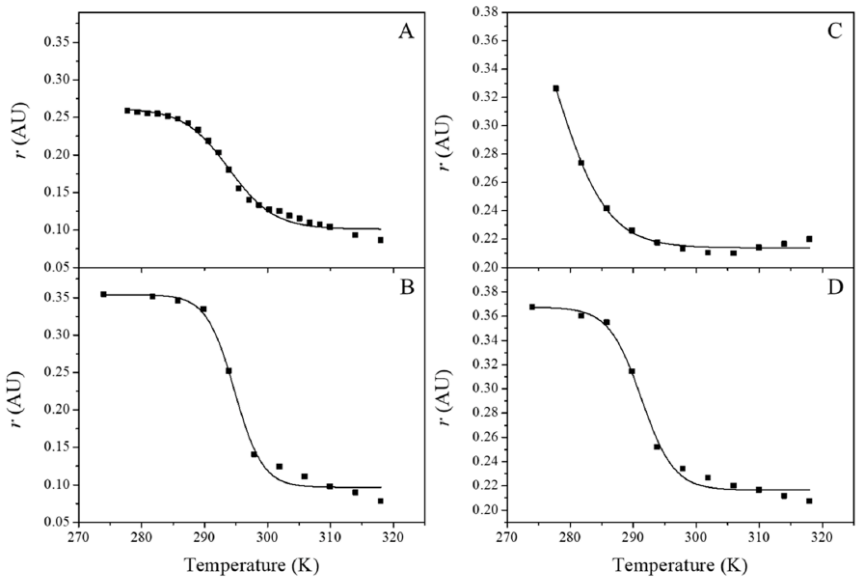

Figure 2. Temperature dependence of the fluorescence anisotropy (*r*) of POPE:POPG (3:1, mol/mol) liposomes incubated with DPH (**A**,**B**) or TMA-DPH (**C**,**D**). (**A**,**C**) Blank liposomes; (**B**,**D**) liposomes incubated with 5 nM G1OLO-L_2OL_2.

According to Equation (2), there were no significant changes in the transition temperature (T_m) of the hydrophobic region (DPH) of the liposomes following their incubation with the peptide (Table 2), whereas with TMA-DPH there was a significant shift towards higher temperatures. For both fluorescent probes, the presence of the peptide increased the cooperativity of the gel (L_β) to liquid-crystalline (L_α) phase transition. At low temperatures, the anisotropy values of DPH were higher in the liposomes in the presence of the peptide than in blank liposomes and very similar to those of TMA-DPH in the liposomes.

Table 2. Values of the experimental parameters obtained by fitting the data in Figure 2 to Equation (2). Measures were made by triplicate.

	POPE:POPG (3:1, mol/mol) Liposomes			
	DPH		TMA-DPH	
	Blank	+G1OLO-L_2OL$_2$	Blank	+G1OLO-L_2OL$_2$
$A_1 \pm$ SE	0.2624 ± 0.004	0.354 ± 0.007	0.44 ± 0.09	0.367 ± 0.005
$A_2 \pm$ SE	0.1011 ± 0.003	0.097 ± 0.006	0.214 ± 0.002	0.216 ± 0.003
$T_m \pm$ SE (K)	293.9 ± 0.4	294.8 ± 0.4	277 ± 3	291.3 ± 0.4
b ± SE	36 ± 3	57 ± 9	30 ± 7	49 ± 7
r^2	0.991	0.990	0.991	0.991

The polarity-sensitive fluorescent probe Laurdan localizes to the glycerol backbone of a lipid bilayer, with its lauric acid tail anchored in the phospholipid acyl chain region. Laurdan is sensitive to the nature of the fluid phase of its lipid environment and is thus used to differentiate lipid microdomains differing in their lipid composition and lipid phase. In this study, Laurdan was used to analyze the induction of lipid microdomains in liposomes exposed to G1OLO-L_2OL$_2$. Figure 3 shows the changes in GP_{ex} as a function of λ_{ex} for the blank (Figure 3 Top) and for liposomes incubated with 5 nM G1OLO-L_2OL$_2$ (Figure 3 Bottom). As the λ_{ex} increased, GP_{ex} decreased at all temperatures studied, indicating a transition towards a more fluid phase for lipid domains that did not differ in their composition.

The changes in the zeta potential indicated that the peptide modifies the effective surface charge of the liposome. The zeta potential is the effective charge at the shear plane between the liposome and the medium. Since the peptide reaches the lipid bilayer surface, we examined the electrostatic modification of the surface potential of the liposomes using the probe ANS. For both the blank liposomes and the liposomes incubated with 5 nM G1OLO-L_2OL$_2$, titration of the sample with ANS increased the fluorescence signal. The amount of ANS bound to the liposome (ANS_B) was calculated using Equation (5). In Figure 4, this value is represented as a function of the free ANS concentration in the presence of blank liposomes (Figure 4A) and liposomes incubated with 5 nM G1OLO-L_2OL$_2$ (Figure 4B).

Fitting the data to Equation (4) (Table 3) yielded two very similar curves (C_{max} and b were not significantly different) but the binding constant was higher in the presence of G1OLO-L_2OL$_2$. According to Equation (6), in the presence of the peptide (5 nM) the surface potential of the blank liposomes increased by 5.06 mV.

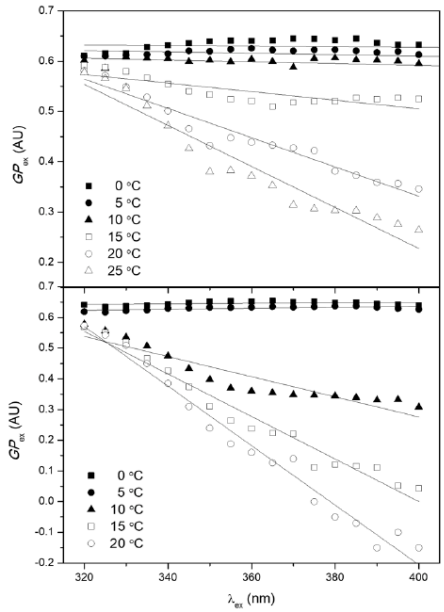

Figure 3. GP_{ex} values as a function of the λ_{ex} for POPE:POPG (3:1, mol/mol) liposomes at different temperatures. (**Top**) blank liposomes; (**Bottom**) liposomes incubated with 5 nM G1OLO-L_2OL_2.

