UNIVERSITAT DE BARCELONA

Discovery, evaluation and optimization of new antimicrobials against multidrug resistant bacteria

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“Discovery, evaluation and optimization of new antimicrobials against multidrug resistant bacteria”

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1 ABBREVIATIONS AND ACRONYMS

AMR	Antimicrobial resistance
AMP	Antimicrobial peptides
CARB-X	Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator
CDC	Centers for Disease Control
CEBA	tetra-ortho-chloro-azobenzene amino acid
ECDC	European Centre for Disease Prevention and Control
EPs	Efflux pumps
FDA	Food and Drug Administration
GARDP	Global Antibiotic Research and Development Partnership
GSK	GlaxoSmithKline
HGT	Horizontal gene transfer
ICU	Intensive care unit
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MDR	multidrug resistant
MIC	Minimum inhibitory concentration
MW	Molecular weight
PASTEUR	Pioneering Antimicrobial Subscriptions to End Up surging Resistance
PBPs	Penicillin binding proteins
PDR	Pandrug resistant
R&D	Research and development
rRNA	Ribosomal RNA
TEE	Transferable exclusivity extension
TEM	Transmission electron microscopy
TNF-α	Tumour necrosis factor α
VAP	Ventilator associated pneumonia
WHO	World Health Organization
XDR	Extensively drug resistant

2 LIST OF ARTICLES INCLUDED IN THIS THESIS

Thesis in compendium of publications format. This thesis consists of one main objective (nine specific objectives), two published articles and one manuscript:

Objective 1 (specific objectives 1, 2, 3 and 4): Manuscript 1

Title: Novel cathelicidin-based peptides with antibacterial activity against MDR Gram-negative pathogens.

Authors

Javier Moreno-Morales, Daniela Kalafatovic, Marko Babic, Salvador Guardiola, Clara Ballesté-Delpierre, Ernest Giralt and Jordi Vila.

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Objective 1 (specific objectives 5, 6 and 7): Paper 1

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Javier Moreno-Morales, Salvador Guardiola, Clara Ballesté-Delpierre, Ernest Giralt and Jordi Vila.

Journal, year, volume (issue): pages

Journal of Antimicrobial Chemotherapy, 2022, (77): 3077-3085.

Impact factor and quartile (year)

5.2, Q1 (2022).

Objective 1 (specific objectives 8 and 9): Paper 2

Title: Controlling Antibacterial Activity Exclusively with Visible Light: Introducing a Tetra-ortho-Chloro-Azobenzene Amino Acid.

Authors

Xavier Just-Baringo, Alejandro Yeste-Vázquez, **Javier Moreno-Morales**, Clara Ballesté-Delpierre, Jordi Vila and Ernest Giralt.

Journal, year, volume (issue): pages

Chemistry-A European Journal, 2021, (27): 12987-12991.

Impact factor and quartile (year)

5.02, Q2 (2021).

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3 THESIS SUMMARY IN SPANISH AND VALENCIAN

Título:

Descubrimiento, evaluación y optimización de nuevos antimicrobianos contra bacterias multirresistentes.

Introducción:

La resistencia antimicrobiana ocurre cuando se producen cambios en microorganismos que reducen la eficacia de los fármacos que usamos para tratar las infecciones que causan. En el caso de bacterias, se estima que la resistencia a los antibióticos estuvo directamente relacionada con 1.27 millones de muertes en todo el mundo tan solo durante 2019 Los antibióticos son un pilar fundamental de la medicina moderna, se usan desde intervenciones simples como tratamiento de heridas infectadas a más complejas como quimioterapia o trasplante de órganos. La aparición y prevalencia de patógenos bacterianos resistentes a los antibióticos pone en riesgo la salud global. Organismos internacionales como la Organización Mundial de la Salud han puesto en valor la necesidad de tomar medidas que puedan reducir la transmisión y la incidencia de infecciones bacterianas resistentes a antibióticos, siendo esencial la búsqueda de nuevos antibióticos efectivos contra bacterias multirresistentes como *Acinetobacter baumannii*, *Pseudomonas aeruginosa* y *Klebsiella pneumoniae*.

Los péptidos antimicrobianos son moléculas pertenecientes al sistema inmunitario que producen los organismos para protegerse en sus interacciones con otros. Están formados por cadenas policatiónicas de aminoácidos, compuestas por algunos residuos hidrofóbicos y cuyo carácter anfipático y naturaleza catiónica (y en algunos casos aniónica) es esencial en su efecto antimicrobiano. Estos péptidos son producidos por insectos, anfibios, plantas e incluso bacterias o mamíferos, donde por ejemplo las catelicidinas y defensinas, se producen en tejidos y membranas mucosas para combatir los microorganismos que las ocupan. Ejercen su acción antibacteriana mediante la disrupción de la membrana de estos patógenos, aunque también son capaces de interferir con funciones intracelulares y tener efecto regulatorio sobre la respuesta inmune. A pesar de que hay descritos cientos de péptidos

antimicrobianos, pocos se han desarrollado en aplicaciones médicas e industriales, por ejemplo, la polimixina B, vancomicina o nisina. Nuevas aplicaciones para el uso de péptidos antimicrobianos como su adición a geles o recubrimientos para dispositivos médicos como catéteres se han propuesto, pero su uso como terapia antimicrobiana se ha visto limitado históricamente debido a sus características de toxicidad y baja estabilidad. Estos péptidos presentan cierta toxicidad debido a su acción antimicrobiana sobre membranas y objetivos intracelulares, además de su complejo efecto inmunogénico. En cuanto a su estabilidad, normalmente sufren degradación mediada por proteasas además de tener baja penetración en la mucosa intestinal y un rápido metabolismo hepático y renal, lo cual limita su biodisponibilidad y tiempo de circulación. Por ello, el uso de los péptidos antimicrobianos de manera tópica ha sido priorizada ya que limita su toxicidad y elude su problemática estabilidad. De todas maneras, existen aplicaciones que permiten reducir la degradación y aumentar la disponibilidad como cambios en la formulación, encapsulación de péptidos o uso de aminoácidos D, así como reducir la toxicidad usando nanoportadores, nanogeles o ciclando péptidos para reducir su hemolisis. Debido a la efectividad de los péptidos antimicrobianos contra bacterias multirresistentes y gracias a estas nuevas estrategias para mejorar su toxicidad y estabilidad, los péptidos antimicrobianos se constituyen como una propuesta prometedora para el desarrollo de nuevos antibióticos.

Por otra parte, tras el uso de antibióticos se produce su liberación al medio ambiente, donde debido a su simple presencia ejercen una presión selectiva sobre los patógenos bacterianos que pueblan reservorios ambientales favoreciendo la aparición de resistencias. En este sentido, la posibilidad de controlar la actividad antibacteriana de los antibióticos supone una ventaja contra el desarrollo y emergencia de resistencia en el medio ambiente. Es a través de la fotofarmacología, es decir la regulación de la actividad de fármacos mediante su exposición a luz, que es posible crear agentes antimicrobianos fotoconmutables: debido a que la estructura de los fármacos es esencial en sus funciones e interacciones con las dianas terapéuticas, mediante el control de su estructura a través de su exposición a la luz, se puede modular la actividad

antimicrobiana de antibióticos que disponen de estructuras fotoconmutables. La estrategia propuesta es el diseño de péptidos antimicrobianos con estructuras fotoconmutables que toman una conformación activa (con efecto antibacteriano) al exponerse a luz roja, pudiendo ser entonces administrados como terapia antimicrobiana, y que, tras su acción y liberación al ambiente, al ser expuestos a luz natural, cambian a una conformación inactiva.

En esta tesis doctoral se han realizado proyectos de diseño, optimización y desarrollo de péptidos antimicrobianos, así como péptidos antimicrobianos con estructuras fotoconmutables.

Hipótesis:

Nuestra hipótesis es que el diseño de dos tipos de péptidos, lineales y cíclicos, produciría estructuras con buena actividad antibacteriana y baja toxicidad *in vitro*. Las estructuras producidas se pueden optimizar mediante cambios en su estructura que mejoren actividad y toxicidad; los péptidos presentarán actividad antimicrobiana al actuar sobre la membrana bacteriana. Finalmente, con el objetivo de estudiar una herramienta que aborde el problema de la liberación de antibióticos al ambiente y su impacto ecológico, tenemos la hipótesis que producir un péptido fotoconmutable (inactivo con luz natural ambiental), será útil para el control de la aparición de resistencias en el medio ambiente.

Objetivos:

Según las hipótesis descritas, los objetivos de esta tesis doctoral son:

Objetivo general:

La caracterización y optimización de los péptidos CAP-18 y PLP-3 (linear y bicíclico, respectivamente) según su actividad antibacteriana y su toxicidad y el desarrollo de una molécula fotoconmutable con actividad antimicrobiana.

Objetivos específicos:

1. Optimización de péptidos derivados de CAP-18 a través del análisis de su actividad antimicrobiana contra una colección de cepas multirresistentes de *A. baumannii* y *P. aeruginosa*.

2. Analizar si la actividad antimicrobiana de los derivados de CAP-18 es bactericida o bacteriostática contra cepas de *A. baumannii* y *P. aeruginosa*.

3. Definir el perfil biológico *in vitro* de los péptidos derivados de CAP-18 contra células eucariotas y eritrocitos humanos para obtener datos de toxicidad.

4. Visualizar los efectos de péptidos derivados de CAP-18 sobre la integridad de las células bacterianas de *A. baumannii* y *P. aeruginosa*.

5. Analizar la actividad antimicrobiana de PLP-3 contra un panel de cepas multirresistentes de *A. baumannii*, *K. pneumoniae* y *P. aeruginosa*.

6. Definir la toxicidad *in vitro* de PLP-3 contra células humanas y eritrocitos.

7. Investigar los efectos de permeabilización de membrana de PLP-3 sobre células de *A. baumannii* y *P. aeruginosa*.

8. Analizar la actividad antimicrobiana de análogos fotoconmutables de tirocidina A contra un panel de cepas multirresistentes Gram positivas y Gram negativas.

9. Obtener valores de toxicidad *in vitro* de análogos fotoconmutables de tirocidina A contra eritrocitos humanos.

Resultados y discusión:

En el primer estudio de este proyecto, Manuscrito 1, se sintetizaron derivados peptídicos de diferentes longitudes incluyendo distintos motivos de la estructura del péptido parental, para posteriormente analizar su actividad contra cepas de *A. baumannii* y *P. aeruginosa* multirresistentes. Los péptidos CAP-18, CAP-18_{31} y sus enantiómeros, D-CAP-18 y D-CAP-18_{31} fueron los péptidos derivados con mayor actividad antimicrobiana, con valores máximos de concentración mínima inhibitoria contra el 90% de cepas testadas (CMI_{90}) de 16 mg/L al ser testados contra un panel de cepas multirresistentes de *P. aeruginosa*, y de exactamente 0,5 mg/L contra un panel de cepas multirresistentes de *A. baumannii*, en este caso para cada uno de los péptidos testados. Siendo estos últimos datos indicativos de su potente actividad antimicrobiana.

Posteriormente se analizó la actividad antimicrobiana de estos derivados contra cepas de *A. baumannii* bajo concentraciones fisiológicas de albúmina humana con el objetivo de estudiar la posible unión inespecífica entre estos derivados catiónicos y una de las proteínas de mayor presencia en el plasma humano como indicativo de su estabilidad y disponibilidad terapéutica. Los valores de concentración mínima inhibitoria (CMI) de los péptidos se vieron ligeramente afectados, aunque se mantuvieron en concentraciones fácilmente alcanzables en la práctica clínica. A pesar de que estos péptidos se unan a albúmina, aún producen inhibición del crecimiento de cepas de *A. baumannii* a concentraciones peptídicas bajas.

Con el objetivo de describir la toxicidad de estos cuatro derivados de CAP-18, se estudió su capacidad hemolítica contra eritrocitos humanos así como la viabilidad de las líneas celulares HeLa y A549 bajo tratamiento peptídico individual. En lo que respecta a su capacidad hemolítica, todos los péptidos

tuvieron valores de hemólisis por debajo del 10% hasta concentraciones de 32 mg/L, indicando una baja toxicidad contra eritrocitos humanos a concentraciones clínicamente relevantes. Por otra parte, los enantiómeros D mostraron menor toxicidad contra la línea A549 que los L; para la línea celular HeLa los valores de toxicidad de estos péptidos fueron menores que contra A549, en este caso los isómeros D fueron más tóxicos contra las HeLa que sus enantiómeros L.

Ensayos de curvas de letalidad se llevaron a cabo para describir el efecto bactericida o bacteriostático de los péptidos contra cepas de *A. baumannii* y *P. aeruginosa*. Todos los derivados testados tienen efecto bactericida contra cepas de *A. baumannii* entre 2 y 4 horas de incubación. Sin embargo, contra *P. aeruginosa* se necesitaron incubaciones de entre 2 a 8 horas para conseguir efecto bactericida y los péptidos CAP-18_{31} y D-CAP-18_{31} no consiguieron mostrar efecto bactericida en los ensayos contra cepas de *P. aeruginosa.*

Finalmente se realizó la visualización de muestras de *A. baumannii* y *P. aeruginosa* tratadas con derivados de CAP-18 mediante Microscopía de Transmisión Electrónica. En las micrografías se pueden ver diferentes estadios de daño celular, con membranas elongadas, dañadas, vesículas vacías, huecos en citoplasma e incluso agregación de componentes intracelulares y células explotadas. Estos resultados nos sugieren que el mecanismo de acción de estos péptidos contra células bacterianas esté basado en una disrupción de la membrana bacteriana como ocurre normalmente con los péptidos antimicrobianos descritos en literatura como son las catelicidinas o defensinas.

En el Artículo 1 de esta tesis doctoral, se describe la optimización de un péptido cíclico basado en la protegrina PG-1. Tras el diseño del péptido PLP-3, se estudió su actividad antimicrobiana contra cepas multirresistentes de *A. baumannii*, *K. pneumoniae* y *P. aeruginosa*, obteniéndose valores de CMI_{90} de 2 mg/L contra *A. baumannii*, 8 mg/L contra *P. aeruginosa* y 16 mg/L contra *K. pneumoniae*.

Para caracterizar las posibles uniones inespecíficas debido a la naturaleza policatiónica de PLP-3, se realizaron ensayos de actividad contra cepas de *A. baumannii* a concentración fisiológica de albúmina humana, viéndose muy ligeramente aumentados los valores de CMI de PLP-3 contra las cepas testadas bajo estas condiciones (aumentando sólo 1 o 2 órdenes de magnitud). Por lo tanto, apenas se produce unión de PLP-3 para que se vea afectada la potencia de su actividad antimicrobiana contra cepas de *A. baumannii*.

Posteriormente se realizaron ensayos de toxicidad. Primero los ensayos de hemólisis contra eritrocitos humanos indicaron que se producen porcentajes de hemólisis bajos hasta concentraciones de 16 mg/L (la máxima CMI_{90} encontrada contra cepas de *K. pneumoniae*) lo cual sugiere niveles seguros a concentraciones clínicamente relevantes de PLP-3. Además, los ensayos de viabilidad celular realizados con células HeLa y A549 indicaron que solo se produce inhibición a la mayor concentración testada (225 mg/L), lo cual ofrece una gran ventana terapéutica considerando los bajos valores de CMI contra patógenos multirresistentes de PLP-3.

Por último y con el objetivo de entender mejor la relación entre PLP-3 y células bacterianas, se hicieron ensayos de permeabilización de membrana (mediante fluorescencia) contra cepas de *A. baumannii* y *P. aeruginosa* tratadas con PLP-3. PLP-3 produce una rápida permeabilización de membrana tan solo 5 minutos después de su inoculación contra *A. baumannii* 19606 llegando a una permeabilización del 55% a una concentración de 16 mg/L contra esta cepa; el efecto contra *P. aeruginosa* 27853 es similar, llegando a los 5 minutos a un 58% también a una concentración de 16 mg/L. Aunque es necesario realizar más ensayos para determinar el mecanismo de acción exacto de PLP-3, creemos razonable pensar a la vista de estos resultados que este mecanismo será similar al de PG-1 y estará relacionado con la disrupción de la membrana bacteriana.

PLP-3 es un péptido con potente actividad antimicrobiana contra patógenos multirresistentes Gram negativos, baja unión inespecífica a albúmina, un perfil hemolítico razonable a concentraciones inhibitorias del crecimiento bacteriano, baja citotoxicidad contra líneas celulares y posiblemente un mecanismo de acción sobre la membrana bacteriana.

Finalmente, el Artículo 2 de la tesis doctoral detalla el desarrollo y caracterización de antibióticos fotocontrolados. Basándonos en tirocidina A y mediante el desarrollo de una pieza fotoconmutable que puede ser utilizada en el diseño de péptidos antimicrobianos, un aminoácido tetra-orto-cloro-azobenceno, se sintetizaron hasta 13 análogos fotoconmutables lineales y cíclicos de esta molécula. Tras la caracterización de tiempos y ratios de cambio entre la forma activa e inactiva mediante luz roja de cada análogo, procedimos al estudio de su actividad antimicrobiana en este caso contra un panel multirresistente de cepas Gram positivas y Gram negativas. Si bien encontramos diferencias moderadas en la actividad antimicrobiana de los análogos entre su estado activo e inactivo, el análogo lineal 2 consiguió una CMI de 8 mg/L en estado activo, una diferencia de más de 3 órdenes de magnitud con respecto a su estado inactivo.

También se realizó la caracterización hemolítica de los péptidos fotoconmutables en sus conformaciones activas e inactivas, siendo en este caso los péptidos más hemolíticos de todos los estudios realizados en esta tesis. El análogo 17 con un valor de hemólisis IC_{50} de 173 mg/L en estado activo, es el menos hemolítico de todos los análogos testados. Este esfuerzo que hemos desarrollado para estudiar péptidos antimicrobianos fotocontrolados supone el comienzo de una estrategia novedosa y útil para el control de las resistencias bacterianas en el medio ambiente mediante la obtención de antibióticos que se inactivan con luz natural usando aminoácidos fotocontrolables que pueden ser aplicados a diversas estructuras peptídicas.

Conclusiones:

1. De todos los péptidos derivados de CAP-18 diseñados y testados, CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} fueron los derivados con mayor actividad antimicrobiana contra cepas multirresistentes de *A. baumannii* y *P. aeruginosa*.

2. El péptido PLP-3 tiene actividad antimicrobiana potente contra cepas multirresistentes de *P. aeruginosa*, *K. pneumoniae* y *A. baumannii*.

3. Acortar el extremo N-terminal de CAP-18 para generar derivados, especialmente en 5 o más aminoácidos, produce péptidos con menor actividad antimicrobiana en comparación con derivados más largos como CAP-18_{31} o D-CAP-18.

4. Concentraciones fisiológicas de albúmina humana afectan a la actividad antimicrobiana de péptidos derivados de CAP-18 aumentando sus valores de CMI. Este efecto puede ser consecuencia de unión inespecífica de los derivados a la albúmina, lo que limitaría la concentración de péptido libre y por tanto se necesitaría mayor dosis de péptidos para ejercer un efecto inhibitorio en patógenos bacterianos. En conjunto, las CMIs de derivados de CAP-18 contra *A. baumannii* en medio suplementado con albúmina humana se mantienen en rangos alcanzables, lo cual sugiere que se puedan alcanzar concentraciones favorables *in vivo* para el tratamiento contra este patógeno.

5. La actividad antimicrobiana de PLP-3 contra *A. baumannii* se mantiene incluso bajo concentraciones fisiológicas de albúmina humana, lo cual indica una baja unión de PLP-3 a esta molécula.

6. Los derivados CAP-18_{31} y D-CAP-18_{31} son los péptidos antimicrobianos menos hemolíticos y con la ventana terapéutica más ancha contra *A. baumannii* de todos los derivados de CAP-18 testados, sugiriendo que su actividad es selectiva contra las células bacterianas en lugar de contra eritrocitos humanos.

7. La hemólisis de eritrocitos humanos causada por PLP-3 es baja en aquellas concentraciones de este péptido que inhiben el crecimiento bacteriano.

8. A pesar de que hay diferencias moderadas en la toxicidad de cada uno de los péptidos derivados de CAP-18 contra células humanas, los valores varían entre los tipos celulares testados al establecer comparaciones entre los estereoisómeros. Los enantiómeros D sobre los péptidos derivados de CAP-18 tienen menor toxicidad contra células A549 mientras que los enantiómeros L CAP-18 y CAP-18_{31} son menos tóxicos que D-CAP-18 y D-CAP-18_{31} contra células HeLa.

9. Ensayos de citotoxicidad de PLP-3 sobre células A549 y HeLa muestran que este péptido no es tóxico contra ninguno de estos tipos celulares e incluso que la citotoxicidad se mantiene baja a concentraciones clínicamente relevantes de PLP-3 que inhiben el crecimiento bacteriano.

10. Los péptidos CAP-18, D-CAP-18, CAP-18_{31} y D-CAP-18_{31} tienen efecto bactericida contra cepas de *A. baumannii* en un rango de 2 a 4 horas de incubación. El efecto bactericida contra *P. aeruginosa* se produjo en tiempos de incubación mayores, de entre 2 a 8 horas, para CAP-18 y D-CAP-18 mientras que CAP-18_{31} y D-CAP-18_{31} mostraron efecto bactericida entre 2 y 8 horas solo contra la cepa *P. aeruginosa* R2 mientras que no fueron bactericidas contra la cepa *P. aeruginosa* 121110.

11. Los ensayos de Microscopía Electrónica de Transmisión realizados sobre péptidos derivados de CAP-18 contra cepas de *A. baumannii* y *P. aeruginosa* muestran alteraciones en las membranas bacterianas externas, vesículas y alteraciones en el citoplasma, agregación y rotura de membrana. Estos fenómenos son consecuentes con que los péptidos tengan la membrana bacteriana como diana e interactúen con la misma para ejercer su efecto, lo cual coincide con el mecanismo de acción descrito para los péptidos antimicrobianos.

12. Los ensayos de permeabilidad sugieren que PLP-3 produce una permeabilización rápida y dosis dependiente de *A. baumannii* y *P. aeruginosa*, lo cual indica la posibilidad de que su mecanismo de acción esté relacionado con la disrupción de la membrana.

13. Los péptidos PLP-3, CAP-18, D-CAP-18, CAP-18_{31} y D-CAP-18_{31} son potenciales candidatos para ser desarrollados clínicamente como antibióticos efectivos contra patógenos bacterianos resistentes a antibióticos.

14. El desarrollo de un nuevo aminoácido tetra-orto-cloro-azobenceno fotoconmutable que pueda ser manipulado mediante luz visible permite ganar el fotocontrol de la actividad antimicrobiana de péptidos.

15. Los análogos fotoconmutables lineales y cíclicos de tirocidina A muestran resultados de actividad antimicrobiana prometedores así como gran fotocontrol, en particular, el análogo lineal 2 contra las cepas de *A. baumannii* testadas.

16. Los análogos fotoconmutables de tirocidina A se vuelven rápidamente a un estado inactivo al ser expuestos a luz natural, lo cual supone una herramienta útil para controlar de manera efectiva la liberación de

antibióticos al medio ambiente, reduciendo la probabilidad de que surjan resistencias a antibióticos en microorganismos que habitan el medio.

17. La investigación sobre análogos fotoconmutables de tirocidina A ofrece una nueva estrategia en la lucha contra bacterias resistentes, al usar luz visible como un estímulo seguro para controlar la actividad antimicrobiana.

Títol:

Descobriment, avaluació i optimització de nous antimicrobians contra bacteris multiresistents.

Introducció:

La resistència antimicrobiana ocorre quan es produeixen canvis en microorganismes que redueixen l'eficàcia dels fàrmacs que usem per a tractar les infeccions que causen. En el cas de bacteris, s'estima que la resistència als antibiòtics va estar directament relacionada amb 1,27 milions de morts a tot el món tan sols durant 2019. Els antibiòtics són un pilar fonamental de la medicina moderna; s'usen en intervencions simples com a tractament de ferides infectades, fins a més complexes, com en quimioteràpia o trasplantament d'òrgans. L'aparició i prevalença de patògens bacterians resistents als antibiòtics posa en risc la salut global. Organismes internacionals com l'Organització Mundial de la Salut han posat en valor la necessitat de prendre mesures que puguen reduir la transmissió i la incidència d'infeccions bacterianes resistents a antibiòtics, sent essencial la cerca de nous antibiòtics efectius contra bacteris multiresistents com *Acinetobacter baumannii*, *Pseudomonas aeruginosa* i *Klebsiella pneumoniae*.

Els pèptids antimicrobians són molècules pertanyents al sistema immunitari que produeixen els organismes per a protegir-se en les seues interaccions amb uns altres. Estan formats per cadenes policatiòniques d'aminoàcids amb alguns residus hidrofòbics. El seu caràcter amfipàtic i naturalesa catiònica (i en alguns casos aniònica) és essencial en el seu efecte antimicrobià. Aquests pèptids són produïts per bacteris, plantes, insectes, amfibis, i fins i tot mamífers, on les catelicidines i defensines, es produeixen en teixits i membranes mucoses per a combatre els microorganismes que les ocupen. Exerceixen la seua acció antibacteriana mitjançant la disrupció de la membrana dels patògens, encara que també són capaços d'interferir amb funcions intracel·lulars i tindre efecte regulador sobre la resposta immune. Malgrat que hi ha centenars de pèptids antimicrobians descrits, pocs s'han desenvolupat en aplicacions mèdiques i industrials. Alguns exemples d'èxit són la polimixina B, la vancomicina o la nisina.

S'han proposat noves aplicacions per a l'ús de pèptids antimicrobians, com l' addició a gels o a recobriments per a dispositius mèdics , però el seu ús com a teràpia antimicrobiana s'ha vist limitat històricament a causa de la seua toxicitat i baixa estabilitat. Aquests pèptids presenten una certa toxicitat per l' acció antimicrobiana sobre membranes i objectius intracel·lulars, a més del seu complex efecte immunogènic. Pel que fa a la seua estabilitat, normalment pateixen degradació mediada per proteases, a banda de tindre baixa penetració en la mucosa intestinal i un ràpid metabolisme hepàtic i renal, la qual cosa limita la seua biodisponibilitat i temps de circulació. Per això, l'ús comú dels pèptids antimicrobians es fa de manera tòpica, ja que limita la seua toxicitat i eludeix la problemàtica estabilitat. De tota manera, existeixen aplicacions que permeten reduir la degradació i augmentar la disponibilitat, com canvis en la formulació, encapsulació de pèptids o ús d'aminoàcids D, així com reduir la toxicitat usant nanoportadors, nanogels o ciclant pèptids per a reduir l' hemòlisi. A causa de l'efectivitat dels pèptids antimicrobians contra bacteris multiresistents, i gràcies a les noves estratègies per a millorar la seua toxicitat i estabilitat, els pèptids antimicrobians es constitueixen com una proposta prometedora per al desenvolupament de nous antibiòtics.

D'altra banda, l' alliberament d'antibiòtics al medi ambient després del seu ús, exerceix una pressió selectiva sobre els patògens bacterians que poblen reservoris ambientals, afavorint l'aparició de resistències. En aquest sentit, la possibilitat de controlar l'activitat antibacteriana dels antibiòtics suposa un avantatge contra el desenvolupament i emergència de resistència en el medi ambient. A través de la fotofarmacologia es pot controlar l'activitat antimicrobiana d'antibiòtics que disposen d'estructures fotoconmutables mitjançant la regulació de la seua estructura, la qual és essencial en les seues funcions i interaccions amb les dianes terapèutiques, a través de la seua exposició a la llum. L'estratègia proposada és el disseny de pèptids antimicrobians amb estructures fotoconmutables que prenen una conformació activa (amb efecte antibacterià) en exposar-se a la llum roja, podent ser llavors administrats com a teràpia antimicrobiana, i després de la seua acció i alliberament a l'ambient, en ser exposats a llum natural, canvien a una conformació inactiva.

En aquesta tesi doctoral s'han realitzat projectes de disseny, optimització i desenvolupament de pèptids antimicrobians, així com pèptids antimicrobians amb estructures fotoconmutables.

Hipòtesi:

La nostra hipòtesi és que el disseny de dos tipus de pèptids, lineals i cíclics, pot produir estructures amb bona activitat antibacteriana i baixa toxicitat *in vitro*. Les estructures produïdes es poden optimitzar mitjançant canvis en la seua estructura que milloren activitat i toxicitat; els pèptids presentaran activitat antimicrobiana en actuar sobre la membrana bacteriana. Finalment, amb l'objectiu d'estudiar una eina que abordi el problema de l'alliberament d'antibiòtics a l'ambient i el seu impacte ecològic, tenim la hipòtesi de que produir un pèptid fotoconmutable (inactiu amb llum natural ambiental), serà útil per controlar l'aparició de resistències al medi ambient.

Objectius:

Segons les hipòtesis descrites, els objectius d'aquesta tesi doctoral són:

Objectiu general:

La caracterització i optimització dels pèptids CAP-18 i PLP-3 (lineal i bicíclic, respectivament) segons la seua activitat antibacteriana i la seua toxicitat, i el desenvolupament d'una molècula fotoconmutable amb activitat antimicrobiana.

Objectius específics:

1. Optimització de pèptids derivats de CAP-18 a través de l'anàlisi de la seua activitat antimicrobiana contra una col·lecció de soques multiresistents de *A. baumannii* i *P. aeruginosa*.

2. Analitzar si l'activitat antimicrobiana dels derivats de CAP-18 és bactericida o bacteriostàtica contra soques de *A. baumannii* i *P. aeruginosa*.

3. Definir el perfil biològic *in vitro* dels pèptids derivats de CAP-18 contra cèl·lules eucariotes i eritròcits humans per a obtindre dades de toxicitat.

4. Visualitzar els efectes de pèptids derivats de CAP-18 sobre la integritat de les cèl·lules bacterianes de *A. baumannii* i *P. aeruginosa*.

5. Analitzar l'activitat antimicrobiana de PLP-3 contra un panell de soques multiresistents de *A. baumannii*, K. peumoniae i *P. aeruginosa*.

6. Definir la toxicitat *in vitro* de PLP-3 contra cèl·lules humanes i eritròcits.

7. Investigar els efectes de permeabilització de membrana de PLP-3 sobre cèl·lules de *A. baumannii* i *P. aeruginosa*.

8. Analitzar l'activitat antimicrobiana d'anàlegs fotoconmutables de tirocidina A contra un panell de soques multiresistents Gram positives i Gram negatives.

9. Obtindre valors de toxicitat *in vitro* d'anàlegs fotoconmutables de tirocidina A contra eritròcits humans.

Resultats i discussió:

En el primer estudi d'aquest projecte, Manuscrit 1, es van sintetitzar derivats peptídics de diferents longituds incloent diferents motius de l'estructura del pèptid parental, per a posteriorment analitzar la seua activitat contra soques de *A. baumannii* i *P. aeruginosa* multiresistents. Els pèptids CAP-18, CAP-18$_{31}$ i els seus enantiòmers, D-CAP-18 i D-CAP-18$_{31}$ van ser els pèptids derivats amb major activitat antimicrobiana, amb valors màxims de concentració mínima inhibitòria contra el 90% de soques testades (CMI$_{90}$) de 16 mg/L contra un panell de soques multiresistents de *P. aeruginosa*, i d'exactament 0,5 mg/L contra un panell de soques multiresistents de *A. baumannii*, en aquest cas per a cadascun dels pèptids testats. Aquestes últimes dades són indicatives de la seua potent activitat antimicrobiana.

Posteriorment es va analitzar l'activitat antimicrobiana d'aquests derivats contra soques de *A. baumannii* sota concentracions fisiològiques d'albúmina humana amb l'objectiu d'estudiar la possible unió inespecífica entre aquests derivats catiònics i una de les proteïnes de major presència en el plasma humà com a indicatiu de la seua estabilitat i disponibilitat terapèutica. Els valors de concentració mínima inhibitòria (CMI) dels pèptids es van veure lleugerament afectats, encara que es van mantindre en concentracions fàcilment assolibles en la pràctica clínica. Malgrat que aquests pèptids s'uneixen a albúmina, encara produeixen inhibició del creixement de soques de *A. baumannii* a concentracions peptídiques baixes.

Amb l'objectiu de descriure la toxicitat d'aquests quatre derivats de CAP-18, es va estudiar la seua capacitat hemolítica contra eritròcits humans, així com la viabilitat de les línies cel·lulars HeLa i A549 sota tractament peptídic individual. Pel que fa a la seua capacitat hemolítica, tots els pèptids van tindre valors d'hemòlisi per davall del 10% fins a concentracions de 32 mg/L, indicant una baixa toxicitat contra eritròcits humans a concentracions clínicament rellevants. D'altra banda, els enantiòmers D van mostrar menys toxicitat contra la línia A549 que els L; per a la línia cel·lular HeLa els valors de toxicitat d'aquests pèptids van

ser menors que contra A549, en aquest cas els isòmers D van ser més tòxics contra les HeLa que els seus enantiòmers L.

Assajos de corbes de letalitat es van dur a terme per a descriure l'efecte bactericida o bacteriostàtic dels pèptids contra soques de *A. baumannii* i *P. aeruginosa*. Tots els derivats testats van tindre efecte bactericida contra soques de *A. baumannii* entre 2 i 4 hores d'incubació. No obstant això, contra *P. aeruginosa* es van necessitar incubacions d'entre 2 a 8 hores per a aconseguir efecte bactericida, i els pèptids CAP-18_{31} i D-CAP-18_{31} no van aconseguir mostrar efecte bactericida en els assajos contra soques de *P. aeruginosa*.

Finalment, es va realitzar la visualització de mostres de *A. baumannii* i *P. aeruginosa* tractades amb derivats de CAP-18 mitjançant Microscòpia de Transmissió Electrònica. En les micrografies es poden veure diferents estadis de mal cel·lular, amb membranes elongades, danyades, vesícules buides, buits en citoplasma i, fins i tot, agregació de components intracel·lulars i cèl·lules explotades. Aquests resultats suggereixen que el mecanisme d'acció d'aquests pèptids contra cèl·lules bacterianes pot estar basat en una disrupció de la membrana bacteriana com ocorre normalment amb els pèptids antimicrobians descrits en literatura com són les catelicidines o defensines.

En l'Article 1 d'aquesta tesi doctoral, es descriu l'optimització d'un pèptid cíclic basat en la protegrina PG-1. Després del disseny del pèptid PLP-3, es va estudiar la seua activitat antimicrobiana contra soques multiresistents de *A. baumannii*, *K. pneumoniae* i *P. aeruginosa*, obtenint-se valors de CMI_{90} de 2 mg/L contra *A. baumannii*, 8 mg/L contra *P. aeruginosa* i 16 mg/L contra *K. pneumoniae*.

Per a caracteritzar les possibles unions inespecífiques a causa de la naturalesa policatiónica de PLP-3, es van realitzar assajos d'activitat contra soques de *A. baumannii* a concentració fisiològica d'albúmina humana, i es van veure lleugerament augmentats els valors de CMI de PLP-3 contra les soques testades

sota aquestes condicions, augmentant només 1 o 2 ordres de magnitud. Per tant, gairebé no es produeix unió de PLP-3 afectada per a afectar la potència de la seua activitat antimicrobiana contra soques de *A. baumannii*.

Posteriorment, es van realitzar assajos de toxicitat. En primer lloc, els assajos d'hemòlisi contra eritròcits humans van indicar que es produeixen percentatges d'hemòlisi baixos fins a concentracions de 16 mg/L (la màxima CMI_{90} trobada contra soques de *K. pneumoniae*),la qual cosa suggereix nivells segurs a concentracions clínicament rellevants de PLP-3. A més, els assajos de viabilitat cel·lular realitzats amb cèl·lules HeLa i A549 van indicar que només es produeix inhibició a la major concentració testada (225 mg/L), la qual cosa ofereix una gran finestra terapèutica considerant els baixos valors de CMI contra patògens multiresistents de PLP-3.

Finalment, i amb l'objectiu d'entendre millor la relació entre PLP-3 i cèl·lules bacterianes, es van fer assajos de permeabilització de membrana mitjançant fluorescència contra soques de *A. baumannii* i *P. aeruginosa* tractades amb PLP-3. Aquest pèptid produeix una ràpida permeabilització de membrana tan sols 5 minuts després de la seua inoculació contra *A. baumannii* 19606 arribant a una permeabilització del 55% a una concentració de 16 mg/L contra aquesta soca; l'efecte contra *P. aeruginosa* 27853 és similar, arribant a un 58% als 5 minuts, també a una concentració de 16 mg/L. Encara que és necessari realitzar més assajos per a determinar el mecanisme d'acció exacte de PLP-3, creiem raonable pensar a la vista d'aquests resultats que aquest mecanisme serà similar al de PG-1 i estarà relacionat amb la disrupció de la membrana bacteriana.

PLP-3 és un pèptid amb potent activitat antimicrobiana contra patògens multiresistents Gram negatius, baixa unió inespecífica a albúmina, un perfil hemolític raonable a concentracions inhibitòries del creixement bacterià, baixa citotoxicitat contra línies cel·lulars i possiblement un mecanisme d'acció sobre la membrana bacteriana.

Finalment, l'Article 2 de la tesi doctoral detalla el desenvolupament i caracterització d'antibiòtics fotocontrolats. Basant-nos en tirocidina A i mitjançant el desenvolupament d'una peça fotoconmutable que pot ser utilitzada en el disseny de pèptids antimicrobians, un aminoàcid tetra-orto-clor-azobenzè, van ser sintetitzats fins a 13 anàlegs fotoconmutables lineals i cíclics d'aquesta molècula. Després de la caracterització de temps i ràtios de canvi entre la forma activa i inactiva mitjançant llum roja de cada anàleg, vam procedir a l'estudi de l' activitat antimicrobiana, contra un panell multiresistent de soques Gram positives i Gram negatives. Si bé trobem diferències moderades en l'activitat antimicrobiana dels anàlegs entre el seu estat actiu i inactiu, l'anàleg lineal 2 va aconseguir una CMI de 8 mg/L en estat actiu, una diferència de més de 3 ordres de magnitud respecte al seu estat inactiu.

També es va realitzar la caracterització hemolítica dels pèptids fotoconmutables en les seues conformacions actives i inactives, sent en aquest cas els pèptids més hemolítics de tots els estudis d'aquesta tesi. L'anàleg 17 amb un valor d'hemòlisi IC50 de 173 mg/L en estat actiu, és el menys hemolític de tots els anàlegs testats. Aquest esforç que hem dut a terme per a estudiar pèptids antimicrobians fotocontrolats suposa el començament d'una nova estratègia pel control de les resistències bacterianes en el medi ambient per mitjà de l'obtenció d'antibiòtics que s'inactiven amb llum natural usant aminoàcids fotocontrolables que poden ser aplicats a diverses estructures peptídiques.

Conclusions:

1. De tots els pèptids derivats de CAP-18 dissenyats i testats, CAP-18, D-CAP-18, CAP-18_{31} i D-CAP-18_{31} van ser els derivats amb major activitat antimicrobiana contra soques multiresistents de *A. baumannii* i *P. aeruginosa*.