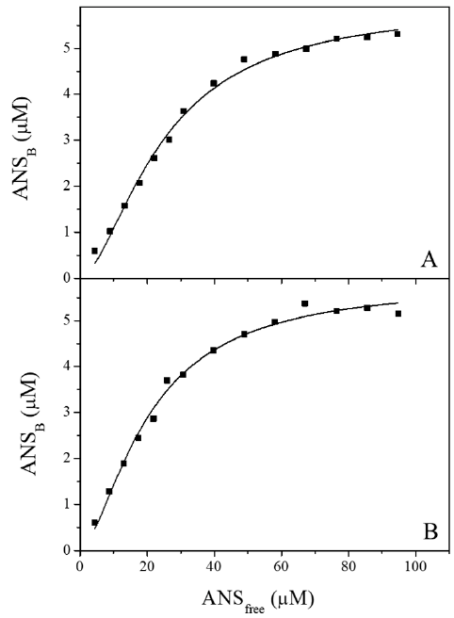

Figure 4. Concentration of ANS bound ($[ANS]_B$) to POPE:POPG (3:1, mol/mol) liposomes as a function of the free ANS concentration ($[ANS]_{free}$). (**A**) Blank liposomes; (**B**) liposomes incubated with 5 nM of G1OLO-L_2OL_2.

Table 3. Values of the experimental parameters based on the ANS binding data using Equation (4).

	POPE:POPG (3:1, mol/mol) Liposomes	
	Blank	**+G1OLO-L_2OL_2**
C (μM)	6.0 ± 0.2	5.8 ± 0.2
k (μM^{-1})	0.040 ± 0.002	0.049 ± 0.003
b	1.67 ± 0.13	1.60 ± 0.14
r^2	0.993	0.990

3.3. Atomic Force Microscopy Studies

Supported lipid bilayers: AFM has been used extensively to visualize the effect of small and large molecules on model lipid membranes. Although individual peptides are too small to visualize directly, their effects on lipid membranes can be followed at a subnanometer resolution using AFM. Figure 5 shows the AFM images (Figure 5A–D) and height line profiles (Figure 5E–H) of a supported lipid bilayer (SLB) of POPE:POPG (3:1, mol/mol) onto the mica surface. The SLB prior to its incubation with the peptide is shown in Figure 5A. From the image, three different regions can be defined: (i) small dark patches, attributable to the uncovered mica surface; (ii) a wide extended region (orange) with a step height difference of 4.61 ± 0.18 nm over the mica surface; and (iii) small yellowish domains protruding 0.66 ± 0.05 nm from the extended region, with a step height of 5.27 nm from the mica surface to the top of the yellowish domains. The heights of the different regions are depicted in the profile line in Figure 5E and were determined along the white line in Figure 5A. Five minutes after injection of the peptide to a final concentration of 5 nM (Figure 5B), the mica surface was fully covered, evidenced by the disappearance of the dark patches seen in Figure 5A, presumably due to fluidification and high lateral mobility of the lower lipid layer. The blurry borders of the higher lipid domains were probably due to the short stabilization time after injection of the peptide into the AFM cell. Following peptide injection, the high lipid domains protruded 1.56 ± 0.15 nm from the lower lipid domain but assumed a shape similar to that seen in Figure 5A. After 20 min. (Figure 5C) the lipid borders of the high lipid domains were well defined, no lipid domain appeared or vanished, and the step height from the bottom of the lower lipid domains to the top of the higher lipid domains was 1.84 ± 0.16 nm. After 35 min (Figure 5D), the step height difference between the lipid domains was 0.74 ± 0.06 nm. However, although high lipid domains retained their shape after peptide addition, areas indicating their degradation (black arrows) were also observed. A similar degradation occurred at shorter incubation times. In both cases, the lipid domains lost their structure, disorganizing from the center of the domain to the margins.

Bacteria: AFM was also used to investigate the effect of G1OLO-L_2OL_2 on planktonic cultures of *P. aeruginosa* (27,853) and *E. coli* (ATCC 25,922). Figure 6 shows the amplitude (Figure 6A–C) and 3D height (Figure 6D–F) of *P. aeruginosa* colonies incubated with different concentrations of G1OLO-L_2OL_2, as determined by AFM. In the control images (Figure 6A,D), the rod-shaped cells adsorbed onto the mica have flat and smooth surfaces, whereas after incubation with 0.02 μM G1OLO-L_2OL_2 (2 × MIC) (Figure 6B,E) the bacterial surface was characterized by a cluster of small round structures 200–300 nm wide. Many of the round structures were as tall as the original bacterium, with the most peripheral being those with a lower step height from the mica surface. A higher G1OLO-L_2OL_2 peptide concentration (4 × MIC) (Figure 6C,F) destroyed the bacterial cells. Roughness (R_a) values can be found in Table 4 where the addition of the peptide increased the R_a value of the bacteria.

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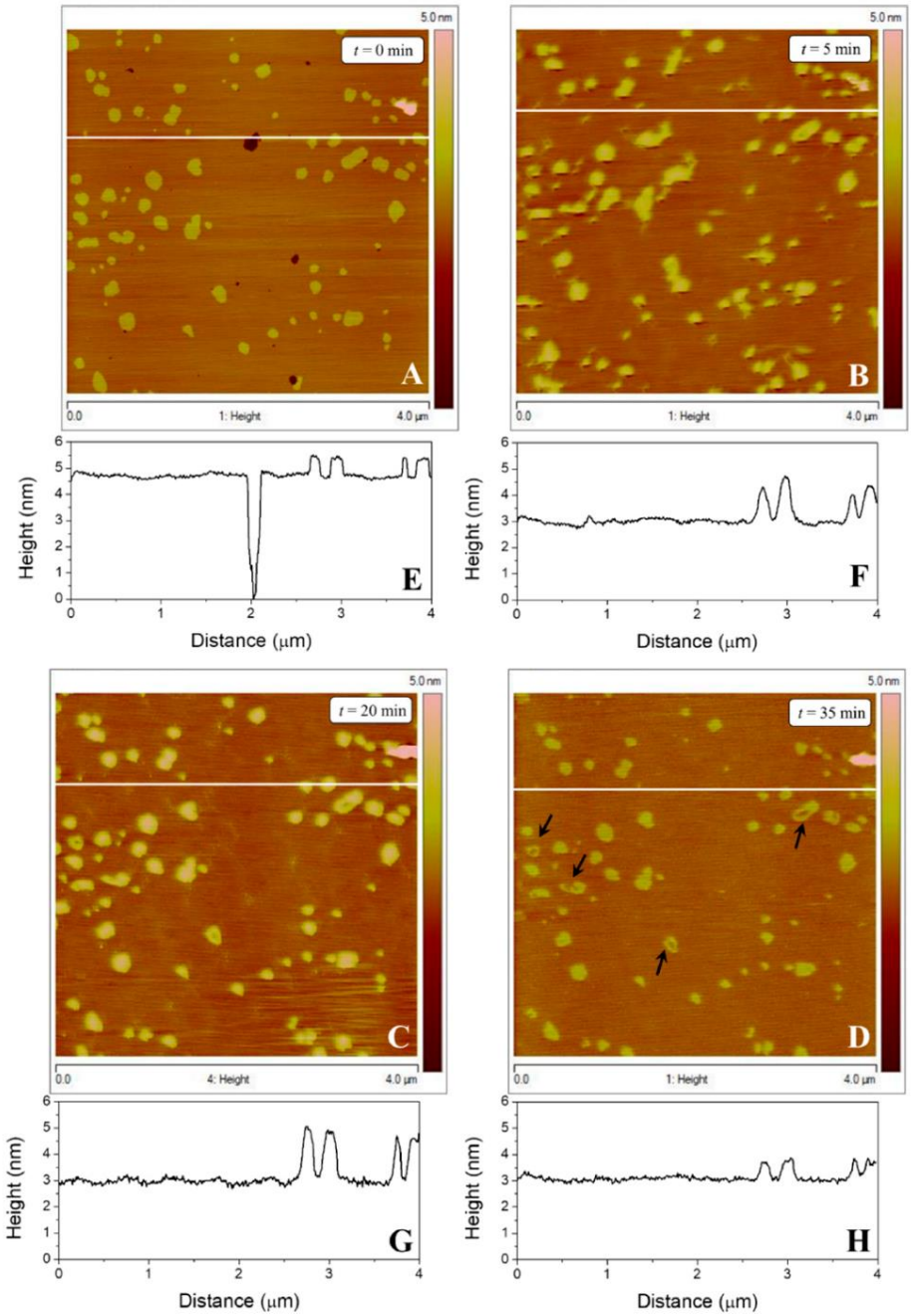

Figure 5. AFM topographic images of a SLB of POPE:POPG (3:1, mol/mol) incubated with 5 nM G1OLO-L_2OL_2 for different incubation times (**A**–**D**). Subfigures show one line topography profile for each image (**E**–**H**) represent the sample height along the white line in the corresponding AFM topographic image. Black arrows in (**D**) point to the degradation of the lipid domain.

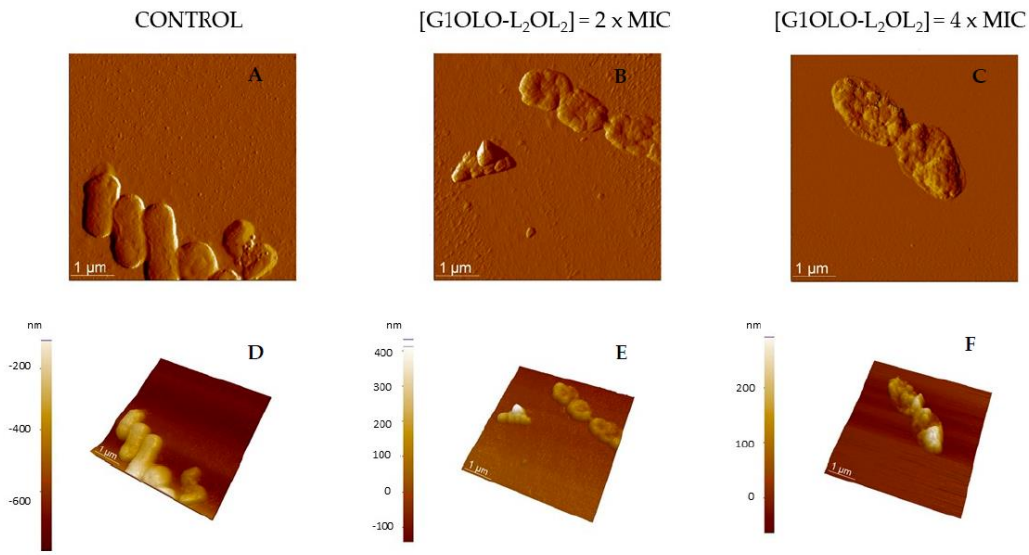

Figure 6. AFM amplitude (**A**–**C**) and 3D topographic images (**D**–**F**) of *P. aeruginosa* (27,853) incubated with different concentrations of the peptide G1OLO-L_2OL_2.

Table 4. Nano-roughness (*Ra*) average values from Figures 6 and 7.