2. El pèptid PLP-3 té activitat antimicrobiana potent contra soques multiresistents de *P. aeruginosa*, *K. pneumoniae* i *A. baumannii*.

3. Acurtar l'extrem N-terminal de CAP-18 per a generar derivats, especialment en 5 o més aminoàcids, produeix pèptids amb menor activitat antimicrobiana en comparació amb derivats més llargs com CAP-18_{31} o D-CAP-18.

4. Concentracions fisiològiques d'albúmina humana afecten l'activitat antimicrobiana de pèptids derivats de CAP-18 augmentant els seus valors de CMI. Aquest efecte pot ser conseqüència de la unió inespecífica dels derivats a l'albúmina, la qual cosa limitaria la concentració de pèptid lliure i, per tant, es necessitaria una major dosi de pèptids per a exercir un efecte inhibitori en patògens bacterians. En conjunt, les CMIs de derivats de CAP-18 contra *A. baumannii* al medi suplementat amb albúmina humana es mantenen en rangs assolibles, fent que es puguen aconseguir concentracions favorables *in vivo* per al tractament contra aquest patogen.

5. L'activitat antimicrobiana de PLP-3 contra *A. baumannii* es manté fins i tot sota concentracions fisiològiques d'albúmina humana, la qual cosa indica una baixa unió de PLP-3 a aquesta molècula.

6. Els derivats CAP-18_{31} i D-CAP-18_{31} són els pèptids antimicrobians menys hemolítics i amb la finestra terapèutica més ampla contra *A. baumannii* de tots els derivats de CAP-18 testats, suggerint que la seua activitat és selectiva contra les cèl·lules bacterianes, en lloc de contra eritròcits humans.

7. L'hemòlisi d'eritròcits humans causada per PLP-3 és baixa en aquelles concentracions d'aquest pèptid que inhibeixen el creixement bacterià.

8. Malgrat que hi ha diferències moderades en la toxicitat de cadascun dels pèptids derivats de CAP-18 contra cèl·lules humanes, els valors varien entre els tipus cel·lulars testats en establir comparacions entre els estereoisòmers. Els enantiòmers D sobre els pèptids derivats de CAP-18 tenen menys toxicitat contra cèl·lules A549, mentre que els enantiòmers L CAP-18 i CAP-18_{31} són menys tòxics que D-CAP-18 i D-CAP-18_{31} contra cèl·lules HeLa.

9. Assajos de citotoxicitat de PLP-3 sobre cèl·lules A549 i HeLa mostren que aquest pèptid no és tòxic contra cap d'aquests tipus cel·lulars, i fins i tot que la citotoxicitat es manté baixa a concentracions clínicament rellevants de PLP-3 que inhibeixen el creixement bacterià.

10. Els pèptids CAP-18, D-CAP-18, CAP-18_{31} i D-CAP-18_{31} tenen efecte bactericida contra soques de *A. baumannii* en un rang de 2 a 4 hores d'incubació. L'efecte bactericida contra *P. aeruginosa* es va produir en temps d'incubació majors, d'entre 2 a 8 hores, per a CAP-18 i D-CAP-18, mentre que CAP-18_{31} i D-CAP-18_{31} van mostrar efecte bactericida entre 2 i 8 hores sols contra la soca *P. aeruginosa* R2, però no van ser bactericides contra la soca *P. aeruginosa* 121110.

11. Els assajos de Microscòpia Electrònica de Transmissió realitzats sobre pèptids derivats de CAP-18 contra soques de *A. baumannii* i *P. aeruginosa* mostren alteracions en les membranes bacterianes externes, vesícules i alteracions en el citoplasma, agregació i trencament de membrana. Aquests fenòmens són conseqüència de que els pèptids tinguen la membrana bacteriana com a diana i interactuen amb la mateixa per a exercir el seu efecte, la qual cosa coincideix amb el mecanisme d'acció descrit dels pèptids antimicrobians.

12. Els assajos de permeabilitat suggereixen que PLP-3 produeix una permeabilització ràpida i dosi dependent de *A. baumannii* i *P. aeruginosa*, indicant la possibilitat de que el seu mecanisme d'acció es relacione amb la disrupció de la membrana.

13. Els pèptids PLP-3, CAP-18, D-CAP-18, CAP-18_{31} i D-CAP-18_{31} són potencials candidats per a ser desenvolupats clínicament com a antibiòtics efectius contra patògens bacterians resistents a antibiòtics.

14. El desenvolupament d'un nou aminoàcid tetra-orto-clor-azobenzè fotoconmutable que puga ser manipulat mitjançant llum visible permet guanyar el fotocontrol de l'activitat antimicrobiana del pèptid.

15. Els anàlegs fotoconmutables lineals i cíclics de tirocidina A mostren resultats d'activitat antimicrobiana prometedors, així com gran fotocontrol. En particular, l'anàleg lineal 2 contra les soques de *A. baumannii* testades.

16. Els anàlegs fotoconmutables de tirocidina A tornen ràpidament a un estat inactiu en ser exposats a llum natural, la qual cosa suposa una eina útil per a controlar de manera efectiva l'alliberament d'antibiòtics al medi ambient, reduint la probabilitat de que puguen sorgir resistències a antibiòtics en microorganismes que habiten el medi.

17. La investigació sobre anàlegs fotoconmutables de tirocidina A ofereix una nova estratègia en la lluita contra bacteris resistents, que utilitza llum visible com un estímul segur per a controlar l'activitat antimicrobiana.

4 INTRODUCTION

4.1 Bacterial antimicrobial resistance public health impact

Antimicrobial resistance (AMR) in bacteria can take place naturally when changes in these organisms reduce the efficacy of the drugs used to treat the infections they cause. International health organisms such as the World Health Organization (WHO) and Centers for Disease Control (CDC) consider AMR one of the current leading public health threats.(1–4)

Until recently, few publications had been published tracking the global burden and highlighting the importance of AMR. Studies have been published within the last two decades with a thorough analysis of the burden of bacterial infections and AMR stratified, for instance, by geographical regions, types of infections or patient age. On a late paper by the Antimicrobial Resistance Collaborators from 2022, it was estimated that 1.27 million deaths were directly caused by bacterial AMR in 2019 (an estimation obtained considering that recorded drug resistant infections would have been susceptible instead) when assessing recorded data of 23 bacterial pathogens and 88 pathogen-drug combinations worldwide.(1) On another study of global mortality linked to bacterial infections across eleven infectious syndromes, 7.7 million deaths associated with thirty-three bacterial pathogens (considering both resistant and susceptible) were estimated in 2019 and more than 6 million deaths were the result of three bacterial infectious syndromes included in the study.(5) From these articles, the AMR burden and age-standardised mortality rate of the bacterial pathogens studied were highest in Sub-Saharan Africa.(1,5) Finally, to illustrate the impact of bacterial AMR on a region comprising high income countries, yet on another recent 2022 study by the European Antimicrobial Resistance Collaborators similar in number of pathogens and pathogen-drug interaction data to that of the above-mentioned article,(1) 133.000 deaths in 2019 were estimated to be attributable to bacterial AMR in the WHO European Region.(6) With such a harmful global impact, affecting even in high income regions, the size of bacterial AMR data is comparable to that of other global diseases like malaria (at 409.000 deaths in 2019) or HIV/AIDS (with 690.000 related deaths in 2019) and would be the second cause of death globally after ischaemic heart disease comparing data from the 1990-2019 Global Burden of Diseases report.(1,7–9) Tailored policies to

prevent infections, improve access to antibiotics, target the most threatening pathogens and to develop novel antibiotics need to be put forward worldwide.

Antibiotics are one of the most transformative drugs in humanity: our life span has increased by almost two decades since their discovery and application in clinical practice.(10–12)

Antibiotics are essential in the practice of modern medicine. They are used in a wide range of interventions, from relatively minor procedures such as treatment of wound infections to complex surgeries, chemotherapy, caesarean sections, or organ transplants as both prophylaxis and treatment. However, antibiotics are far from being the perfect drug: their effectiveness diminishes with use since the very pathogens they target become resistant to their action. This situation plus antibiotic misuse and abuse accelerate the rate of AMR in bacteria, and are the reason why we are in constant need and search for novel molecules.(10,12) Aside from the loss of effectiveness associated with resistance, antibiotics use in clinical practice can be limited by other problems such as side effects and toxicity (fluoroquinolones and colistin), difficulties on dosing regimens and restrictions on use, particularly for children.(13–15)

4.1.1 Bacterial AMR and One Health

The effects of antibiotic consumption, accelerating bacterial AMR, does not only affect human health, but it also has wider consequences that can be better understood through a ‘One Health’ perspective. Many definitions of One Health can be found in literature, each with its own nuances based on the field of science defining it. The term has been quite relevant in the climate change and AMR fields, but more recently it has spread to the public because of the zoonotic nature of the COVID-19 pandemic. Having a unanimous definition of the term would enable proper development of global health policies, thus, the following has been proposed: One Health is a comprehensive approach that aims to balance and optimize the health of people, animals, and ecosystems, recognizing the

interdependence of human health, the health of domestic and wild animals, plants, and the environment.(16,17)

One Health supports working from different disciplines, sectors and communities with the ultimate objective of ensuring well-being, while respecting equity between sectors, maintaining a socioecological equilibrium, putting forward proper resource stewardship and promoting transdisciplinary collaboration.(16)

Some authors have proposed a distinction between the concepts of One Health and Global Health, to provide better understanding of the policies and relations that would help when specifically addressing the topic of bacterial AMR. Whereas One Health relates to the characteristics of geographically close ecosystems when spreading AMR (e.g., at a regional or national level), Global Health refers to the worldwide conditions that demand interaction of global actors such as countries or international organizations. Even if the two seem connected in the tools they propose, One Health interventions seem attainable compared to those of Global Health, since the latter needs deep international and intercultural efforts.(18,19)

Bacterial AMR can be understood through a One Health perspective by defining the entities involved in AMR and their relations (Figure 1). Antibiotic resistant bacteria are characterized by carrying resistance genes, which might produce antibiotic resistant infections in patients. The spread of these resistant bacteria not only depends on the relations stablished between the individuals harbouring them or just at the hospitals where patients infected with them are treated but also between different communities carrying these resistant bacteria and within hospitals treating these patients. It could be argued that hospitals that have significant rates of bacterial AMR could be classified as ‘antibiotic resistant hospitals’, and such view could be extended to environments, where polluted rivers or farms could constitute ‘resistant environments’ too. Bacterial AMR is therefore understood as the result of the relations between bacteria present in human, animals and their environments, where the resistance genes they harbour can be spread between them and give raise to resistant pathogens.(18)

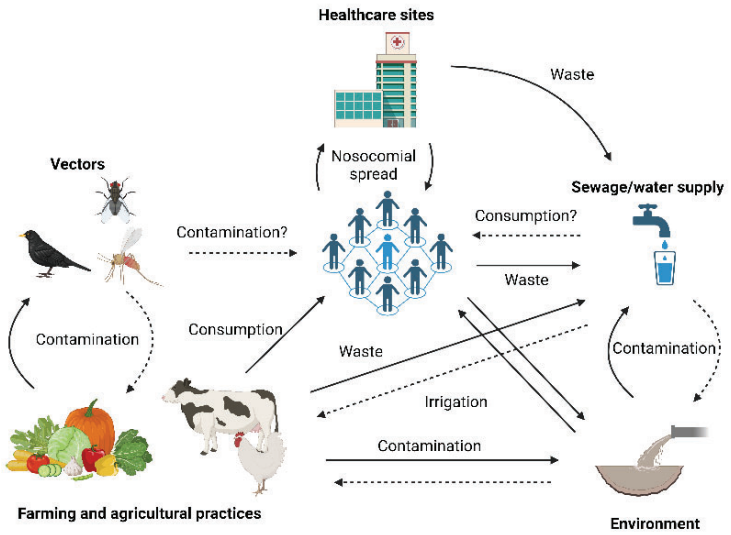

Figure 1. Entities involved in AMR through a One Health perspective. Dashed lines indicate possible transmission paths. Adapted from (20), created with BioRender.com.

As mentioned before, antimicrobial use is not only limited to humans but also to animals. Antimicrobial use in livestock has increased parallel with growing global demand for animal protein since its use facilitates the practice of intensive animal production.(21,22) Sub-therapeutic concentrations of antibiotics can increase significantly the production of poultry and livestock (e.g., less occurrence of subclinical disease, less morbidity and mortality, increase of growth rate, decrease of feeding costs, improvement of reproduction and improvement of meat quality among others).(23–29) In order to track progress of the policies and measures addressing AMR, data of antimicrobial use in animals needs to be accurate and its access granted. Although antimicrobial use varies between regions and countries, data registering global antimicrobial use helps understanding the current situation. A study from 2017 points that 73% of all antimicrobials used worldwide were exclusively used in animals, and a recent 2023 estimate shows that the global use of veterinary antimicrobial during 2020 was at 99,5 tonnes.(21,22) With such data, some countries have decided to limit or ban use of certain antimicrobials as growth promoters based on their relevance or use in human medicine. For instance, the European Union declared illegal to use antibiotics as growth promoters for livestock since 2006 and planned to ban imports of dairy and meat produced using antibiotic growth promoters from 2022.

However, use of some antibiotics such as fluoroquinolones (classified by WHO and the Food and Drug Administration, FDA, as critically important antimicrobials for medical use) is still licensed in the UK and many European countries: in 2021 around 146.4 tonnes of fluoroquinolones were sold in 31 European countries including UK (without including antibiotic tablets) mainly in food producing animals, while data of total fluoroquinolone sales in the USA in the same year was around 24,3 tonnes (even lower than Spain's value of 27.4 tonnes).(21,23,30–33)

The release of therapeutic antibiotics, e.g., as wastewater from both the animal industry and human consumption, into the environment contributes to AMR, since it can select for resistance, and poses a threat to water reuse. Moreover, although the exact impact that global warming has on antimicrobial resistance has yet to be quantified, it is believed to be exacerbating the interaction between microorganisms, vectors, animals, and humans by creating new environments and conditions for these interactions to take place.(18,34,35)

It seems we are just recently beginning to understand some of the political, economic, social and cultural components that affect how AMR spreads. Globalization, the increase in production of agricultural and animal products (enhanced by the use of antimicrobials in farming) and global warming definitively have had an impact on environmental health and the dissemination of AMR. (18,22,36–38) Regarding efforts put forward, a recent analysis of 114 countries' response to antimicrobial resistance has revealed that the global response falls far short of what is needed to effectively combat this threat. The authors of the analysis highlight the significant disparities in efforts between the countries studied and emphasize the need for better monitoring and evaluation of data in order to gain a comprehensive understanding of the impact of AMR at both a regional and international level. Improvement in key areas such as financing, accountability, the mechanisms of feedback, education, and ensuring equal access to antimicrobial treatments need to be done to enhance AMR response. These will ultimately lead to improved regional, international and overall collaborative policies, and more effective implementation strategies.(39)

4.1.2 History of antibiotic discovery and the importance of platforms for discovery

Using microbes that produce antibiotics for prevention of diseases and treatment of wounds dates to almost 2000 years ago; evidence has been found in China, Greece or Egypt pointing towards the utilization of mouldy bread cataplasms for treatment of wounds. However, the concept of chemotherapy and the development of what could be considered anti-infectives, are quite recent.(12)

Paul Ehrlich, a German physician and scientist from the late 19th century, studied staining with methylene blue, trypan red and atoxyl with the objective of using these dyes to find a substance that would specifically kill microbes while leaving human cells unharmed, what in his works he would define as a 'magic bullet'. After it was discovered that the arsenical compound atoxyl had activity against trypanosomes, Ehrlich engaged with a team of chemists lead by Alfred Bertheim to obtain a series of arsenical derivatives that could achieve sterile cultures from animals after a single dose. He also coined the term chemotherapy.(40,41)

In 1905, the zoologist Fritz Schaudinn, dermatologist Erich Hoffmann and bacteriologist Fred Neufeld (a disciple of Robert Koch) identified the etiological agent of syphilis, a spirochaete initially denominated '*Spirochaeta pallida*'. By then, syphilis affected a significant percentage of the population. This discovery promoted syphilis research during the coming years. Motivated by this research, Alfred Bertheim and his team synthesized arsphenamine in 1907, an arsenical which was then tested on spirochaetes by Ehrlich's lab assistants. This derivative was analysed and labelled as useless, so it was put aside. However, Sahashiro Hata, another assistant at Ehrlich's lab, retested arsphenamine and found that it was quite superior to any of the other drugs they had screened previously. Just after two years, by 1910, arsphenamine was marketed as Salvarsan. These experiences represent the first systematic screenings for drug discovery using a library of compounds. Ehrlich's team would then resume their efforts in analysing substances, which would result in the discovery of Neosalvarsan, which was more soluble than Salvarsan.(12,40,42,43)

Another scientist, Bayer's bacteriologist Gerhard Domagk, continued Ehrlich's work on sulpha drugs and discovered the sulphonamide prodrug Protonsil, for which he was awarded the Nobel Prize for Physiology or Medicine in 1939. Sulphonamides were the first effective, broad-spectrum antimicrobials in clinical use, but their usage was replaced after Florey, Chain and Heatley purified Alexander Fleming's penicillin developing it into a drug (by researching on mass producing processes) almost a decade after its discovery. Further efforts resulted in the discoveries of tyrocidine.(12,41,44–47)

4.1.2.1 Waksman and the first antibiotic discovery platform

Even though some antibiotics had been discovered, the field was lacking a proper platform for antibiotic discovery, but it was not for long. The first strategy designed to systematically discover antibiotics was set up by Selman Waksman in the late 1930s. The platform was based on screening streptomycetes from soil samples for antibiotic activity by coculturing them with a susceptible microorganism; antibiotic candidates were detected if growth inhibition zones were seen in the co-cultured plates. Although the methodology was similar to that of Alexander Fleming's discovery of penicillin in 1928, this screening platform set up for constant discovery of antibiotics.(11,12,45,48–50)

The first antibiotic discovered using Waksman's platform was streptomycin in 1943, which later earnt Waksman a Nobel prize in Physiology or Medicine (although this discovery was disputed by his Ph.D. student, Albert Schatz). This aminoglycoside was also the first effective drug against tuberculosis.(48,51) The pharmaceutical industry joined the 'antibiotic rush' through this platform with quite success. Such was the popularity of this method that pharmaceutical company workers were even encouraged to bring soil samples from their travels for later screening. Soon more antibiotics were discovered and developed into effective drugs: erythromycin, isolated in 1948 by a Filipino scientist of Eli Lilly, (52,53) vancomycin, found in a dirt sample sent by a missionary in Borneo in 1953 to a friend and scientist also at Eli Lilly,(54) or rifampicin, developed in Dow-Lepetit

Research Laboratories from a 1957 soil sample from the French Riviera and named after Rififi, a popular movie at that time (1955),(55,56) to name a few.

The antibiotic pipeline was quite prolific initially. New drugs or derivatives with better properties were constantly being brought into clinical practice with great success, as most pathogens were susceptible to them (apart from intrinsic resistances found in some species of microorganisms).(10,48) However, under selective pressure from antibiotic use, resistance was already detected even in these early days.

Rapid discovery and development of novel antibiotics had served as a way to fight emergence of resistant pathogens initially, but it could not hold long. (10,48,57) Research on active analogues of the newly discovered antibiotics, for which resistance already arose, was proposed as a solution. Although structural optimization was not an easy task, it resulted into some great synthetic antibiotics: research efforts upon the structure of nalidixic acid led to the discovery and production of fluoroquinolones.(11,48)

Waksman's platform had a flaw: constant rediscovery of known compounds. Due to this, the antibiotic discovery industry took a toll slowly after two decades of continuous exploitation. By the end of the 1960s the antibiotic pipeline began drying, marking the end of the 'Golden Era'.(12,48)

4.1.2.2 The Omics revolution; a promising platform for discovery and development of antibiotics

From the 1970s onwards, the industry turned into the aforementioned research on synthetic antibiotics. But by the 1990s, different biomedical disciplines began implementing target-focused strategies and research on antibiotics was soon to follow. A novel platform for antibiotic discovery was assembled around genomics, combinatorial chemistry, high throughput screening (HTS), rational drug design, the new technologies that facilitated production of target proteins in convenient models, such as *Escherichia coli,* and technologies to disclose 3D structure of

protein targets such as nuclear magnetic resonance and x-ray crystallography. Chemical libraries full of compounds were ready to be screened.(10,12,48)

Sequencing of *Haemophilus influenzae* was completed in 1995, and was soon followed by sequencing of other species (such as *Streptococcus pneumoniae*, *Staphylococcus aureus* or *E. coli*).(13) This changed how discovery of novel antibiotics was made, bacterial genes could be identified, analysed and regarded as new targets for antibiotics;(13,48) this genomic approach was as follows: highly conserved genes, or candidate genes, were identified by comparing the genomic information obtained from sequenced pathogens.(13) It was hypothesized that these genes ubiquity was due to them being essential for bacterial survival. The final *in silico* step of this approach was comparing these genes to the human genome, so that those candidates that had higher homology with human genes were discarded.(13) To test the essentiality hypothesis, targeted mutation of the candidate genes was produced in bacterial strains so that absence of growth indicated, but not guaranteed, that the gene was required for viability. Mutants were produced and tested in different species. Also, comparing the growth variation with the gene expression level was used as a double check of the importance of the candidate gene for survival.(13) This platform's other assumption, aside from the essentiality of the identified genes, was that the proteins encoded by the gene candidates would be potential targets for HTS of chemical libraries or rational drug design of antibiotics.(13) Because of the nature of this approach, it was easier to determine the mode of action of the future antibiotics acting against these candidate genes.(13,48)

Pharmaceutical companies embraced the target-based approach. For instance, GlaxoSmithKline (GSK) evaluated more than 300 genes based on their potential as targets for novel antibacterial agents. After the screening, 160 genes were considered essential (thus possible targets) and over 70 HTS comprising individual targets, macromolecular synthesis pathways and whole-cell screens were performed against GSK's collections of synthetic compounds in an effort to find novel antibiotics either with a broad-spectrum activity or active against Gram-positive bacteria.(12,13,58) Only 5 leads were obtained from GSK's HTS (from which the enoyl-acyl carrier protein reductase, FabI, inhibitor lead has recently

reached phase II trials)(59) and the success rate of the genomics approach GSK undertook was almost 5 folds lower than for targets from other therapeutic areas between 1995 and 2001.(13) After spending around 1 million USD per HTS campaign in a span of 6 years, GSK abandoned target screening through this approach and completely changed their antimicrobial research in 2002 since even the leads found in the program did not meet the required spectrum requirements the company had designed for these programmes. (13) Starting on 2002, GSK focused on novel chemical structures and lead optimization to improve molecules' antibacterial and developability properties.(13)

Although it was a successful strategy in other biomedical fields such as cancer research, the genomics and HTS approach was not so efficient in discovering new antibiotics.(10,13) Different weak points were revealed when this strategy started being applied:(13) i) target genes identified might only be present in the particular strains for which sequence comparisons are produced;(60) ii) different genes encoding for enzymes that share an essential role (such as the case with some Gram-positive bacteria harbouring double *murA* genes, where both Mur enzymes produced by the genes must be inhibited to elicit a lethal effect) are not easily identified due to the screening design; (61) iii) careful interpretation of genomic analysis should be done when selecting targets for broad-spectrum antibiotics due to differences in the essentiality of a gene among strains or species.(62) Lastly and aside from the previous weaknesses, the lack of penetration through the bacterial membranes of the discovered compounds that aimed at identified targets was a common flaw in this approach.(48,58)

With increasing availability of genomic datasets, the genomics plus HTS approach has been revisited and revamped, now including subtractive genomics, analysis of metabolic pathways and using bioinformatics to study genomic and proteomic data.(63) We currently sit in the dawn of the "big data era" for drug discovery, fusing novel genomic tools with proteomics and transcriptomics.(63) And even more recent informatic tools include machine learning approaches, based on training neural network on antibiotic candidate growth inhibition datasets to obtain *in silico* predictions of new molecules. Such is the case of

abaucin, a novel antibiotic with *in vitro* and *in vivo* activity against *Acinetobacter baumannii*, and a mechanism of action that disturbs lipoprotein traffic.(64)

4.1.2.3 **Relation between the rise of AMR and the stall of antimicrobial discovery platforms**

The lack of progress in producing novel compounds from the antimicrobial discovery platforms is alongside the rise of AMR. This is a natural process that has been exacerbated after continued uncareful antibiotic use and abuse over decades, favouring the growth of pathogens resistant to previously effective drugs. Today, finding infections caused by bacterial pathogens resistant to all clinically available antibiotics is not a rare sight. Research has elucidated the mechanisms through which resistance arises. Microorganisms can turn resistant both vertically, thanks to errors in their genetic material replication (which are then passed on to their offspring), and horizontally, since some individuals can acquire mobilized resistance genes on plasmids and/or transposons from others among the same or between different species (known as horizontal gene transfer, HGT).(10)

Although these mechanisms can take place simultaneously because of the nature of microorganisms, usually resistance is achieved mainly through one or the other depending on the characteristics of the antibiotic and the microorganism. For example, mutations can be the main cause of resistance for fluoroquinolones or for organisms such as *Mycobacterium tuberculosis* which does not often engage in HGT. Nonetheless, acquisition of resistance genes is arguably the most concerning mechanism of resistance for antibiotics because of the properties of mobile genetic elements. Plasmids and transposons often contain several resistance genes to different antibiotics. This means that if resistance is selected by the presence of just one antibiotic, it can translate into the acquisition of resistance to other antibiotics even if they are not used. Because of the mixture of pathogens in both the environment and healthcare settings, HGT is a great tool for exchanging resistance genes between microorganisms. This cumulative process has taken place during several decades, up to modern times when it is common to find pathogen isolates with

multidrug (MDR), extensively drug (XDR) and even pandrug (PDR) resistant phenotypes.(10,65)

The inability to produce new antibiotics by both approaches, from the Waksman' platform and library screening, the increasing costs of drug development and the low return on investment for antibiotics, made larger pharmaceutical companies to flee the field leaving a research gap which from then on was filled by smaller companies and academia.(10,11,66,67) All in all, past research efforts and the aforementioned platforms have led us to our current antibiotic arsenal (Figure 2).

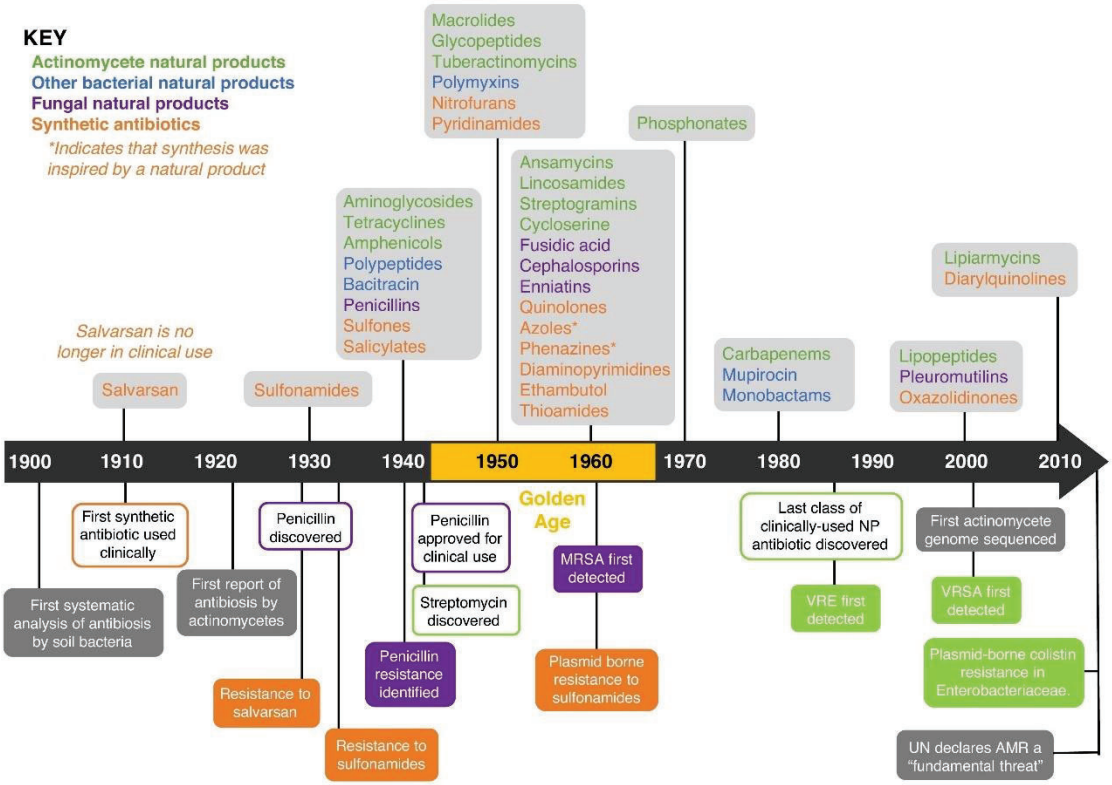

Figure 2. Timeline of clinical use of antibiotic classes. Dates of antibiotic discovery and antimicrobial resistance detection added at the bottom of the timeline. Adapted from (12).

Although around 200 essential proteins have been identified in bacteria, the amount of exploited antibiotic targets remains small. The most successful antibiotics attack conserved pathways: the ribosome, cell wall synthesis and DNA gyrase or topoisomerase (Figure 3).(48)

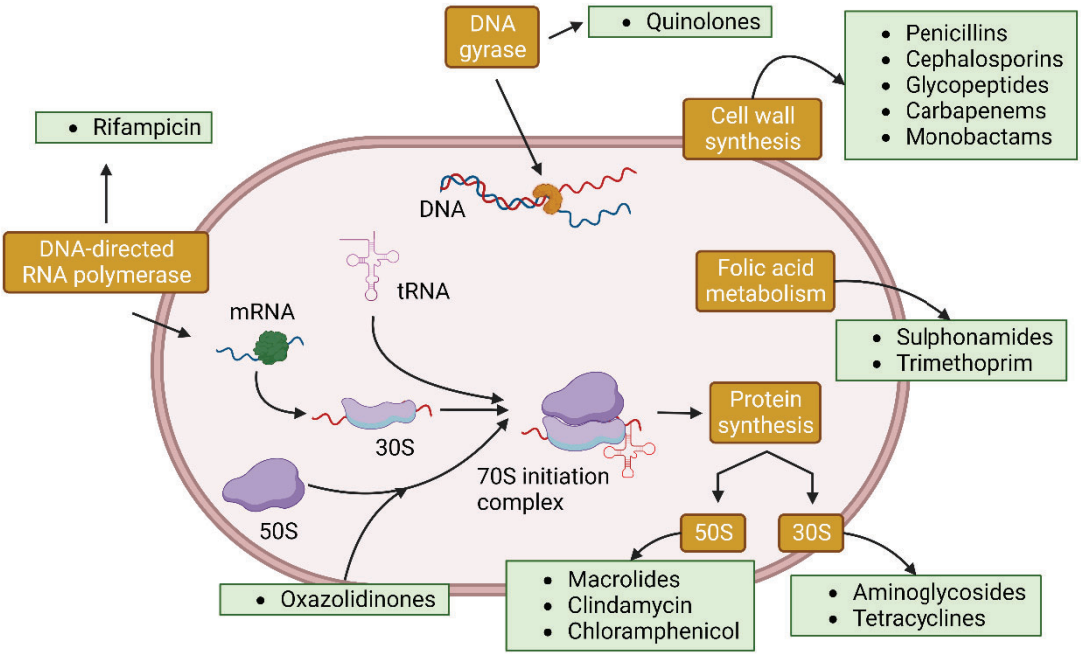

Figure 3. Depiction of conserved antibiotic targets and pathways (yellow) and some examples of antibiotics acting on them (green). Adapted from (48), created with BioRender.com.

Despite all the advances in research and discovery of antibiotics in history there are still many gaps in our understanding of them, which could be summarized in two points: i) identifying the targets of all the natural antibiotics that have already been discovered and ii) understanding the mechanisms through which antibiotics penetrate bacteria, and more precisely the rather selective walls of Gram-negative pathogens.(48)

4.2 Current status of antimicrobial R&D in the pharmaceutical industry

4.2.1 Antimicrobial research and development industry

Antibiotics are essential in our lives; their use is ubiquitous in medicinal and veterinary practices but also extremely important in agriculture. Unfortunately, it is a fact that most large pharmaceutical and biotechnological companies have

fled the antimicrobial research field based on commercial analysis and forecasting, because of the low return on investment on the antibiotic discovery area compared to other diseases.(10,11,13,66–73) Some pharmaceutical companies that have sadly halted their efforts on antibiotic research in the past years are AstraZeneca (2016), Novartis and Sanofi (both in 2018) or, on recent news and although yet to be officially addressed, Johnson & Johnson (2023).(67,68,74)

The traditional pharmaceutical business model stablishes economic rewards upon the total volume of drugs sold and is held responsible for the scarcity of antibiotic development by some experts. It seems that within this traditional model, the two main objectives of the antibiotic discovery industry, developing new antibiotics to tackle AMR while restricting use of antibiotics (ensuring proper stewardship), collide.(69) Antibiotic developers cannot recover R&D (Research and Development) costs since the very agents they have developed and marketed will have low sales in the first years after market entry, a situation further discouraging research on novel molecules.(66,75) The tensions of the current market are specially damaging against antibacterials due to their frequency of use and because of the existence of prior generations of these agents that have declining effectiveness but are still on use.(76)

The traditional pharmaceutical market is specially challenging for antimicrobial developing companies for three reasons: i) new antimicrobials agents need to compete with already available antibiotics which are relatively cheap (and usually generics), ii) antimicrobial treatment duration is usually short, especially when compared to chronic conditions, so volume of sales is low and iii) due to antimicrobial stewardship, use of novel agents is restricted to reduce the emergence of resistance.(66,75)

Reflecting on how the antibiotic market has behaved from the beginning of the XXI century, global revenue for branded antibiotics was at its highest in 2001 with a total of 21 billion USD. By 2021, this number had decreased significantly to 8 billion USD. While this trend was seen in markets of high-income regions such as Europe, it was particularly steep in the USA. Such reduction might be

explained due to a shift towards use of generic antibiotics because of their cheaper prices compared to branded antibiotics, plus the effect of antibiotic stewardship programmes. Generic antibiotics accounted for most of the global usage and spending in 2021.(77)

In contrast to the increase in global antibiotic use in the last 20 years, high-income countries such as the USA, UK, Italy, France, Germany and Spain saw a reduction. Far from equally augmenting with global antibiotic use, the cost per standard antibiotic unit slightly decreased in the last two decades; antibiotic access improved but this situation also raises concerns about their misuse and abuse. Another trend recorded was the decline in benefits of on-patent antibiotics over generics, in 1999 the benefit was over 1.06 USD but by 2021 it had decreased to 0.47 USD, and while the price for on-patent antibiotics got cheaper, it got more expensive for generics.(77)

A shift towards generics is clear and it might have hindered research on novel antibiotics because, theoretically, sales of branded antibiotics could fund research and development of novel and antibiotics.(77)

Regarding current costs, developing an antibiotic is extremely expensive. Estimations show that the cost is around 1.5 billion USD (using 2011 US prices for reference) while the average revenue from its sales per year would be close to 46 million USD.(69) Considering preclinical development as a single phase in the development process, it represents the greatest share of the capitalized development cost,(69) amounting around 45% of the total R&D budget in a single phase.(68) The reason why preclinical development is so expensive is because the failure rate at early stages of research is extremely high.. (66,68,69)

After ‘big pharma’ left the antibiotic discovery field, smaller companies stood up trying to bring new agents to the pipeline. Small and medium-sized enterprises such as Achaogen (plazomizin), Melinta (delafloxacin), Tetraphase (eravacycline) or Entasis (zoliflodacin) that had continued research on antibiotics, found that after discovery and FDA approval the results they obtained were either bankruptcy, merging under financial pressure or the redistribution of their assets

at low prices. Again, this was a consequence of low profit margins and failure at the market entry level (due to antimicrobial stewardship) that are characteristic of the traditional market model.(10,12,66,68,72,73,75,78)

4.2.2 Current antimicrobial pipeline

Despite efforts to create antibacterial drugs with distinct mechanisms of action, the majority of recently approved antibacterial agents fall into one of the already established categories: inhibition of cell envelope biogenesis, inhibition of protein synthesis, and disruption of DNA and or RNA homeostasis.(79). The 'latest' successful approach taken to develop novel agents has been the structural modification of already commercialized antibiotics in order to obtain derivatives with improved efficacy. While novel derivatives obtained by modification may improve efficacy temporarily, the basic interaction with the target often remains unchanged, and it is important to acknowledge that the underlying resistance mechanisms (e.g., enzyme mediated, target-based or through efflux pumps) can still persist in the environment.(13) The ideal scenario for the discovery of new antibacterials would be the development of drugs with novel pharmacophores, which are regions of a molecule responsible for a specific biological or pharmacological activity. Having two antibacterial drugs with the same mode of action but different pharmacophores is possible.(79)

International organisations have produced reports to guide development of novel antimicrobials and guide stewardship by raising awareness about the most important antimicrobials for medical use, for instance, the WHO priority pathogen list, or its analysis of the pipeline of antibacterial agents in clinical and preclinical development along with Pew Charitable Trusts' work. (80–82) With the release of the first priority pathogen list in 2017, WHO aimed to put focus on the development of novel antimicrobials effective against the most dangerous pathogens.(83) Since then, 12 new antibacterial drugs have been approved. Of those, only the boronate β-lactamase inhibitor vaborbactam (that is used in combination with meropenem for treatment of *Enterobacterales* infections) belonged to a new antibacterial class, understood as having a new bacterial drug-

related pharmacophore. It is also worth mentioning cefiderocol (commercialised as Fetroja): this β-lactamic drug (cephalosporin derivative) has an iron-chelating siderophore that allows its entrance into Gram-negative bacteria, and is active against carbapenem-resistant *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacterales*. The other 10 antibacterial agents approved had pharmacophores shared with previously described antibiotics, therefore belonging to existing classes.(79–81)

Out of the 76 antimicrobial agents in clinical trials by June 2021, 45 fell into 'traditional' (understood as direct acting antimicrobial candidates) and 31 into the 'non-traditional' (candidates with alternative modes of action) classes. Most of these agents were in early stages of clinical development, with 12 in phase III and only 4 having submitted a new drug application/market authorization application to a regulatory body (as to get authorization for human use for the first time in any region). Factors that affect general medicines in clinical trials, such as lack of efficacy or toxicity, are responsible for the concentration of antimicrobial agents in the early stages compared to the late ones, however, how traditional market characteristics relate to the nature of antibiotics and resistance could also hinder the advance of antimicrobial candidates to the later stages.(79,80)

Only 18 antimicrobial agents out of the 76 in clinical trials have new pharmacophores (Table 1); 9 target mycobacteria, 4 *Clostridioides difficile*, 2 *S. aureus*, 2 *E. coli, 2 N. gonorrhoeae* (one of these agents also acts against *E. coli* and was included before) and 1 targeting Gram-negative bacteria. And from the 18 with new pharmacophores, only 4 agents have modes of action not previously exploited by commercialized antibiotics: BVL-GSK098 (in phase I, acts inhibiting ethionamide-acquired resistance), GSK 2556286 (phase I, is suggested to be involved in *M. tuberculosis* cholesterol catabolism) and two agents targeting virulence, fluorothyazinon (tested in combination with cefepime in phase II, and acting inhibiting the Gram-negative type III secretion system) and GSK 3882347 (in phase I, acts as antagonist of the Gram-negative type 1 pilus adhesin FimH).(79,80)

Table 1. Antibacterials in clinical trials with new pharmacophores; pathogenic by colour: tuberculosis, non-tuberculosis mycobacteria, *S. aureus*, *C. difficile*, *N. gonorrhoeae*, Gram-negative bacteria and *E. coli*. Adapted from (79).