	Ra (nm)		
	Control ± SE	**2 × MIC (4 h) ± SE**	**4 × MIC (4 h) ± SE**
E. coli ATCC (25,922)	7.37 ± 0.53	24.07 ± 2.64	27.40 ± 2.35
P. aeruginosa ATCC (27,853)	8.25 ± 0.69	23.39 ± 2.55	30.62 ± 2.89

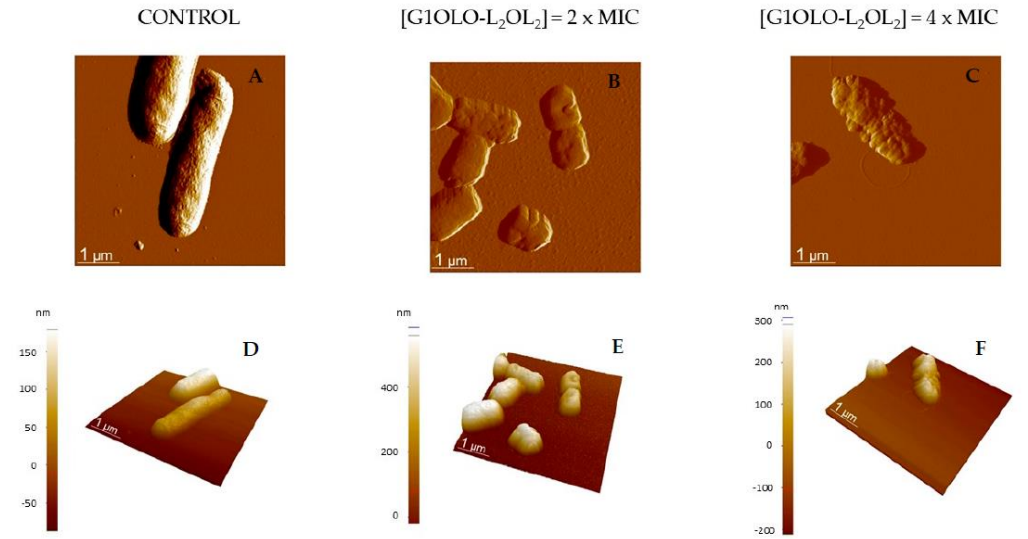

Figure 7. AFM amplitude (**A**–**C**) and 3D topographic (**D**–**F**) images of *E. coli* (ATCC 25,922) incubated with different concentrations of G1OLO-L_2OL_2.

Similar studies were performed on *E. coli*. Figure 7 shows the AFM amplitude (Figure 7A–C) and 3D height (Figure 7D–F) of *E. coli* in the absence or presence of G1OLO-L_2OL_2 at different concentrations. In the absence of the peptide, *E. coli* assumed their typical rod-shaped structure, with each cell having a length of 1–2 µm. However, incubation of the cells with 0.01 µM G1OLO-L_2OL_2 (2× MIC) (Figure 7B,E) resulted in a considerable reduction in cell size and a slight modification of the cell surface. At a concentration of 0.02 µM G1OLO-L_2OL_2 (4 × MIC) (Figure 7C,F), the shape of the bacteria was largely maintained but many small round structures appeared over the cell surface. Nano-roughness values of bacterial surface (treated and untreated) are reported in Table 4, showing a large increase in the initial values of Ra when increasing the peptide concentration.

3.4. Synergy Study

A possible synergistic effect of G1OLO-L_2OL_2 and imipenem on imipenem-resistant *P. aeruginosa* was examined to determine the effect of the peptide on the bacterial membrane. Specifically, we determined whether G1OLO-L_2OL_2 acted as a pore opener or disturbed membrane integrity, such that imipenem was then able to penetrate the bacteria, with lethal consequences. The FICi values obtained from the synergy studies are shown in Table 5. The values of FICi obtained in the checkerboard analysis of the potential synergy between G1OLO-L_2OL_2 and imipenem in MDR (imipenem-resistant) *P. aeruginosa* demonstrate indifference which is consistent with the biophysical data. Together, these results show that, while G1OLO-L_2OL_2 alters the bacterial outer membrane, it is unable to open channels or induce membrane discontinuities that would allow imipenem to penetrate imipenem-resistant *P. aeruginosa*.

Table 5. MIC and FICi data from the synergy study of G1OLO-L_2OL_2 and imipenem.

	MIC				FICi
	Imipenem		G1OLO-L_2OL_2		
	µg/mL	µM/L	µg/mL	µM/L	
PA 846 VH	16	0.053	>64	0.046	1.1875
PA 356 SJD	16	0.053	>64	0.046	1.1875

4. Discussion

In a previous work [16] we described a new AMP family, grouped under the name super-cationic peptide dendrimers (SCPDs). Although all members exert antibacterial activity, some of them were shown to be selective for Gram-negative species but with virtually no cytotoxicity in HepG2 and HEK293 human cells. These results suggested that SCPD peptides could serve as a valuable class of AMPs. Among the SCPDs, G1OLO-L_2OL_2 was one of the most promising candidates for the development of an antibacterial agent against Gram-negative bacteria. The most prominent difference between Gram-negative and Gram-positive bacteria is the presence in the former of an outer membrane that acts as a permeability barrier—although the mechanism that ultimately kills bacteria may act on the internal (cytoplasmic) membrane. Our final aim was to decipher the mechanisms by which G1OLO-L_2OL_2 is able to kill Gram-negative MDR and, particularly in this work we aimed to gain insights on its interaction with model membranes and living cells. Using biophysical approaches, we examined the interaction of G1OLO-L_2OL_2 with model membranes mimicking the inner membrane of *E. coli* (POPE:POPG 3:1, mol/mol) [19]. When G1OLO-L_2OL_2 is incubated with liposomes in suspension, it preferentially interacts with the latter's phospholipid head groups, as can be seen by the shift towards a higher melting transition temperature detected with the TMA-DPH probe. Something that does not occur with the DPH probe that resides in the core of the bilayer. This indicated an increase in the rigidity of the headgroup region of the liposome due to its interaction with the peptide. In their interactions with liposomes, peptides can either be adsorbed onto the membrane or be partially absorbed into the lipid-water interface, close to the upper portion

of the fatty acyl chains, where TMA-DPH tends to localize. However, it cannot be excluded that this increase in the Tm, might be attributed to a charge screening selective effect due to the interaction of the peptide with the negatively charged POPG. The fluorescence results showed that the peptides were not found in the hydrophobic core region of the liposome, although their ability to permeate the membrane by forming pores cannot be excluded. In spite of the structural differences, a similar behavior was observed for a series of three nine residue peptides that contained unnatural amino acids in the primary sequence [20]. Nevertheless, the addition of G1OLO-L_2OL_2 to reconstituted black lipid bilayers did not generate noticeable electrophysiological phenomena, thus suggesting that the peptide was unable to generate true transmembrane channels (data not shown).