Target class	Target	Antibacterial name	Phase	Antibacterial class
Cell Wall	Decaprenylphosphoryl-β-d-ribose 2′-epimeraseDprE1	OPC-167832	II	3,4-dihydrocarbostyril
		TBA-7371	II	azaindole
		macozinone	II	benzothiazinone
		BTZ-043	I	benzothiazinone
	Enoyl-acyl carrier protein reductase FabI	afabicin	II	pyrido-enamaide
	Cell division protein FtsZ	TXA709	I	difluorobenzamide
	Unknown	ridinilazole	III	*bis*-benzimidazole
Protein synthesis	Leucyl-tRNA synthetase	GSK3036656	II	oxabolore
	Methionyl-tRNA synthetase	CRS3123	II	diaryldiamine
DNA	GyrB	zoliflodacin	III	spiropyrimidinetrione
	GyrA	gepotidacin	III	triazaacenaphthylene
	DNA polymerase IIIC	ibexapolstat	II	substituted guanine
	DNA groove binding	MGB-BP-3	II	distamycin
	GyrB and ParE	SPR720	II	ethyl urea benziamidazole
Respiratory complex	Complex bc_1	telacebec	II	imidazo[1,2-α]pyridine amide
Virulence	Type III secretion system	fluorothyazinone	I	thyazinone
	Fimbrin D-mannose specific adhesin antagonist	GSK3882347	I	new class
Miscellaneous	Reversing ethionamide-acquired resistance	BVL-GSK098	I	amido piperidine
	Possibly cholesterol catabolism	GSK2556286	I	new class

Comparing R&D fields provides better understanding of the current antimicrobial pipeline situation with respect to others. While in 2021 there were around 76 antimicrobials in clinical trials, just in 2020 there were around 1784 immune oncology therapeutics from phases I to III.(75,84,85) Another illustrative comparison, from all the new antibacterial agents marketed since the beginning of 2010, the total sales in the USA until June 2020 were 714.3 million USD for all agents (18 agents in total), which roughly is what a single oncology product makes in the same period.(73,86)

These data, showing few candidates in trials with respect to other fields, the concentration of agents in early stages and the low number of candidates with new modes of action and pharmacophores, suggest that although the current clinical pipeline of antimicrobials is getting stronger, it is not robust enough yet to provide agents to overcome the challenge of AMR. International efforts promoting an enhancing antibiotic R&D are needed with urgency now so that in the following decades, this scenario will have changed.(80)

4.3 Promoting antimicrobial R&D

4.3.1 International partnerships and organizations

Government and international organizations (Table 2) are proposing push and pull policies to promote antibiotic R&D: push measures usually provide funding to reduce the early development costs, while pull measures are directed towards late stages of development and try to make viable market demand for sponsors.(66,79)

Among these incentives and policies, programmes and public-private partnerships aimed to fight the public health problems associated with a lack of investment in antibiotics have been stablished in the last decade. In 2016, government agencies of the USA, Germany, UK and the Wellcome and Bill & Melinda Gates Foundation created the partnership Combating Antibiotic-

Resistant Bacteria Biopharmaceutical Accelerator, CARB-X. With an initial programme of 500 million USD, by 2022 data CARB-X had funded 92 early phase antimicrobial and diagnostics projects. Other examples of these efforts are Novo Holdings' finance package: Replenishing and Enabling the Pipeline for Anti-Infective Resistance (REPAIR) with over 165 million USD.(68,79)

As an alternative from partnership and private or commercial efforts, non-profit approaches have also been developed. The Global Antibiotic Research and Development Partnership, GARDP, was funded in 2016 by the WHO and the Drugs for Neglected Diseases initiative. With a focus on addressing public health needs that WHO presented through the priority pathogen list and other guidelines, GARDP's purpose is to deliver 5 new or improved treatments for: neonatal and child sepsis (also improving and optimizing formulation of antibiotics), sexually transmitted infections and drug resistant infections. It has collaborated with companies developing a novel, first in class treatment against *N. gonorrhoeae* (zoliflodacin, by Entasis), cefepime-taniborbactam (against complicated urinary tract infections). GARPD's efforts include providing access to scientific knowledge, resources and connections through REVIVE, a website for seminars and other resources, so that this and the coming scientific generations will have access to them.(79,87,88)

The industry also starts to show some signs of responding to the economic challenges of antibiotics. The International Federation of Pharmaceutical Manufacturers and Associations launched in 2020 the AMR Action Fund. This initiative is composed of 24 companies. It aims to support late phase development of 2 to 4 new antibiotic candidates by investing in small biotech companies and providing industry expertise to aid in clinical development, so these candidates can be brought to markets by 2030; the AMR Action Fund had committed around 1 billion USD upon its creation and is currently the world's largest public-private partnership that invests on new antimicrobial therapeutics.(68,79,89)

Table 2. International organizations and partnerships involved in promoting antibiotic R&D.

Partnership/Organization	Founders	General focus	Budget
CARB-X	USA-Germany-UK governments, the Wellcome and the Bill & Melinda Gates Foundation	Supporting early phase antimicrobial and diagnostics projects	500 million USD
REPAIR	Novo Holdings	Investing in companies in early stages of antimicrobial R&D against resistant organisms	165 million USD
GARDP	WHO and Drugs for Neglected Diseases Initiative	Addressing public health needs from WHO's priority pathogen list through late-stage antibiotic development, granting global access to antibiotics and focusing on drug development projects targeting priority pathogens.	104.7 million EUR(90)
AMR Action Fund	International Federation of Pharmaceutical Manufacturers and Associations	Bringing 2-4 new antibiotic candidates to the market	1 billion USD

4.3.2 Delinked strategies

4.3.2.1 Antibiotic subscription model

Aside from public and private efforts, economic experts in the antimicrobial and pharmaceutical field have proposed a revamp of the antibiotic market model with a focus on adjusting of the commercialization system to the characteristics of antimicrobial resistance and the value of antimicrobials. This is an insurance market model, a change that delinks revenue from volume of use (eliminating marketing), that theoretically would ease stewardship in doing so, and that is

ultimately directed to promoting antibiotic R&D since it rewards companies' innovation through a series of incentives and obligations, thus, building back a healthy pipeline. The payments to antibiotic manufacturing and developing companies would be based on the added value that novel agents they produce to the healthcare system and their future discretional use, with payments understood as investments to cover the economic costs of the expensive preclinical research. (66,68,69,75,76)

The UK has become the first country to implement such a delinked antibacterial subscription system (also popularized as the Netflix model), the USA follows with legislation such as the 'Pioneering Antimicrobial Subscriptions to End Up surging Resistance' (PASTEUR) Act and in Europe, Sweden, Germany and France also plan to develop reimbursement systems. (66,68,73,75,76)

Between 2019 and 2020, the UK government planned a subscription program to antibiotics: initially two contracts would be assigned to pharmaceutical companies, providing economic coverage of the early and expensive stages of R&D. Also, a point-based guidance stablishing the basics on which characteristics qualify drugs for subscriptions was designed to guide antibiotic developing companies on what requirements are needed be met to receive these contracts. These UK contracts (at 10 million pounds/year per agent) started in July 2022: one for an existing antibacterial, Pfizer's ceftazidime-avibactam, and one for a new-to-market agent, Shionogi's cefiderocol. Adding other two to three agents per year was planned. Criticism arose since the contracts do not directly act on global stewardship and access but nonetheless, these contracts set the foundation for future agreements and legislation. Other critics are concerned about the subscription program not effectively encouraging innovation and argue that approval through non-inferiority trial assessments is not as valid. These last comments have also been addressed by scientific and regulating experts: although the first two agents signed under contract on the UK have structures close to beta-lactams, chemical structure novelty is not the only factor that affects clinical relevance and both agents represent effective therapeutics that meet the high standards set by the UK analysis; regarding approval based on non-inferiority trials, these critics are considered to oversee the complexity of

performing superiority trials in infectious diseases because of challenges in patient recruitment plus the ethical implications of comparing a new drug to an existing agent to which the pathogen is resistant (reason why most studies of antibacterials are non-inferiority trials).(67,68,70,75,76,78,91,92)

If governments applying antimicrobial subscription models want to be successful, transparency in their pull and push incentives needs to be the pillar of their model and legislation, so that the public health necessities will be met while ensuring access to antibiotics and stewardship. Otherwise, the antimicrobial pipeline will just suffer political consequences on top of the problems it already has.(66,76)

4.3.2.2 Transferable exclusivity extension vouchers

As part of the measures to encourage antibiotic R&D, during March 2023, the European Commission is considering the use of transferable exclusivity extension vouchers (TEEs). Antimicrobial developing companies that successfully launch new antibiotics would be given TEEs that grant the patent extension of any medication (not necessarily related, e.g., immune-oncology therapeutics) in European countries for up to 12 months. Developers could also sell this voucher to other pharmaceutical companies for them to use. Due to the easiness of implementing TEEs compared to other measures, European commission policy makers deem them a viable tool. (71,93)

However, European scientists and organizations have raised concerns about TEEs; worries about the pharmaceutical companies applying the vouchers to their most expensive and high selling medicines to increase their benefits have been expressed. On the other hand, the pharmaceutical industry has publicly welcomed this idea, stating the advantages of TEEs: no upfront government funding would be needed, vouchers are deemed an attractive economic incentive and since the vouchers can be sold, they argue their use could potentially benefit all pharmaceutical companies independently of their size.(71,93,94)

The estimated consequences of TEEs and their critics are many. Total cost of TEEs upon the healthcare systems of European countries is difficult to estimate

yet but is expected to be substantial, considering the sheer size of the pharmaceutical market. As a comparison, an economic analysis by Rome & Kesselheim in 2020 of the total cost of possible TEEs based on FDA approved antimicrobials from 2007 to 2016, matched to the highest revenue generic entry 4 years after antimicrobial approval, estimated a total median excess spending by using TEEs of 4.5 billion USD over 10 years; finally the authors of this analysis also agree with European critics stating that use of TEEs could also delay an improvement in medicine access that comes after generic market entry. The fact that TEEs could turn into a significant incentive is not completely clear: compared with other market entry rewards that have already been applied (such as advanced market commitments for pneumococcal conjugate vaccines) there is doubt about the extent to which TEEs will promote antibiotic R&D. TEEs do not secure antibiotic access since there developers could just commercialize new antibiotics in member states where benefits would be expected. Compared to the subscription market model, TEEs still would fall into the traditional market failures, not properly stablishing the clinical value of antibiotics to the size of the reward received but rather to the value of the high selling drug TEEs would be used upon.(71,93,94)

4.3.3 Current challenges of the antimicrobial R&D field

Although economic challenges are viewed by many as principal in the antibiotic R&D field,(66) they are not the only challenges faced by scientists and developers. Due to the lack of new opportunities, there is a generation and knowledge gap between the senior scientists that have worked in the sector for over the past 4 decades and young scientists that are discouraged from a career in infectious diseases research because of a lack of incentives.(10)

Requirements for individual research experience are stringent and getting new professionals from related research fields (e.g., biochemistry or biomedicine) is difficult. It is widely known that funding mechanisms available for researchers are cumbersome and bureaucratic and the antibiotic discovery and development field

is not an exception to this situation. On top of this, translation between academia and industry is feeble, delaying novel advances and discoveries in the sector.(95)

‘Antibiotic researchers’ are scarce, in a 2017 estimate around 500 professionals (not counting graduate students) were identified in over 50 institutions globally; for comparison, there were 364 professionals (excluding Ph.D. students) working just at the Spanish National Cancer Research Centre, CNIO, in 2021 in Madrid. Top talent is attracted to other fields because of few career opportunities and the state of the industry. Efforts are needed to create a robust research ecosystem with diverse science professionals that will allow present and coming generation of young scientists to ingrain and flourish.(95,96)

Global antibiotic development goals are being stablished with the publication of international guidelines, reports on global progress of novel antimicrobials, surveillance of resistant pathogens and emerging resistance mechanisms. It is still early to see robust results, the efforts produced in the last decades will have a higher impact in the coming years.(95,97)

There are different challenges ahead on our road to fight bacterial AMR, some easier to define and achieve while others require of synergy between research, politics and economic. First of all, there is the challenge of discovering novel effective antibiotics against present and emerging MDR pathogens, with low toxicity and appropriate pharmacokinetic and pharmacodynamic parameters (having a novel mode of action would be the best scenario possible). Following this, finding new antibiotics for use in neonates and children since dosing, formulations and drug regimens need to be quite precise for their treatment, and guidelines or evidence-based treatments for paediatric infections are scarce. Clinical trials with new antibiotics also represent a challenge: antibiotic manufacturing, proper trial design and achieving enough candidates to represent the target population. Since old antibiotics are still relevant as treatments in everyday global clinical practice, there is the challenge of global access to antimicrobials, where antibiotic availability, supply and pricing are key concerns that still restrict access for already treatable or common bacterial infections, these elements affect the rise of resistant bacteria and the outcome of patients. On top

of this situation there is a need for strong and sustained political and governmental support and commitment that allows scientists to overcome economic barriers in their research. Finally, there is the case of market models, also related to policy and economy; market dynamics need to be improved focusing on enhancing the development and approval of innovative and effective antibacterial treatments.(66,79)

4.4 Bacterial pathogens

Bacterial mechanisms of resistance to antimicrobials are closely related to their structures. Therefore, it is important to outline the structural differences and characteristics bacteria have in order to understand modern approaches for discovery and development of antimicrobials.

One element that differentiates bacteria is their cell wall. This structure is key in how antibiotic and, specifically as it will be later explained, antimicrobial peptides interact with pathogens since it is the barrier that separates bacteria from their environment. The Danish bacteriologist Hans Christian Gram developed a staining procedure to differentiate between Gram-positive and Gram-negative bacteria (Figure 4).(98,99)

Gram-negative bacteria are enveloped by a double membrane that isolates and protects the cell. In these double membranes, the inner membrane is rich in phospholipids while the outer membrane has fewer phospholipids mostly located in the innermost leaflet, being rich instead in glycolipids such as the lipopolysaccharide (LPS).(99–101) Between the inner and outer membranes sits a thin and rigid layer of a polymer of N-acetylglucosamine and N-acetylmuramic acid, the peptidoglycan. With the outer membrane, the peptidoglycan layer helps stabilization and prevents cell lysis buffering the high pressure found inside the bacterial cell. Under the peptidoglycan layer and before the inner membrane, the periplasm holds many enzymes responsible of cell wall maintenance.(98,99) The phosphate groups from LPS provide Gram-negative bacteria an overall negative charge.(99,102)

Gram-positive bacteria lack a double membrane but have a thick outer layer of peptidoglycan around a single, and inner, membrane. The thick layer is plagued by anionic glycopolymers involved in the stability of the membrane, its function and many intercellular interactions. The most notable of these polymers are wall teichoic acid and lipoteichoic acid (LTA), providing Gram-positive bacteria a negative charge to their surface as Gram-negative bacteria have.(99,100,102–104)

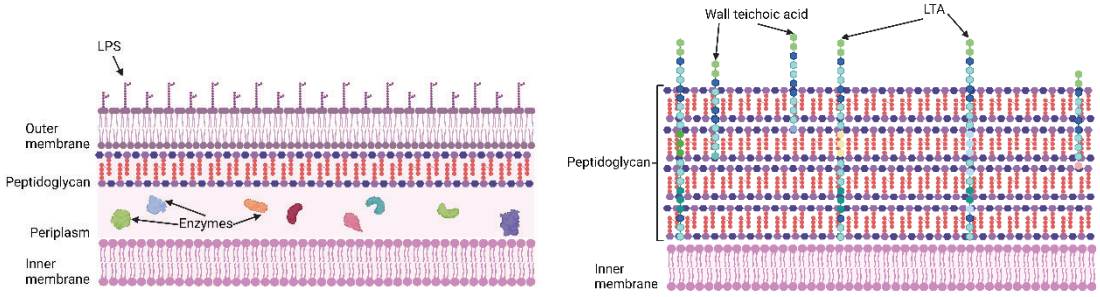

Figure 4. Left, Gram-negative and right, Gram-positive bacterial cell walls. Adapted from (99), created with BioRender.com.

4.4.1 Antimicrobial resistance mechanisms

Elucidating the molecular mechanisms through which bacterial AMR occurs as well as analysing which of these mechanisms are most prevalent or relevant are key not only for appropriate antibiotic prescription but also to guide design of novel treatments or agents.

Antibiotic resistance mechanisms are usually classified into intrinsic or acquired. However, some authors propose adaptive mechanisms to this classification.(105) Intrinsic resistance is regarded as the result of the inherent characteristics or mechanisms of microorganisms countering the action of antibiotics. Some examples that better illustrate intrinsic resistance are the low permeable outer membranes of *P. aeruginosa* or *A. baumannii* or most efflux pumps systems.(105,106)

In the case of acquired resistance, an initially susceptible microorganism or species gains new genetic material (e.g., plasmids, integrons, transposons or naked DNA) or suffers mutations, that in either case provide new capacities for survival in the presence of antibiotics. In comparison with acquired mechanisms, intrinsic mechanisms rely on bacteria making use of genes they already had to overcome antibiotic presence.(105,106)

Lastly, adaptive resistance are the mechanisms through which a microorganism gains a temporary increase in tolerance to an antibiotic as a result of gene or protein expression alterations due to exposure to environmental triggers (e.g., stress, presence of nutrients or subinhibitory concentrations of antibiotics). Contrary to intrinsic or acquired, adaptive resistance mechanisms are unstable and cannot be transmitted vertically, usually reverting upon removal of the trigger or stress.(105)

Clinically relevant mechanisms of bacterial resistance have been identified and are now generally better understood (Figure 5). (48,106)

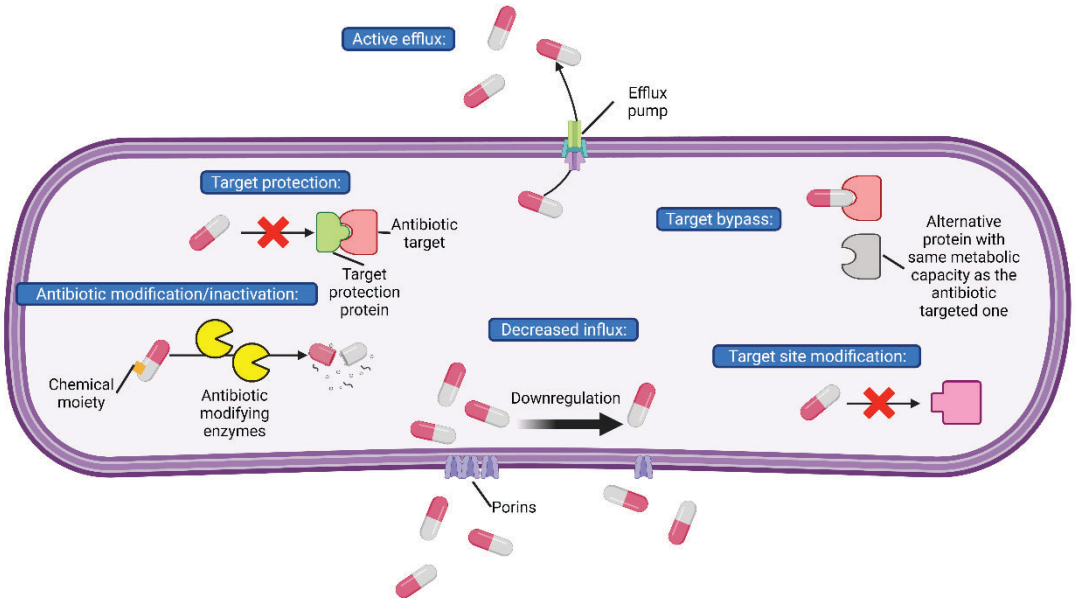

Figure 5. Clinically relevant mechanisms of antibiotic resistance (blue) depicted in a Gram-negative schematic model. Adapted from (106), created with BioRender.com.