In the AFM experiments, G1OLO-L_2OL_2 was injected in situ inside the AFM liquid cell at the same concentration used with liposomes in solution. In the absence of injected peptide, the AFM images revealed two lipid domains in the SLB surfaces, in concordance with our previous study [21]. Since the Laurdan fluorescence provided no evidence of the existence of domains of different lipid composition on the liposomes, these lipid domains may have been: (i) domains induced by changes in temperature of the same lipid composition, in which lipids in the taller domains were in a more rigid phase than those in the shorter domains or (ii) domains of different lipid composition in a different lipid phase, in which the presence of the mica surface decreased the lateral diffusion of the lipids, thus promoting the formation of segregated lipid domains differing in their lipid composition. The work performed in this study and in previously published work [22] indicated that the taller lipid domains were POPG enriched, and the more extended domains POPE enriched.

When added to the SLBs, G1OLO-L_2OL_2 interacted with their surfaces, without formation of pores, in agreement with the synergy studies. According to these observations, the peptide, at the concentrations studied, was able to adsorb onto or be partially absorb into the surface of the SLBs. However, a dose-dependent effect of the peptide was also observed, as higher concentrations (Figure 8) induced the erosion and solubilization of the SLBs. A similar effect was observed in the fluorescence experiments (data not shown), in which higher G1OLO-L_2OL_2 concentrations induced the erratic behavior of the liposomes, most likely attributable to their destabilization.

In agreement with these observations, determinations of the zeta potential of the liposomes were consistent with the incorporation of G1OLO-L_2OL_2 into the vesicles. However, to confirm that the peptide was in close contact with the lipid membrane of the liposome and not located in the hydration layer (where the zeta potential is actually measured), the surface potential was determined in an ANS fluorescence assay, which also showed that the peptide was present on the liposome surface.

Finally, the effects of G1OLO-L_2OL_2 on living bacteria were evaluated by AFM. The peptide had a more destructive effect on *P. aeruginosa* than on *E. coli*. While at 2 × MIC *P. aeruginosa* was destroyed, while *E. coli* retained its shape and cell integrity to a certain degree. However, it should be noted that AFM reveals only the topography of the bacterial surface, not bacterial viability. It is therefore possible that the bactericidal effect was similar in *P. aeruginosa* and *E. coli*, but the destruction of the lipid outer membrane differed. Studies of the differences in the membrane lipid composition in the two species in model membranes could help to explain the differences in the observed behaviors. In fact, although membrane permeabilization is the main mechanism of action of AMPs against pathogens, additional mechanisms have been described in detail. This includes membrane destabilization, inhibition of macromolecular synthesis and intracellular translocation and inhibition of the biosynthesis of nucleic acids and proteins [13]. The bacterial cytoplasm possesses a high osmotic potential that is maintained by the function of bacterial envelopes. The alteration of the membrane and/or the cell wall may determine a water influx and generate hydrostatic pressures incompatible with bacterial growth and even with survival. This is known as osmotic stress and has in bacteria some characteristics clearly different from those in the eukaryotic cells [23]. Here, the presence of low concentrations of G1OLO-L_2OL_2 at the outer membrane surface could induce osmotic stress and thereby facilitate a

destabilization of cell integrity at higher peptide concentrations. In spite of this, the clear effect of G1OLO-L_2OL_2 on bacterial membranes, and its action on other targets cannot be ruled out and should be further investigated.

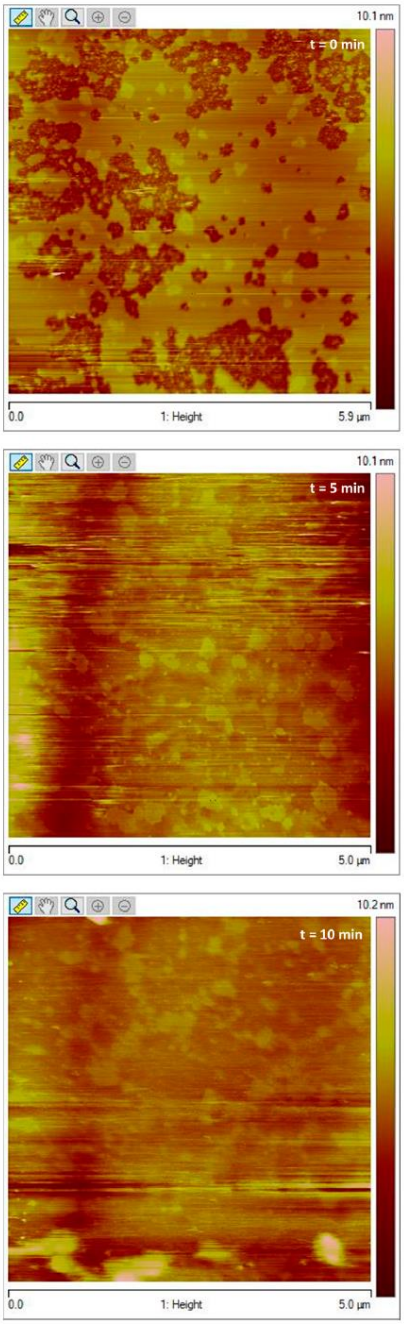

Figure 8. Erosion and solubilization of the SLBs at high peptide concentrations.

5. Conclusions

This study evaluated the interaction of the SCPD peptide G1OLO-L_2OL_2 with model membranes, liposomes, and SLBs using biophysical and microbiological approaches. The results consistently pointed to a surface effect of G1OLO-L_2OL_2 on the model lipid membranes, not only the adsorption or a close proximity of the peptide to the surface, but to some extent its association with or absorption into the more hydrophilic region of the phospholipids.