New technologies have provided a better understanding of complex and even multi-layered molecular mechanisms involved in bacterial AMR. These advances have helped identifying the underpinning role of efflux systems supporting other resistance mechanisms or even in detailing these systems so that inhibitors can be developed.
(106) A brief review of each of these mechanisms is detailed in the next sections.

4.4.1.1 Selective permeability

Structural differences between Gram-negative and positive bacteria explain why some antibiotics might be effective against one or the other, for instance, some antibiotics need to cross the bacterial envelope to reach their target and exert their activity.(106)

The double membranes of Gram-negative bacteria can turn them intrinsically resistant to some or many antibiotics; this is why there is a major challenge when developing membrane-crossing novel antimicrobials against Gram-negative pathogens. Modifications in the structures of bacterial envelopes relate to AMR emergence because they can have an effect upon the penetration of antimicrobials into bacteria cells.(106)

The outer membrane of Gram-negative bacteria is complex and selective, providing protection while allowing passing of nutrients. One of the most notable characteristics, related to resistance, of this membrane, is that it is decorated with porins: β-barrel protein channels that present different functions and structures. Generally speaking, porins in Gram-negative bacteria allow influx of hydrophilic compounds of less than 600 Da, which makes some antibiotics such as vancomycin or daptomycin excluded due to their molecular weight. This generalization seems to be based upon the average molecular weight of Gram-negative antibacterials. Compounds like azithromycin (at 749 Da) or polymyxin B1 (at 1203 Da) are exceptions to this assumption, but both belong to classes with special permeability properties that promote their penetration.(105,107,108) It was previously thought that porins were substrate specific but this idea has

been refuted in recent studies, suggesting diffusion occurs in a passive and spontaneous way.(109) Logically and as mentioned earlier, modification to porin's structures can lead to resistance, for instance, an double amino acid insertion of Gly115-Asp116 into the structure of the porin OmpK36 tightens its pore and represents one of the mechanisms through which carbapenem resistance occurs in *Klebsiella pneumoniae*.(110) Some pathogens like *P. aeruginosa* or *A. baumannii* generally lack large channel porins. In turn, they are equipped with an array of specific porins that make their membranes quite impermeable (particularly against hydrophilic molecules) only allowing passing of molecules up to 200 Da.(106,111–113)

Even though the role of porins is essential in controlling membrane selectivity and resistance in bacteria, e.g., loss of OprD porins has been suggested as a carbapenem resistance mechanism in *P. aeruginosa* (plus providing increased *in vivo* fitness for mutants lacking *oprD* in mice) since OprD mediates entrance of carbapenems in this species,(114–117) it is often paired with other resistance mechanisms. Synergism between porins and other mechanisms to provide antibiotic resistance is principal, for instance, in *P. aeruginosa,* inactivation of all porins did not result in complete blockage of drug entry.(106,113)

Regarding Gram-positive bacteria, these bacteria lack double membranes making them theoretically more permeable to antibiotics. Nonetheless, changes in the fluidity of the cytoplasmic membrane also reduce permeability to antibiotics: differences in cell membrane phospholipid and fatty acid composition relate to development of resistance against daptomycin in enterococcal strains.(106,118)

4.4.1.2 Active transport

There are other mechanisms through which bacteria can coordinate entrance and concentration of different kinds of compounds and nutrients aside from membrane selectivity. Bacteria can actively export antibiotics through efflux. This process is mediated by transmembrane proteins that transport a wide array of toxic compounds (not only antibiotics) by spending energy. These proteins, efflux

pumps (EP), are present in efflux systems in all bacteria but they are of particular importance as mediators of resistance in Gram-negative bacteria. EP systems collaborate with other mechanisms such as membrane selectivity, to make pathogens intrinsically resistant to antibiotics. Although the importance of EP systems changes depending on the antibiotic, they are principal in antibiotic resistance and allow other mechanisms of resistance to have a greater impact.(106,119) Efflux systems are classified into superfamilies based on the kind of transporter protein their EP possess at their base (in the inner membrane). These superfamilies are the ATP-binding cassette, the major facilitator superfamily, the multidrug and toxin extrusion, the small multidrug resistance, the proteobacterial antimicrobial compound efflux and the resistance-nodulation-division (RND).(120,121)

Members of the RND superfamily are among the most clinically relevant EPs in Gram-negative bacteria because they export many different antibiotics and their overexpression contributes to MDR in clinical isolates.(106,120–122) RND transporters array of binding pockets enable them to specifically bind to several substrates, but the molecular basis of this poly-specificity is not completely understood. Thanks to their huge substrate range, RND EP contribute to MDR phenotype in dangerous pathogens such as *E. coli, P. aeruginosa* and *A. baumannii*.(106,123,124) Research to elucidate the mechanisms through which RND EP work can help the rational design of EP inhibitors aimed at restoring susceptibility to antibiotics.(106,125–127)

Going back to EPs, their expression is controlled by transcriptional regulators (usually repressors) located adjacent to EP genes. Mutations on these repressors are found in clinical isolates that present pump overexpression.(106,123,124,128) For instance, environmental signals act as triggers for several local and global transcription regulators that in turn induce the expression of RND components. Moreover, these inducers are not exclusively acting on EP regulation and are involved in other activities such as membrane integrity, DNA repair, formation of biofilms, virulence or quorum sensing. (106,129–132) This indicates that the expression of EP is intertwined within a network of genes promoting stress survival and management in

bacteria.(129,131) The genuine features of these regulatory processes, once identified, could constitute a therapeutic target to combat MDR clinical pathogens.(106,133)

4.4.1.3 Antibiotic target modification and protection

The selective toxicity of most antibiotics goes hand in hand with their specificity for targets essential to exert bacterial functions or involved in their growth. Since many antibiotics bind a target involved with essential cellular functions with high affinity, logically, resistance can emerge through structural modification or protection of that target since the modification leads to decreased antibiotic binding. (106,134) Such is the case for some mechanisms of resistance to quinolones (which bind close to the active site of the topoisomerase), where amino acid substitutions of topoisomerases result in lower antibiotic binding efficiency but still allowing the enzyme function, (135) or resistance to β-lactams, by mutations in genes coding for penicillin-binding proteins (PBPs).(106,136)

Point mutations on antibiotic target sites can accumulate during growth and become dominant upon antibiotic pressure. Also, if various alleles of target genes are present two situations can occur: i). recombination among them can produce mutant alleles (e.g., linezolid resistance can arise in Gram-positive species as the result of mutation and recombination of alleles of the 23S ribosomal RNA, rRNA, gene)(137)and ii). transformation can take place, where related species share alternative alleles generating mosaic genes by recombination (e.g., common in competent species such as the *Neisseria* genus).(106,138)

The addition of modifications to antibiotic targets can result in target protection that prevents binding of the antibiotic. This is the case for some mechanisms of resistance to macrolides, streptogramins, lincosamines and aminoglycosides: where their 16S rRNA target is protected by methylation,(106,139–141) or for colistin, where modifications of its target LPS that change its overall charge confer protection and thus, resistance.(142–145) As seen with ‘transferable mechanisms of quinolone resistance’, encoding a family of proteins protecting

bacterial topoisomerases from quinolone inhibition and usually passed through plasmids in Gram-negative species, protection of a target might produce a mild increase in the minimum inhibitory concentration (MIC) of the antibiotic, but it is the combination with other mutations of the target site can in turn provide higher MICs.(106,146)

Target protection can even occur without avoiding antibiotic binding, in such a way that the drug reaches the target, but its action is alleviated. Fusidic acid acts upon translation by binding the elongation factor G; but in fusidic acid-resistant *S. aureus*, FusB proteins are expressed. These proteins rescue translation upon fusidic acid presence, their zinc finger domain by promotes the dissociation of the complex between fusidic acid and the elongation factor G.(106,147)

4.4.1.4 Antibiotic modification or inactivation

Enzymatic modification or inactivation of antibiotics is a common mechanism of resistance that spreads through mobile gene elements. These processes (modification and inactivation) are advantageous compared to other resistance mechanisms, e.g., antibiotic target mutations, since they usually offer a lower fitness cost to the pathogen.(106)

Resistance mediated by modifying enzymes that add chemical groups to antibiotics is a mechanism affecting different classes: macrolides, aminoglycosides or phenicols among others. In the case of aminoglycosides, the amino or hydroxyl groups are modified by different transferases, reducing the affinity of these antibiotics to their target. Aminoglycoside modifying enzymes have been identified both in mobile genetic elements and chromosomes, and they are equally present in Gram-positive and negative bacteria.(106,148–150)

The degradation or damaging of the structure of an antibiotic leads to loss effectiveness and is a quite concerning mechanism of AMR. β-lactamases are the classic example to illustrate this mechanism. These enzymes hydrolyse the β-lactam rings present in β-lactam antibiotics and have been studied since the

start of the Golden Era of antibiotic discovery. Over 7800 β-lactamases have been recorded in the Beta-Lactamase DataBase by January 2023.(151,152) Yet another worryingly example of this mechanism of resistance are carbapenemases; carbapenems represent one of the most powerful classes of antibiotics we have, but resistance mediated either by carbapenemases or combined action of extended spectrum β-lactamases and loss of porins threatens their clinical effectiveness. Spread of both carbapenemases and β-lactamases is facilitated by plasmids and has been reported worldwide.(106,153–155)

4.4.1.5 Target bypass

Bacteria can circumvent antibiotic action with the acquisition and later expression of genes encoding for an alternative target for said antibiotic, but which is not efficiently inhibited. Such is the case for methicillin-resistant *S. aureus*: methicillin binds PBP inhibiting the transpeptidase domain, but by acquiring the alternative PBP2a (homologous to PBP but with lower affinity for methicillin and encoded in mobile genetic elements) *S. aureus* becomes resistant to methicillin. Here, binding of methicillin to the alternative target site of PBP2a does not inhibit transpeptidase activity as with original PBP.(106,156,157)

4.4.2 Gram-negative drug resistant pathogens of interest

Efforts to guide antimicrobial/antibiotic developers have been produced and condensed in reports by national bodies(158) and international health agencies such as the WHO and CDC as previously mentioned in this text. In the late 2000's the term ESKAPE started being used to refer to problematic pathogens escaping the action of our arsenal of antibiotics; this acronym was used to refer to *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and (what was termed back then) *Enterobacter* species.(158) WHO's priority pathogen list(83) was produced in order to gather efforts in developing antibiotics active towards the most worrying pathogens and ordered in priority tiers. Carbapenem resistant *A. baumannii, P. aeruginosa* and *Enterobacterales* (also third-generation cephalosporin resistant of the latter), all Gram-negative drug

resistant members of the ESKAPE group, were included as critical pathogens in WHO's list. The work in this thesis is focused on the discovery of novel antimicrobials towards species of this critical priority list and a brief introduction of these species, including their main characteristics, is presented in the following sections.

4.4.2.1 *Acinetobacter baumannii*

The genus *Acinetobacter* is composed of over 50 species, as of 2019, with 15 species having just a tentative description.(159,160) *Acinetobacter* cells are Gram-negative coccobacilli of different sizes and shapes (Figure 6), they are also strict aerobic, oxidase negative, catalase positive, non-fermenting and non-motile(159,161) (hence their name, from Greek "*Akinetos"* meaning not mobile, although *A. baumannii* has shown twitching motility).(159,162) Although *Acinetobacter* species such as *Acinetobacter nosocomialis* and *Acinetobacter pitti* are dangerous pathogens that cause outbreaks in intensive care units (ICU), *A. baumannii* is usually considered the most clinically relevant species in nosocomial settings.(163)

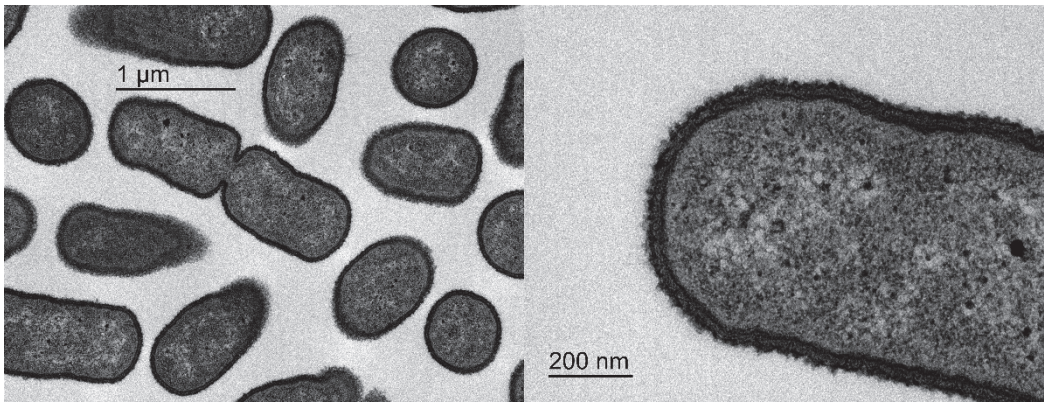

Figure 6. Transmission electron microscopy (TEM) of *A. baumannii* CR17 cells, left image 40000X and right image 150000X. Images obtained at the Electronic Microscopy Unit, Medicine Faculty, University of Barcelona.

A. baumannii's spread as a nosocomial pathogen is explained by its capacity to persist in stressful environments and to resist the action of multiple

antibiotics.(159,164) In fact, after becoming resistant to most first-line antibiotics during the last decades, only more toxic drugs such as colistin remain for treating infections by MDR *A. baumannii*.(165)

Virulence factors are principal for *A. baumannii*'s nosocomial spread, which include:(159) outer membrane proteins (e.g., OmpA, CarO),(166,167) the cell envelope (LPS and *A. baumannii'*s capsule),(168) desiccation resistance thanks to capsular polysaccharides,(169,170) biofilm production, twitching motility,(162) and protein secretion systems (e.g., toxin injection through type VI).(171)

Several risk factors are related to the acquisition of *A. baumannii*: previous hospital/ICU stay, long ICU stay, prior antimicrobial therapy, use of devices (e.g., catheters and endotracheal tubes), older age, major surgery, birth prematurity, dialysis, prolonged parenteral nutrition and mechanical ventilation.(159,172–175) The latter particularly concerning considering the high mortality rate caused in critically ill patients suffering ventilator associated pneumonia (VAP) by MDR *A. baumannii;*(176) also, there is a high incidence of community-acquired pneumonia caused by *A. baumannii,* which even has become prevalent in some parts of Asia and Oceania.(159,177,178)

Aside from these severe respiratory infections, *A. baumannii* also causes bloodstream infections in healthcare settings, skin and soft tissue infections in patients with burns and wounds, and even nosocomial meningitis.(159,179)

Regarding AMR in *A. baumannii*, this species is intrinsically resistant to aminopenicillins and first/second generation cephalosporins.(159) Moreover, and as briefly mentioned before, the rise in MDR strains of *A. baumannii* has raised the alarms of international health bodies.(180) Antibiotics such as carbapenems were the standard for treating VAP caused by 'susceptible' *A. baumannii*(181) but with the rise of carbapenem resistance rates(178,180) other agents such as colistin or polymyxin B (associated with nephro/neurotoxicity),(181,182) minocycline,(183) tigecycline(184) or even combination therapy (although there is no definitive clinical data to support its use since clinical trials are limited)(185) have to be used.(159)

To have a glimpse of the current situation, epidemiological reports with published data about *Acinetobacter* spp. in the last years point that the situation is critical. The European Centre for Disease Prevention and Control (ECDC) on its 2021 AMR Annual Epidemiological Report(180) published that *Acinetobacter* spp. accounts for the largest increase in number of reported cases (+43%) between 2020 and 2021 and remarks that this increase is not a feature of improved reporting since data from laboratories consistently reporting from 2017 backs this trend. Countries reporting greatest increases in number of cases and AMR percentages of *Acinetobacter* spp. were those that had already reported high AMR percentages in prior years. On average there has been a +121% increase in the number of reported cases of *Acinetobacter* spp. resistance phenotype to all 3, carbapenems, fluoroquinolones and aminoglycosides in 2021 than the average was from 2018 to 2019.(180) *A. baumannii* remains one of the most dangerous pathogens we are currently facing.

4.4.2.2 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod-shaped motile bacterium (Figure 7). It is a facultative aerobe and can be found in many environments such as soil, water reservoirs, sewage and at healthcare sites.(186) This nosocomial pathogen, causes pneumonia, surgical site infections urinary tract infections and bacteraemia, and is usually associated with chronic infections in patients suffering cystic fibrosis (having high morbidity and mortality).(187,188)

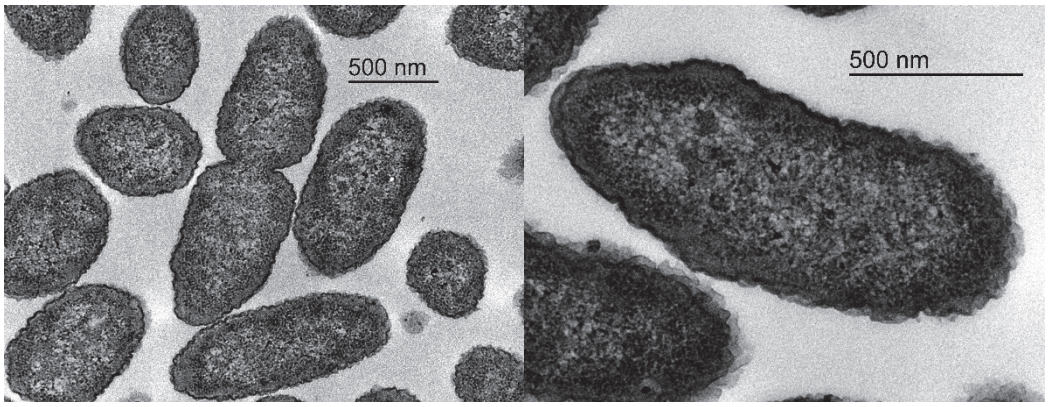

Figure 7. TEM of *P. aeruginosa* 121110 cells, left image 60000X and right image 150000X. Wrinkly cell membranes might be a result of chemical fixation and dehydration steps during sample processing.