Author Contributions: Conceptualization, J.H.B., M.V., B.G.d.l.T. and F.A.; methodology, J.M.S. and, Ò.D.; investigation, I.P.-G., A.B.-C. and M.T.M.; writing—original draft preparation, I.P.-G. and A.B.-C.; writing—review and editing, M.V. and J.H.B.; AFM visualization, A.M. and Ò.D.; supervision, F.A., M.V., J.H.B.; project administration, M.V.; funding acquisition, M.V. and F.A. All authors participated in the discussion and evaluation of the results. All authors have contributed substantially to the work. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Marató TV3 foundation Project BARNAPA (MV & FA).

Data Availability Statement: Not applicable.

Acknowledgments: The technical support given by Sonia Suarez is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AMR: antimicrobial resistance; MDR: multidrug-resistant; AMPs: antimicrobial peptides; SCPDS: super-cationic peptide dendrimers; TMA-DPH: 1-(4-trimethylammoniumphenyl)-6-phenyl 1,3,5-hexatrienep-toluenesulfonate; DPH: 1,6-diphenyl-1,3,5-hexatriene; ANS: 1-anilino-8-naphthalene sulfonate; AFM: atomic force microscopy; POPE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG: 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]; TMA-DPH: 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-trimethylammoniumphenyl)-6-phenyl1,3,5-hexatriene p-toluenesulfonate; ANS 1-anilinonaphthalene-8-sulfonic acid; MLVs: Multilamellar vesicles; MHBCA: Muller-Hinton broth cation-adjusted; CFU: colony-forming units; PBS: Phosphate Buffer Saline; FICi: fractional inhibitory concentration index; MIC: minimum inhibitory concentration; SLB: supported lipid bilayer; HepG2: human liver cancer cell line; HEK293: human embryonic kidney cells grown in tissue culture.

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DISCUSSION

The increasing rise of AMR, as well as the ineffectiveness to develop new antibiotics is a cause of much distress worldwide. Therefore, creating new strategies of development in order to fight this global threat is indispensable.

One path to address this serious problem is focusing on the synthesis of new compounds as an alternative therapy, like synthetic antimicrobial peptides (AMPs).

The studies were carried out in collaboration with the research groups lead by Prof. Fernando Albericio and Prof. Jordi H. Borell, from Dept. of Organic Chemistry at University of KwaZulu-Natal (South-Africa) and Dept. of Physical Chemistry, at the University of Barcelona, respectively.

In the present work a newly design AMP family, named the Super-Cationic peptide dendrimers (SCPDs) was studied for its antimicrobial effect.

These peptides were synthetized based on the cationic AMPs antimicrobial properties, being the hydrophobicity and the positively charge features essential ones due to its relevance in order to boost the interaction to the negatively charged components of the microorganisms, specifically the bacterial cell membrane on Gram-negative bacteria.

The 10 new molecules showed activity range of MIC and MBC between 8 and 256 μg/ml; Even though the *in vitro* testing is broadly done and relies on infections treatments, its correlation with *in vivo* efficacy should be questioned, as previously addressed in other studies (57,58). Therefore, it was considered and implemented in order to widen the factors in consideration the usage of human serum, which had already been studied and shown to alter the antimicrobial capabilities of the molecules in study (59–61). To make a more realistic scenario and consider the patient factor MIC with 50% human serum where performed, although no effect in any of the bacterial strains tested was observed.

Through the comparison of the MIC values, it was estimated that the different levels of branching do not significantly affect its antimicrobial activity, and the N-termini acylation do not improve the activity. In contrast, if the acyl chain of the molecule was longer it seems to decrease the antimicrobial activity, which correlates with the AMP design strategy review recently published (62).

The MIC values obtained from testing resistant isolates were not significantly different form the ones determined on susceptible strains; denoting that the mechanisms of resistance that might affect SCPDs would be different to those of conventional antibiotics.

It seems that SCDPs behave as broad-spectrum antimicrobials since they show activity against both Gram-positive and Gram-negative bacterial strains, which MIC values are close. Consequently, this observation suggests that there are no differences in the mode of action of the molecules regardless of the bacteria. Thus, SCDPs antimicrobial activity is not affected by permeability, a main barrier specifically in Gram-negative bacteria in preventing antimicrobial action (63).

Moreover, taking into account that vancomycin is the last treatment resort for methicillin-resistant *Staphylococcus aureus* (MRSA) infections which resistance is spreading, the new SCPDs may be emerging promising molecules or molecule scaffolds since they act over vancomycin resistant strains, nevertheless an specific study regarding these molecules and vancomycin-resistant *Staphylococcus aureus* (VRSA) should be done focusing on their molecular mechanism of action, like it has been done for a high number of AMPs like Ctriporin, Formicin C, Cecropin, etc (64).

An essential characteristic to become a candidate as an antimicrobial is the capability to cause bacterial cell death without damaging host cells. The molecules, easier to evaluate if divided in three groups (G3, G2 and G1) from most to less branched respectively, were studied using two cell lines (HepG2 and HEK293). G3 group was considered unsuitable as a

therapeutic option, whereas groups G2 even though toxic and G1, not toxic to the tested cell lines, continued to be candidates to study.

Another relevant characteristic to acknowledge is the effect these molecules may have over time; thus, it is suitable to do a kinetic profile (action-interaction-time) of the bacteria in the presence of these compounds. TKC and GC may provide relevant information. It has been shown that the data obtained from these studies usually has a huge correlation with treatment (65–67).

The experimental procedure was done using those of group G2 and G1 with the lowest MIC and highest IC_{50} values and thus with a potentially better therapeutic index (TI).

It was observed that these molecules had negligible effect over *S. aureus* in both TKC and GC, and did not correlate with the MIC values obtained, which should be addressed as a result of the methods themselves. Bacterial growth is highly favorable in a Falcon tube in constant shaking than in the wells of the microtiter plates.

In contrast, for Gram-negative bacteria like *Pseudomonas aeruginosa*, characterized by its intrinsic resistance to many antimicrobials the effect of the different compounds was less dramatic.

For TKC, G2RLR-L_2RL_2 reduced the bacterial population to almost zero and no regrowth was detected, even though at ¼ MIC, the bacterial population remained partially inhibited at the beginning of the exposure. G2OLO-L_2OL_2 killed all bacteria within the first hour at MIC concentration of exposure. For all the tested concentrations regrowth or growth of the bacterial population not totally prevented was observed but at a slower rate than in the control which may suggest that at low concentrations, this peptide acts as a bacteriostatic agent. Moreover a phenomena referred as paradoxical effect was observed called Eagle effect (1948) (68,69) described as the concentration of antibiotic used that killed fewer colonies *in vitro* when the dose was increased beyond a threshold concentration already known to be effective. It was observed that for G2OLO-L_2OL_2 and G1OLO-L_2OL_2 ¼ MIC concentrations causes a reduction of the bacterial population greater than ½ MIC concentration, or even more than MIC concentrations in the case of G1OLO-L_2OL_2 which should not be the expected outcome, this also was seen studding amoxicillin on *Corynebacterium diphtheriae* (70).

For GC the results lead us to believe that a concentration-dependent effect occurs in G2OLO-L_2OL_2 and a similar effect also occurs in G2KLK-L_2KL_2 where the higher the concentration the greater the delay of regrowth of the bacterial.

In addition, for both TKC and GC of *E. coli* all the compounds were able to kill the bacteria with no regrowth after 24h with devastating action regardless of the concentration used.

The results obtained from *E. coli* encouraged us to calculate de potential TI of each compound. Basically G2OLO-L_2OL_2 showed a TI of 70 and 100 for HepG2 and Hek293 cells, respectively which would allow this compound to be studied further, or even it could be possible to improve its therapeutic index as wanted to do with the Lubelisin peptide (71), or done in Magainin Derivatives introducing proline residues and positive charges (72). For compounds G1KLK-L_2KL_2 and G1OLO-L_2OL_2 not toxicity appear for HepG2 and Hek293, only G1OLO-L_2OL_2 had a TI of 235 for Hek293. On the basis of our results, G1KLK-L_2KL_2 and G1OLO-L_2OL_2 emerge as the best candidates as new antimicrobial agents against *E. coli*.

The obtained results lead us to wonder the impact these peptides have over *E. coli*. An important characteristic once a new molecule in study has shown antimicrobial effect is to clarify how they interact with the cell. One of the barriers that these molecules encounter before being able to enter to the cytoplasm is the bacterial cell membrane, a lipid bilayer where proteins are embedded. The efflux pumps have gained all the attention in drug resistance (46,73) but simple diffusion of molecules through is also a significant pathway of cell destabilization.

Even though it is particularly difficult and challenging to identify interactions between AMPs and the cell, generally models are built to examine specific interactions despite the differences or lack of many important features.

Here, in order to study the effect of peptide G1OLO-L_2OL_2 synthetic liposomes made of POPE/POPG lipids, which mimic *E. coli* membranes were done (74), like for studying AMP Maculatin 1.1 (75) or protein lactose permease (LacY) (76).

The fact that cationic AMPs interact electrostatically with the charged membrane surfaces is a fact commonly reported (77–80) thus it is not surprising that G1OLO-L_2OL_2 was able to increase the zeta potential value of blank liposomes in nearly 5 mV, from approximately -31 mV to -26 mV evidencing the incorporation of the peptide to the vesicles. It is worth to acknowledge that zeta potential is the effective charge at the shear plane of the liposome with the medium. This result permits to stablish that the presence of the peptide modifies the effective surface charge of the liposome.

In order to be sure that the peptide was in close contact with the lipid membrane of the liposome and not in the hydration layer where zeta potential was measured it was important to evaluate the electrostatic modification of the surface potential of the liposome. Using ANS, a negatively charge molecule as probe it was estimated that the presence of the 0.005 μM of peptide

increases in + 5.06 mV the surface potential of the blank liposomes.

ANS emits a higher fluorescence in lipid than in aqueous media, therefore observing an increase of its binding indicates a reduction of the positive charges on the surface, it has been used for studying interactions with lipids in ciprofloxacin (81), AMP G15 (82) or oritavancin (83).

The results obtained also corroborated the presence of the peptide in the surface of the liposomes.

The changes in lipid fluidity as a result of the presence of the peptide in the lipid membrane were assessed with DPH and TMA-DPH fluorescence anisotropy measurements. The study with these probes allows to analyze the effect of the peptide properties along the full length of the membrane due to their different location and orientation in the membrane.

G1OLO-L_2OL_2 when interacting with liposomes in solution generates a significant shift towards higher melting transition temperature (Tm) detected with TMA-DPH probe, which charge is at the lipid–water interface. In the hydrophobic region (DPH) no significant changes can be seen. The information obtained is indicative of a rigidification of the headgroup region of the liposome due to the peptide interaction.

Nevertheless, it cannot be excluded that the rise in the Tm might be attributed to a charge screening selective effect due to the interaction of the peptide with the negatively charged POPG.

The fluorescence study showed that G1OLO-L_2OL_2 was not found in the more hydrophobic region of the liposome. Yet, it could not be confirmed if G1OLO-L_2OL_2 was able to pass through the membrane by forming pores.

As a matter of fact, peptides could be adsorbed onto the liposome membrane, or be partially absorbed in the lipid-water interface close to the upper portions of the fatty acyl chains,

Taking into account these results G1OLO-L_2OL_2 should be primarily located at the lipid–water interface influencing the fluidity of the phospholipids forming the liposome, like the studies done for StAsp-PSI (84) which seem to share its location in the liposomes with G1OLO-L_2OL_2. In addition, despite their structural differences, a similar behavior was observed for a series of three nine residue peptides that contain unnatural aminoacids in the primary sequence (85).