Regarding clinical outcomes and incidence, nosocomial pneumonia caused by *P. aeruginosa* has been reported to have worse patient outcomes than other pathogens,(187,189–191) and it is the etiological agent of between 10 and 20% of VAP cases.(192) Chronic *P. aeruginosa* infection leads to biofilm production and to a mucoid phenotype (through production of alginate), which has been reported to increase mortality as patients' lung function declines.(193–195)

P. aeruginosa's virulence factors include: LPS, porins (e.g., OprF, OprH and OprD superfamily) and other outer membrane proteins, lipoproteins, production of biofilm (and alginate), protein secretion systems (e.g., type III secretion system and exotoxins), type IV pili and flagellum, proteolytic enzymes, quorum sensing systems, pyocyanin and siderophores (pyoverdine and pyochelin).(196,197)

And as for risk factors for *P. aeruginosa* infections, these include: structural lung disease, haematological malignancy/neutropenia, transplantation, burn wounds, use of catheters, previous antimicrobial therapy, long hospitalization and mechanical ventilation, aside of course from cystic fibrosis.(187)

Management of *P. aeruginosa* infections through monotherapy or combination is in debate. (198–200) A combination of antipseudomonal agents is often given to severely ill patients since providing a rapid diagnosis and adequate treatment is related to a better outcome. (187,201–203)

Finally, and to get a picture of the current situation regarding *P. aeruginosa* incidence in Europe, we can use data from ECDC 2021 Annual Epidemiological Report. Percentages of AMR isolates of *P. aeruginosa* have increased during the 2021 period: at least 18,7% of all isolates were resistant to at least one antimicrobial group under surveillance and combined resistance to agents and or groups was at 13% of *P. aeruginosa* isolates. Although a higher incidence of *P. aeruginosa* cases was expected during the COVID-19 pandemic (as reported with *A. baumannii*) since infections are also linked to environmental sources and rate of ventilator use among hospitalized patients (and COVID-19 cases), there was no such trend during 2021. This effect could be explained through changes in hospital stays lengths and greater shielding of patients at risk of COVID-19 and *P. aeruginosa* infection (e.g., cystic fibrosis patients). However, the ECDC report does not track lower respiratory tract infections that might be more frequent for *P. aeruginosa*. The most common resistance phenotypes for this pathogen were piperacillin-tazobactam and fluoroquinolone resistance, each at 18,7%, followed by carbapenem resistance.(180)

4.4.2.3 *Klebsiella pneumoniae*

K. pneumoniae is a member of the *Enterobacterales* family, including *Salmonella* and *Escherichia*.(204,205) *K. pneumoniae* is a Gram-negative encapsulated bacterium, which is non-motile and is facultatively anaerobic.(206,207) It has a varied phenotypic and genetic diversity and can be found in a myriad of host-associated niches and in the environment (soil, water and other surfaces).(204,206,208,209)

This species can colonize the upper respiratory tract and the gut in humans (the latter a major reservoir for transmission and infection),(206,208,210,211) a situation that can later turn into a infection when host immune system is altered or cannot control pathogen growth; that is the case of patients that suffer diabetes, that are on glucocorticoid therapy or that have received an organ transplant.(206)

Due to its colonization abilities, *K. pneumoniae* is considered a dangerous opportunistic pathogen that is associated with nosocomial infections, known by leading to respiratory tract infections developing into severe pneumonia and multiorgan infections; it is also capable of producing urinary tract infections, meningitis, biliary tract infections in hospitalized patients, sepsis and has been reported colonizing and infecting patients through use of contaminated respirators, atomizers or catheters.(206,208)

The spread of hypervirulent *K. pneumoniae* strains is a great cause of concern, and cases have already been reported in Asia, Europe and the USA.(212) These strains are characterized by a high virulence (a 50% lethal dose is achieved in animal models as low as at 10^3 colony forming units)(213) and can cause infections in relatively healthy individuals even in community settings. Reports of clinical manifestations of hypervirulent strains indicate an increasing prevalence; the earliest clinical clues include liver abscess and bacteraemia in patients with positive cultures and due to the lethality of these strains, early diagnosis and intervention is principal in patient outcome. (206)

Regarding resistance, as it is the case with other menacing Gram-negative pathogens, *K. pneumoniae* has become resistant to carbapenems too. Different mechanisms mediate carbapenem resistance in *K. pneumoniae* but production of carbapenemases such as the *K. pneumoniae* carbapenemase (KPC), metallo β-lactamases (NDM, VIM or IMP) or oxacillinases (OXA), are the main ones.(206,214) Overexpression of EP, decreased membrane permeability and

production of β-lactamases also have been reported for carbapenem resistant strains of *K. pneumoniae*.(215,216) Emergence of hypervirulent carbapenem resistant strains of *K. pneumoniae* named ST11 CR-HvKp threatens our capacity to control infections caused by these pathogens.(217) Again as with previously described Gram-negative MDR pathogens, more toxic agents such as colistin, or others like tigecycline and intravenous fosfomycin are recommended for treating infections by carbapenem producing strains of *K. pneumoniae*. (206)

Virulence factors that aid in the infectivity of *K. pneumoniae* comprise the bacterium's capsule and production of LPS, adhesin and siderophores (206,209)

Regarding *K. pneumoniae*'s prevalence, ECDC's Annual Epidemiological Report for 2021 points out that there has been a continuous increase in cases of *K. pneumoniae* resistant to carbapenems from 2017, with +20% more cases in 2021 than 2020.(180) *K. pneumoniae* was the third most commonly reported bacterial species in this report at a 11.9%, after *E. coli* and *S. aureus,* in the European Union and the European Economic Area. Although the most common resistance phenotype reported for isolates of *K. pneumoniae* based in 2021 data was third-generation cephalosporin resistance, at 34.3%, combined resistance to third generation cephalosporins, fluoroquinolones and aminoglycosides represented 21.2% samples tested.(180)

4.5 Novel and alternative antimicrobials

The scarcity of approved novel antimicrobials with new mechanisms of action plus the inevitable emergence of resistance mechanisms, even to those new agents, pushes forward efforts towards finding novel and alternative therapeutics.(218)

The following narrative review was produced and published alongside my thesis directors at the journal 'Clinical Microbiology and Infection' during 2019. Its aim

was to compile the most relevant and latest approaches for developing new antimicrobials, gathering information about new antibacterial agents with novel protein targets, virulence blockers, nanoparticles, antimicrobial peptides, phage therapy and enzybiotics and antisense oligonucleotides inhibiting essential bacterial genes.

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Narrative review

Current landscape in the discovery of novel antibacterial agents

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ABSTRACT

Background: Standard treatments against bacterial infections are becoming ineffective due to the rise of antibacterial resistance worldwide. Classical approaches to develop new antibacterial agents are not sufficient to fulfil the current pipeline, therefore new strategies are currently being devised in the field of antibacterial discovery.
Objectives: The objective of this narrative review is to compile the most successful strategies for drug discovery within the antibacterial context that are currently being pursued.
Sources: Peer-reviewed publications from the MEDLINE database with robust data addressing the discovery of new antibacterial agents in the current pipeline have been selected.
Content: Several strategies to discover new antibacterials are described in this review: (i) derivatives of known antibacterial agents; the activity of a known antimicrobial agent can be improved through two strategies: (a) the modification of the original chemical structure of an antimicrobial agent to circumvent antibacterial resistance mechanisms and (b) the development of a compound that inhibits the mechanisms of resistance to an antibacterial agent; (ii) new antibacterial agents targeting new proteins; (iii) inhibitors of virulence factors; (iv) nanoparticles; (v) antimicrobial peptides and peptidomimetics; (vi) phage therapy and enzybiotics; and (vii) antisense oligonucleotides.
Implications: This review intends to provide a positive message affirming that several different strategies to design new antibacterial agents are currently being developed, and we are therefore confident that in the near future some of the most promising approaches will come to fruition. **J. Vila, Clin Microbiol Infect 2020;26:596**

Introduction

The development of new therapeutic strategies seems to have reached a dead end. Despite the urgent need to find new antibacterial products, many pharmaceutical companies, including a significant number of large companies, have abandoned new antibiotic research programmes, investing their research and development resources in other therapeutic areas [1]. Besides private efforts, research groups at the hospital or academic level outside the industry may play an important role in discovering new antibiotics. This narrative review describes the major strategies implemented to design and develop new antibacterial agents.

Improving known antibacterial agents

The activity of a known antimicrobial agent can be improved through two strategies. First, modification of the basic chemical structure of an antimicrobial agent, such as tigecycline, which circumvents antibacterial resistance mechanisms. It is a derivative of minocycline with a 9-tert-butyl-glycylamido side chain added to the D ring at the ninth position of the molecule, which avoids the effect of specific tetracycline efflux pumps or ribosomal protection, two of the mechanisms of tetracycline resistance [2]. Cefiderocol can also be considered a cephalosporin-derivative as it has been linked to a siderophore that helps to reach the periplasmic space and has enhanced stability to β-lactamases. It shows good activity against *Enterobacteriaceae* and non-fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, and it is currently in Phase III studies (Table 1). Second, compounds inhibiting the mechanisms of resistance to an antibacterial agent; in this regard,

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Table 1
New antibacterial agents interacting with new targets in clinical phases

Antibacterial agent	Type of drug	Target	Phase	Pharmaceutical Co.
CRS3123[a]	Diaryldiamine	Methionyl-tRNA synthetase	I	Crestone Inc.
MGB-BP-3[a]	Distamycin	DNA minor groove binder	I	MGB Biopharma
CG400549[a]	Benzyl-piridinone	Biosynthesis fatty acids (FabI)	II	Crystal Genomics
Afabicin[a]	Benzofuran naphthyridine	Biosynthesis fatty acids (FabI)	II	Debipharm Int.
Murepavadin[b]	Peptidomimetic	LptD	III	Polyphor AG
Cefiderecol[c]	Siderophore-cephalosporin	PBP	III	Shionogi Co.

[a] These drugs are not active against bacteria from the ESKAPE group.
[b] This drug is specific for *Pseudomonas aeruginosa* and is discussed in the subheading concerning peptides and peptidomimetics of this review.
[c] Hybrid molecule active against bacteria in the ESKAPE group. It is not actually a new antibacterial agent.

several approaches such as new β-lactamase inhibitors are being used [3]. Two main groups of β-lactamase inhibitors are being developed: the diazabicyclooctane group (i.e. avibactam or relebactam) and the boronate β-lactamase inhibitors group. The diazabicyclooctane group inhibits class A, class C and some class D enzymes, but does not show inhibition of metallo-β-lactamases, class B β-lactamases; however, the combination with aztreonam also covers metallo-β-lactamase-producing *Enterobacteriaceae*, because aztreonam has activity against the bacteria producing metallo-β-lactamases. The main example of the boronate β-lactamase inhibitors group is vaborbactam, which also does not show inhibition of class B and D β-lactamases and is combined with meropenem. Inhibitors of efflux pumps allowing the antibiotic to accumulate in the bacterial cell are being developed. Some examples include phenylalanine-arginine-β-naphthylamide or the most recent indole-2-carboxamides. Nevertheless, none of these inhibitors have reached the clinical trial stage, mainly due to toxicity. Another area of research is the development of inhibitors of RecA, which plays an important role in the SOS response and has been shown to potentiate antibiotic activity and block the evolution of antibiotic resistance [4,5].

New antibacterial agents targeting new proteins

Although 30 antibacterial agents are currently in the pipeline [6], few are actually considered new (Table 1). It was thought that the advent of bacterial genomics would open the door to the discovery of new antibiotics. However, although it is true in part that the search for essential targets using computational analysis is feasible, finding an *in vitro* inhibitor of these protein targets is difficult and faces development hurdles such as limitations in penetrating the bacteria. Therefore, there has been no success with this approach.

The traditional pathway of identifying microorganisms from a rich ecological niche that produce an antibiotic as a secondary metabolite still has potential for the discovery of new antibiotics. Moreover, some authors are trying to find microorganisms producing antibiotics from recondite niches such as marine samples (invertebrates or algae), insects and invertebrate organisms (e.g. symbionts and plants) [7]. An alternative to this approach is searching for new antibacterial compounds from the metabolism of microorganisms present in the human microbiota or from the microbiome of different sources; in this regard lugdunin, a macrocyclic thiazolidine peptide antibiotic produced by *Staphylococcus lugdunensis*, has been shown to be active against a group of Gram-positive pathogens including *Staphylococcus aureus* [8]. However, the mode of action is unknown. Regarding the microbiome, there are two approaches: (a) the capture of biosynthetic gene clusters from whole metagenomic DNA, and (b) the prediction of natural product structures from primary sequence data by means of bioinformatic tools and their production by chemical synthesis. These approaches have led to the discovery of two molecules: tetarimycin and humimycin. The former is a tetracyclic antibiotic that is active against methicillin-resistant *S. aureus* from the soil microbiome and the latter inhibits lipid II flipase and shows activity against Gram-positive bacteria including *S. aureus* and *Streptococcus pneumoniae* and interesting synergy with some β-lactam antibiotics [9,10].

Virulence blockers

An alternative to the classical approach of drug development is to affect pathogenicity by targeting specific virulence factors involved in this process. This strategy aims to prevent the bacterium developing resistance and so contain the spread. Molecules interfering with virulence factors will disarm the pathogen, thereby allowing bacterial clearance by the host immune system. There is a myriad of factors involved in bacterial virulence that are being investigated as targets for new agents including the following categories:

1. Determinants involved in host cell attachment inhibiting access and translocation into the host tissue. Molecules targeting fimbria, such as the FimH antagonist mannosides and the antibody scFv-Fc KP3 targeting type 3 fimbrial subunit [11–13], have shown good *in vivo* effects in mouse models (Table 2). Pilicides, pili formation inhibitors, and the glycosylated molecules mucins are in the discovery phase [11,12,14].

2. Actors involved in host immune modulation. Lipid A inhibitors include LpxC-1 [15], the substituted sulphone-based hydroxamates with good *in vitro* efficacy [16] and ACHN-975, having failed Phase I [17] (Table 2). Another molecule, erianin, a Sortase A inhibitor that interferes in host immune recognition and attachment in host surfaces, affects virulence in *S. aureus* murine infections [18].

3. Biofilm modulation (limiting adhesion, affecting the extracellular matrix and disturbing mature biofilm). A number of small molecule inhibitors have been identified and recently reviewed [19]. Agents of natural origin such as flavonolignans and streptorubin B [20], cyclosporine and its derivative valspodar, have been shown to be good antibiofilm agents [14,21]. AR-105 entered the Phase II clinical phase as an adjunctive treatment [6,22,23] (Table 2).

4. Global regulators of virulence. These include anethole and SE-1, tested *in vivo* and *in vitro*, respectively [24,25] (Table 2). Inhibition of two-component systems has also been shown to block the pathogenesis of clinically relevant bacteria [14], although only savirin and LED209 have shown good *in vivo* results [26,27] (Table 2).

5. The quorum sensing network, which mediates bacterial communication and is key in the infection process. Quorum sensing quenching includes the acyl-homoserine lactone lactonases effective against *P. aeruginosa*. The main advantage of this approach is

Table 2
Description of virulence blockers in the research pipeline of new antibacterial agents

Virulence categories	Agent	Action	Bacterial target	Infectious disease targeted	Current stage	References
Cell attachment	Pilicides (bicyclic 2-pyridones)	Inhibition of pili formation/biogenesis and regulation	Uropathogenic *Escherichia coli* (UPEC)	Urinary tract infections caused by UPEC	Discovery	[11,12]
	Mannosides (FimH antagonist)	Host receptor analogues inhibiting FimH component of type 1 fimbriae	Uropathogenic *E. coli*	Urinary tract infection caused by UPEC	Preclinical[a]	[74]
	ScFv-Fc KP3 (synthetic antibody)	Targeting type 3 fimbrial subunit in *Klebsiella pneumoniae*	*K. pneumoniae*	*K. pneumoniae* infections	Preclinical[b]	[13]
	Mucins	Interference with bacterial adhesins (mimic host cell receptor glycosylation)	*E. coli*, *Salmonella* spp., *Helicobacter pylori*, *Staphylococcus aureus* and *Bacillus subtilis*	Several Gram-positive and Gram-negative infections	Discovery	[14]
Immune modulation of the host	LpxC-1	Inhibition of the lipid A biosynthetic enzyme LpxC	*Acinetobacter baumannii*	*A. baumannii* infection	Preclinical[c]	[15]
	ACHN-975	Inhibition of the lipid A biosynthetic enzyme LpxC	*K. pneumoniae*, *Pseudomonas aeruginosa* and *E. coli*	*K. pneumoniae*, *P. aeruginosa* and *E. coli* infections	Phase I (interrupted)[d]	[17]
	Substituted sulphone-based hydroxamates	Inhibition of the lipid A biosynthetic enzyme LpxC	*K. pneumoniae*, *E. coli*, *Enterobacter aerogenes* and *Citrobacter freundii*	Infections caused by *K. pneumoniae*, *E. coli*, *Enterobacter aerogenes* and *Citrobacter freundii*	Preclinical[e]	[16]
	Erianin	Sortase A inhibitor (Sortase A anchors cell surface molecules involved in pathogenesis in Gram-positive bacteria)	*S. aureus*	*S. aureus* infections	Preclinical	[18]
Biofilm modulators	Hydnocarpin-type flavonolignans (isolated from Silybummarianum)	Inhibition of the icaADBC-dependent biofilm formation pathway	*S. aureus*	*S. aureus*-mediated biofilm infections	Discovery	[75]
	Streptorubin B (isolated from actinobacteria)	Unknown	Methicillin-resistant *S.aureus*	*S. aureus*-mediated biofilm infections	Discovery	[20]
	Cyclosporine and valspodar (cyclosporine-derivative)	Inhibition of the Rgg2/Rgg3 regulatory system	*Streptococcus pyogenes*	*Streptococcus pyogenes* infections	Discovery	[21]
	AR-105 (monoclonal antibody)	Blockage of the polysaccharide alginate (surface polysaccharide of *P. aeruginosa* involved in biofilm formation and adhesion	*P. aeruginosa*	Ventilated-acquired pneumonia caused by *P. aeruginosa* (Adjunctive treatment)	Phase II	[6,22,23]
Global regulators	Anethole (natural compound)	Repression of the production of the cholera toxin and the toxin co-regulated pilus	*Vibrio cholerae*	*Vibrio cholerae* infections	Preclinical[f]	[24]
	SE-1	Inhibition of VirF expression	*Shigella flexneri*	Shigellosis	Discovery[g]	[25]
	Savirin	Inhibition of the transcriptional regulator AgrA (affects the regulatory cascade including *hla*, *psm alpha*, *pvl (lukS)*, *agrA* and *agrC*)	*S. aureus*	Skin and soft-tissue infections caused by *S. aureus*	Preclinical[h]	[26]
	LED209	Blockage of autophosphorylation of the sensor kinase QseC (involved in the regulation of virulence gene expression as motility via *flhDC* operon in *E. coli* or regulation of the pathogenicity island LEE in enterohaemorrhagic *E. coli* and involved in virulence in *Salmonella* Typhimurium and *Francisella tularensis*)	*E. coli*, *Salmonella* Typhimurium and *Francisella tularensis*	Infections caused by *E. coli*, *Salmonella* Typhimurium and *Francisella tularensis*	Preclinical[i]	[14,27,76,77]
Quorum-sensing network	Acyl-homoserine lactone lactonases	Targeting the acyl-homoserine lactones (quorum sense signals)	*P. aeruginosa* and *A. baumannii*	*P. aeruginosa* -mediated biofilm infections	Discovery	[78]
Toxins	MEDI4893 (monoclonal antibody)	Binding to α-toxin of *S. aureus*	*S. aureus*	Diabetic foot ulcers infected by *S. aureus*	Completed Phase II[j]	[29]

Table 2 (*continued*)

Virulence categories	Agent	Action	Bacterial target	Infectious disease targeted	Current stage	References
	Bezlotoxumab (monoclonal antibody)	Binding to toxin B from *Clostridium difficile*	*C. difficile*	Prevention of recurrent *C. difficile* infections (in combination with current therapy)	Undergoing Phase III[k]	[79]
Bacterial functional membrane microdomain-associated proteins	Zaragozic acid	Sterol synthesis inhibitor	Methicillin-resistant *S. aureus*	*S. aureus* infections	Preclinical	[28]
Type three secretion system	Licoflavonol	Regulation of transcription of *sicA/invF* and transportation of SipC	*Salmonella* Typhimurium	*Salmonella* Typhimurium infection	Discovery	[31]
	Salicylidene acyl hydrazides	Targeting T3SS	*Salmonella* Typhimurium and *Chlamydia trachomatis*	Infections caused by *Salmonella* Typhimurium and *Chlamydia trachomatis*	Preclinical	[32]
Toxin traps	CAL02	Liposomes acting as a toxin trap	*S. aureus* and *Streptococcus pneumoniae*	Severe community-acquired *Streptococcus pneumoniae* infection	Phase I[l]	[33]

Abbreviations: UPEC, uropathogenic *Escherichia coli*.