Moreover, we wanted to determine if the presence of the peptide induces lipid microdomains. As it has already been proven, the Laurdan probe (86), a polarity sensitive fluorescent molecule commonly located at the glycerol backbone of the bilayer with its lauric acid tail anchored to the phospholipid acyl chain region,

is especially useful in studies on lipid systems. It will be able to differentiate lipid microdomains of different lipid composition (87) by the presence of the G1OLO-L_2OL_2 in the liposomes.

The study using Laurdan of different peptides like Hylin a1 (88), peptide derivatives of *Galleria mellonella* cecropin D-like (89), LAH_4 (90) or Mettilin (91) has been done to evaluate the effect of these peptides on membrane binding, membrane disruption or model membranes.

For G1OLO-L_2OL_2 it was observed that GPex values visibly decrease as the excitation wave length increases at all temperatures studied, indicating a transition towards a more fluidic phase without the existence of lipid domains of different composition.

Our results of checkerboard experiments in order to explore the eventual synergistic action of G1OLO-L_2OL_2 and Imipenem in MDR (Imipenem-resistant) *P. aeruginosa* strains provide data demonstrating that no interaction occurred when both antimicrobials were acting together and simultaneously. This is consistent with the biophysical data, and confirms that in spite the major effect of G1OLO-L_2OL_2 is the alteration of OM, it is unable to open truly channels or discontinuities that may allow Imipenem to penetrate outer membrane of Imipenem-resistant *P. aeruginosa*.

In order to have direct evidence of how the peptide has an effect over the bilayer membrane, AFM microspopy was the next approach.

This technique has been used extensively in order to observe the effect of different drugs on model lipid membranes because it can follow the effect at a sub-nanometer length.

Firstly, a supported lipid bilayer (SLB) of POPE/POPG liposomes on mica surface is observed in order to evaluate it before its exposure to the peptide. Three different regions appear: small patches of dark regions that are uncovered mica surface, then a wide extended region with an orange coloration over the mica surface, and small yellowish domains protruding from the extended region.

Since Laurdan fluorescence did not show the existence of domains of different lipid composition, the nature of these could be attributed to: i) thermal domains of the same lipid composition, where lipids in the taller lipid domains were in a more rigid phase than the ones in the shorter lipid domains or, ii) domains of different lipid composition in a different lipid phase where the presence of the mica surface decreased the lateral diffusion of the lipids promoting the formation of segregated lipid domains of different lipid composition.

Experimental studies show that (92) the taller lipid domains are POPG enriched lipid domains while the more extended one is a POPE enriched domain.

Our observations show evidence that demonstrates the interaction of the G1OLO-L_2OL_2 at a surface level without formation of pores which correlates with the synergy results. This information suggests that G1OLO-L_2OL_2 at the studied concentration adsorbs or partially absorbs to the surface of the SLB.

G1OLO-L_2OL_2 injected *in situ* inside the AFM liquid cell, has the ability (at 0.0005 μM concentration) to induce fluidification of the lower lipid layer resulting in the mica surface to be fully covered being the dark patches banished. Moreover, after the injection of the peptide the high lipid domains protrude from the low lipid domain but with similar shape and after 35 minutes although the shape of the high lipid domains was quite conserved, it was possible to observe a degradation of the lipid domain which seem to lose their structure disorganizing from the center of the domain to the margins.

The peptide seems to have a dose dependent effect where higher concentrations induced the SLB erosion and solubilization, also observed with the fluorescence experiments at higher G1OLO-L_2OL_2 concentrations. Maybe this could be attributed to the destabilization/destruction of the liposomes.

It is relevant to observe the effect on model membranes but is even more essential to be able to study the effect *in vivo* over bacteria.

All evidence points to a surface effect of G1OLO-L_2OL_2 peptide on model lipid membranes, not only adsorbed or close to the surface, but associated/absorbed to some extent to the more hydrophilic region of the phospholipids. The presence of low concentration of peptide at the surface level could be responsible for osmotic stress that could facilitate the destabilization of the cell integrity at higher peptide concentrations

Exposing *P. aeruginosa* (27853) and *E. coli* ATCC (25922) strains for 4 hours to G1OLO-L_2OL_2 at different concentrations showed a more destructive effect in *P. aeruginosa* than in *E. coli.* Nevertheless, it is important to take into account that AFM only visualize the topography of the bacterial surface, not its viability; consequently, the possibility that despite the bactericidal effect may be similar, the destruction of the OM occurs in *P. aeruginosa* whereas in *E. coli* only a partial destabilization of the membrane happens, though in both cases enough to kill the bacterium.

The explanation of these different behaviors may be related with the differences in membrane lipid composition of both species, which should be studied in model membranes.

CONCLUSIONS

1. - We have designed a set of branched peptides to obtain constructs that we have called super-cationic peptide dendrimers (SCPDs) and tested them as potential antimicrobial agents.

2. - Determination of MIC and MBC values, revealed that almost all the compounds studied showed some antibacterial activity, with no significant differences between Gram-positive and Gram-negative microorganisms. Thus, these molecules appear to be broad-spectrum antibacterial compounds.

3. - The compound G1KLK-L_2KL_2, was selective for Gram-negative bacteria (narrow spectrum).

4. - Time–kill kinetics revealed considerable differences in the action, showing higher activity against Gram-negative bacteria; growth curves reinforced this conclusion.

5. - The cytotoxicity study in HepG2 and HEK293 cell lines revealed that the higher the toxicity the higher branched are the peptides.

6. - Despite their good antibacterial activity, the calculated Therapeutic Index make some molecules unsuitable as therapeutic agents.

7. - Compounds G1KLKL_2KL_2 and G1OLO-L_2OL_2 strong lead candidates for the future development of an antibacterial agent against Gram-negative bacteria.

8. - Biophysical experiments demonstrate a surface effect of G1OLO-L_2OL_2 on the model lipid membranes.

9. - Interaction of the molecule with the lipid bilayers involves not only the adsorption or a close proximity of the peptide to the surface, but to some extent also its association with, or absorption into, the more hydrophilic region of the phospholipids.

10. - The main objective of this thesis was to contribute to the perspective of the research of new synthetic antimicrobials that may contribute to minimize the devastating effects of antimicrobial resistance.

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