[a] Mannosides have shown significantly decreased colonization levels in UPEC and the ST131 multidrug-resistant clinical strain in murine infection models [74].

[b] scFv-Fc KP3 has been shown to inhibit biofilm formation and reduce bacterial burden in a mouse lung infection model [13].

[c] LpxC-1 has shown to strongly attenuate *Acinetobacter baumannii* virulence in mice [15].

[d] Phase I interrupted due to inflammation at the injection site. Undesired effects were due to the presence of essential pharmacophores [80].

[e] Shown to have problematic *in vivo* pharmacokinetic properties [16].

[f] Anethole shown to reduce fluid accumulation of *Vibrio cholerae* in *the in vivo* infection model of rabbit ileal loop [24].

[g] SE-1 has been demonstrated to significantly reduce invasion of eukaryotic cells infected with *Shigella flexneri* [25].

[h] Efficacy has been reported in a murine *Staphylococcus aureus* wound model, interestingly not affecting the commensal *Staphylococcus epidermidis* [26].

[i] *Salmonella* Typhimurium-infected mice with LED209 24 h post infection (oral administration) substantially increased survival rate over the non-treated group (80% survival versus 30%, respectively), and similar results were obtained with *Francisella tularensis* [27].

[j] The efficacy of MEDI4893 was compared with active immunization with a non-toxigenic anti-toxin in diabetic and non-diabetic mice models with *S. aureus*-infected wounds and showed similar therapeutic effect in wound healing promotion with a greater decrease in the bacterial burden in diabetic mice indicating a possible advantage of MEDI4893 [81].

[k] In terms of efficacy, results of the Phase II study indicated a significant reduction in the recurrence rates (7% in the treated group versus 25% in the placebo group) [82]. Currently undergoing a Phase III clinical trial in children with *Clostridium difficile* infections.

[l] Although efficacy outcomes are not conclusive due to the small sample size, a study in a mice model of severe pneumonia (*Streptococcus pneumoniae* infection) and bacteraemia (*Staphylococcus aureus* and *Streptococcus pneumoniae*) showed that a combined therapy of CAL02 with antibiotics substantially improved survival outcomes [34].

that modulation of one quorum sensing system allows interference in other systems [28].

6. Toxins secreted by pathogenic bacteria required for bacteria–host interactions and evasion of the immune system. The anti α-toxin antibody *S. aureus* (MEDI4893) that completed the Phase II trial in 2018 (results not yet available) is promising [29] https://clinicaltrials.gov/ct2/show/NCT02296320 (Table 2). Another interesting case is the monoclonal antibody targeting toxin B from *Clostridioides difficile* (Bezlotoxumab) the first US Food and Drug Administration-approved anti-virulence agent to be used in combination with current therapy to prevent recurrent *C. difficile* infections [30].

7. Bacterial functional membrane microdomain-associated proteins related to signalling networks. Small molecules interfering with the metabolic pathway of polyisoprenoid lipid biosynthesis have been shown to attenuate bacterial virulence. Zaragozic acid alters oligomerization of the penicillin-binding protein PBP2a in methicillin-resistant *S. aureus* reverting the resistant phenotype [28].

8. Type three secretion system, a major Gram-negative virulence factor that allows secretion of effector proteins involved in pathogenicity. Inhibitors of this system include licoflavonol in *Salmonella* Typhimurium [31] and salicylidene acylhydrazides active against infections of *Chlamydia trachomatis* [32] (Table 2).

9. Liposomes interfering in the progression of infection. CAL02 has completed the Phase I trial [33] and improved outcomes were shown as a combination therapy in mice [34] (Table 2). One of the advantages of anti-virulence agents is the preservation of the host's microbiome as commensal bacteria often lack the features targeted by these agents. In terms of drug development, although to date no anti-virulence agent has entered clinical study phases, it is most likely that larger clinical trials will be needed to prove their therapeutic efficacy as adjuvants (as may be the case for other agents under other approaches also discussed in this work) of current antibiotic treatments when effective treatments are available. Additionally, it is expected that the administration of a combination of several anti-virulence agents will be required and will effectively attenuate the bacteria. Another hurdle lies in the fact that administration of the anti-virulence drug must be in concordance with the time at which the targeted factor is expressed. Finally, as one of the features of these molecules is that the effectiveness is dependent on the immune host response, these therapies will not be adequate to treat immunocompromised patients.

Nanoparticles

Nanoparticles (NPs) are defined as particles or materials within the nanometer scale [35]. Although some metals like silver or copper have antibacterial activity in their bulk form, others only have it as NPs against bacteria. The mechanisms of action of these particles have not been completely described, but three processes are hypothesized to occur concomitantly: induction of oxidative stress, non-oxidative mechanisms and in a minor way, interaction of released metal ions with functional groups of proteins and nucleic acids [36,37].

Specific factors such as size, zeta potential (electrokinetic potential), charge, surface morphology and crystal structure determine metal NP antimicrobial activity [37]. NPs can both disrupt bacterial membranes and hinder the formation of biofilms. Smaller NPs provide greater biofilm inhibition (e.g. Ag, ZnO, Mg or NO NPs)

and rod-shaped NPs are better at inhibiting biofilms than spherical NPs [38].

Cytotoxicity of NPs is a drawback and must be carefully regarded. ZnO and Ag NPs have been described as cytotoxic at bacterial inhibitory concentrations. To overcome this issue, it has been proposed that NPs must be delivered locally at the infection site to confine the NPs and their harmful effects to eukaryotic cells [36].

Antimicrobial peptides and peptidomimetics

Antimicrobial peptides (AMPs) are ubiquitous immune effectors that aid the host in fighting pathogens. Although the classically proposed mechanism of action is membrane permeabilization, other mechanisms, including inhibition of protein, DNA and RNA synthesis, and gene material degradation, also take place. Their activity is based on their composition and secondary structure [39].

The AMPs can be classified based on their secondary structure into α-helical AMPs (e.g. cathelicidins), β-sheet-containing AMPs (e.g. α- or β-defensins), AMPs with a β-hairpin or loop stabilized by a single disulphide bond or cyclization of the peptide chain (e.g. thanatin) and short AMPs with extended conformations (e.g. indolicidin) [40].

As a result of their mechanism of action, it has been proposed that these molecules are synergistic in combination with antibiotics that have difficulty in penetrating bacteria or when the resistance mechanism to that antibiotic is related to membrane modification [41].

Antimicrobial peptides usually fail preclinical studies because of low stability or high *in vivo* toxicity. In the last decades few natural AMPs have been commercialized, none of which was a linear peptide [40]. Most of the AMPs that continue in clinical trials are for topical use. The following examples represent promising AMPs that have undergone clinical trials with different applications: OP-145 completed Phase II [40,42–44], two AMPs targeting *C. difficile*, surotomycin which was discontinued after two Phase III studies [45–47] and NVB-302, has completed Phase I [40,48–50].

Peptidomimetics are defined as sequences purposely designed to mimic a peptide or its function but no single α-amino acid makes up the backbone structure. These sequences usually have enhanced *in vivo* stability and lower toxicity than usual α-helical AMPs [40].

Among the different peptidomimetics, ceragenins are resistant to proteases and are easy to produce on a large scale [51]. Two of the most active are: CSA-131, which is active against colistin-resistant *A. baumannii*, *P. aeruginosa* and *Klebsiella pneumoniae* strains and anaerobic bacteria [52–55] and CSA-13 with good antibiofilm activity [56,57] (Table 4).

Murepavadin, which belongs to a novel class of outer membrane protein [6], is of special interest, although it was recently halted in Phase III [41,58–60] (Table 4).

Although it has been suggested that there is little to no resistance to AMPs (and/or to peptidomimetics), cross-resistance can arise when experimentally exposing *S. aureus* against pexiganan [61].

Table 3
Description of antibacterial agents in the research pipeline following alternative strategies

Category	Agent	Action	Bacterial target	Infectious disease targeted	Current stage	References
Antimicrobial peptides and peptidomimetics	OP-145 (AMP60.4Ac or P60.4Ac; based on LL-37)	Hypothesized to inhibit bacterial adherence	Gram-positive	Chronic middle ear infection	Phase II	[40,42–44]
	Surotomycin	Membrane depolarization	*Clostridium difficile*	Infectious diarrhoea associated with *C. difficile*	Discontinued Phase III [a]	[45–47]
	NVB-302 (lantibiotic; polycyclic peptide containing thioether amino acids)	Inhibition of cell wall biosynthesis by lipid II binding	*C. difficile* and wide range of Gram-positive bacteria	*C. difficile* infection	Completed Phase I	[48–50]
	Murepavadin (POL7080; cyclic protegrin analogue)	Outer membrane biogenesis	*Pseudomonas aeruginosa*	Ventilator-associated bacterial pneumonia (*Pseudomonas* infections)	Discontinued Phase III [b]	[41,58–60]
	CSA-131 (ceragenin)	Charge driven cell membrane destabilization	*Acinetobacter baumannii*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Bacteroides* spp., *Prevotella* spp., *Peptostreptococcus* spp. and *C. difficile*	Infections caused by *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, *Bacteroides* spp., *Prevotella* spp., *Peptostreptococcus* spp. and *C. difficile*	Discovery	[52–55]
	CSA-13	Antibiofilm activity; bacterial membrane	Mixed *P. aeruginosa* and *Staphylococcus aureus* biofilm and streptococci biofilms	Infections caused by *P. aeruginosa*, *S. aureus* and streptococci biofilms	Discovery	[56,57]
Antisense oligonucleotides (Gene expression inhibitors)	CPP-PMO conjugate	Gene expression inhibition of *gyrA*	*Enterococcus faecalis and S. aureus* (*gyrA*)	*Enterococcus faecalis* and *S. aureus* infections	Discovery	[69]
	CPP-PNA conjugate	Gene expression inhibition of *rpoA*	*Listeria monocytogenes* (*rpoA*)	*L. monocytogenes* infection	Preclinical	[70]
	PNA	Gene expression inhibition of *polA*	*Brucella suis* (*polA*)	*B. suis* infection	Discovery	[71]
	PNA conjugate	Gene expression inhibition of *ftsZ*	*S. aureus* (*ftsZ*)	*S. aureus* infection	Discovery	[72]

[a] Surotomycin did not show superiority for clinical response or sustained clinical response versus vancomycin and failed to achieve non-inferiority for clinical cure at end of treatment [46,47].
[b] Two Phase III studies were suspended due to renal toxicity [59,60].

Table 4
Description of phage therapies and enzybiotics currently available and under development

Research strategy	Name	Agent	Bacterial target	Infectious disease targeted	Current stage	References
Phage therapy	Intestibacteriophage	Phage cocktail	*Salmonella*, *Shigella*, *Escherichia coli*, *Proteus*, *Pseudomonas*, *Enterococcus* and *Staphylococcus*	Intestinal disease	Commercialized	[83]
	Pyobacteriophage	Phage cocktail	*Staphylococcus*, *Streptococcus*, *Pseudomonas*, *E. coli* and *Proteus*	Surgical wound infections	Commercialized	[84]
	Phagoburn study	Cocktail of 12 natural lytic anti-*P. aeruginosa* bacteriophages	*Pseudomonas aeruginosa*	Burn wound infected with *P. aeruginosa*	Phase 1/2[a]	[62]
Enzybiotics	Cpl-711	Endolysin (degradation of peptidoglycan)	Multidrug-resistant *Streptococcus pneumoniae*	*Streptococcus pneumoniae* infections	Preclinical 11[b]	[65]

[a] Results from a Phase 1/2 trial involving 27 patients with burn wound infected with *P.aeruginosa* indicated that although a decrease in bacterial burden was observed using phage therapy the standard of care still showed better results indicating that higher doses of phages should be used in future studies [62].
[b] Cpl-711 showed greater protection than Cpl-711 in animals that had received endolysin intraperitoneally 1 h after infection (*Streptococcus pneumoniae* injected intraperitoneally) [65].

Phage therapy and enzybiotics

The use of lytic phages has been restricted to Eastern European countries, particularly Georgia and Poland where phage cocktails are commercially available (Table 4). Regarding Western European countries, a study called Phagoburn was conducted in Belgium, France and Switzerland from 2013 to 2017 to evaluate phage therapy for treating burn wounds infected with *E. coli* and *P. aeruginosa* [62] (Table 4). Additionally, the ambitious Phage 4 cure project, is currently ongoing in Germany and includes the development of inhalable bacteriophages to treat *P. aeruginosa* infections from manufacturing to preclinical studies following international quality standards [63,64] (Table 4).

Another antibacterial approach, the so-called enzybiotics, involves the use of phage-derived enzymes to specifically attack different species or even bacterial serotypes. These lysins were first described in the 1960s and act by degrading peptidoglycan and inducing bacterial lysis by osmotic imbalance and have shown good antibacterial activity. Endolysins, in particular Cpl-711, have shown good results when administered in mice previously challenged with *Streptococcus pneumoniae* [65] (Table 4). Alternatively, polysaccharide depolymerases are also currently being studied as they degrade the carbohydrates of bacterial membranes. Hence, the use of this family of enzymes in the disruption of biofilms and against encapsulated bacteria has generated enormous interest [63,65,66].

Although phage therapy is seen as a potentially promising alternative to fight against antimicrobial-resistant pathogens, there are still several hurdles to overcome. One is pharmacokinetics, as high doses of phages are needed to eliminate a bacterial population (even small communities) because they have to replicate inside the host cell to exert their bactericidal effect. In terms of host response, considerations regarding immune reaction, through neutralizing antibodies, derived from the action of bacteriophages, must also be considered. Finally, the threat of the rise of bacterial resistance to bacteriophages should be taken into account and one strategy to overcome this issue lies in the combination of phages with classical antibiotics. Regarding enzybiotics, the main limitation is their weak stability and lack of solubility, requiring the need for chemical engineering.

Antisense oligonucleotides

Oligonucleotides can be used to inhibit gene expression both in eukaryotes and prokaryotes. These molecules act on different levels in the gene expression regulation pathways. Depending on their mechanism these molecules are classified as transcription process inhibitors (e.g. triplex-forming oligonucleotides aimed against DNA), translation process inhibitors (e.g. antisense oligonucleotides, small interfering RNAs, ribozymes and microRNAs; aimed at mRNA) and oligonucleotides blocking protein activity (e.g. aptamers or decoy oligonucleotides for transcription factors).

Antisense oligonucleotides are single-stranded DNA mimicking oligomers of around 20 nucleotides that bind mRNA to modulate gene expression but do not affect nucleotide translation [67]. The most commonly investigated antisense oligonucleotides are: (a) phosphorothioate oligodeoxynucleotides (S-oligos); (b) locked nucleic acids; (c) peptide nucleic acid (PNAs); and (d) phosphorodiamidate morpholino-oligomers (PMOs) [68]. Antisense oligonucleotides can be used to fight antimicrobial resistance by inhibiting essential gene expression through RNA silencing. The main drawback of this strategy is achieving high enough concentrations inside the bacterium, which has been addressed using cell-penetrating peptides (CPPs) that aid in the effective intracellular delivery of the oligomers.

The potential of antisense oligonucleotides as antimicrobials has been shown by different research groups (e.g. CPP-PMO [69], CPP-PNA [70], PNA targeting *polA* [71] and PNA conjugates [72] (Table 3).

Conclusion

Although as seen in this review there are currently several strategies being carried out for the discovery of new antibacterial agents, the time is not yet ripe for complacency. Therefore, more traditional and non-traditional approaches are needed to ensure a future with effective treatments against infectious diseases caused by multidrug-resistant bacteria. To make this possible, more funding opportunities are needed for public research in the field (current programmes such as Carb-X and ENABLE have been shown to be insufficient) and new incentives are necessary to induce the industry to return to the discovery of antibacterial agents. In this sense, a 'subscription' style payment model, such as the one that the United Kingdom recently announced [73], could be an interesting strategy to be followed up.

Transparency declaration

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4.5.1 Antimicrobial peptides

Since the work on developing novel antimicrobials performed in the current thesis focusses on antimicrobial peptides (AMPs), an updated introduction to these agents further expanding what was detailed in the previous narrative review, is presented down below.

The complex relationship stablished between organisms (from single to multicellular species) in their natural environments can make them interact with each other in a harmful manner; AMPs belong to the defensive strategies organisms produce in these interactions. AMPs have been present in nature for millions of years. The fact that these peptides are still present and effective seems to have relation with the slow rate at which resistance to AMPs arises compared to traditional antibiotics. Slow resistance rate to AMPs could be explained due to AMPs' diverse mechanisms of action (including the targeting of the cell membrane), and also because of some characteristics AMPs have that differ from usual antibiotics: AMPs have a steeper dose dependant killing curve and killing rate (generally, higher AMP concentrations quickly act upon sensitive bacterial populations) and they do not increase mutation rate and recombination in bacteria.(218–223)

4.5.1.1 Characteristics, sources and classification

AMPs are short polycationic chains of amino acids ranging from 8 to 50 residues in length, with low molecular weight, amphipathic, comprised of several hydrophobic amino acids and of cationic or anionic nature.(222,223) They are secondary metabolites of many organisms and they belong in the defence frontline of hosts as part of the innate immune system. They are produced by eukaryotes and prokaryotes and, in multicellular organisms, their expression can be constitutive (storing in granules for later release) or induced because of infectious or inflammatory stimuli such as pathogen-associated molecular patterns or cytokines.(224–227) In mammals, AMPs like cathelicidins and defensins are found in tissues and mucous membranes, where epithelial cells

and phagocytes synthesize them to police the numerous microorganisms that plague these surfaces. AMPs are usually encoded in clusters, which results in the simultaneous expression of many peptides that accumulate locally; also, many AMPs are produced as precursors or proforms which need the intervention of proteases to turn active, so AMP expression is not only dependent on expression of the AMP gene product but also on the abundance of useful proteases. These molecules have been widely described, they are quite versatile and usually present a broad spectrum of activity, acting against bacterial, fungal, protozoal and viral organisms.(218,222,225–230)

Although there are thousands of described AMPs and many more under research for further characterization, only few peptide antibiotics, such as polymyxin B, gramicidin S, vancomycin, daptomycin and nisin, (all of bacterial origin) have been used in medical and industrial applications.(231,232) Researchers have composed AMP data bases to facilitate descriptions and further studies, with some collecting over 40,000 entries.(233) Such a diverse group of molecules have been categorized based on their origin, biological function, structural conformation and biochemical properties.(218) There are lots of AMPs based on their source(225,234,235), for instance, mammalian,(236) insect,(237) arachnids,(238) amphibian,(239) fish,(240) plant(241) and even bacterial AMPs.(224,242) Classification based on their biological functions relates to their defensive antimicrobial activity, finding antibacterial, antifungal, antiparasitic and antiviral AMPs; and this classification also considers other activities, finding then even anti-tumour peptides.(225) Regarding their structures, AMPs have been classically divided into: α-helical (LL-37), β-sheet (human β-defensin 1) and extended structures rich in Pro/Trp/His/Lys/Arg amino acid residues (indolicidin) which can be stabilized and looped by disulphide bridges (bactenecin).(223,234,243,244) Finally, AMPs can also be classified according to their biological and chemical properties aminoacidic sequence, length, net charge and hydrophobicity.(218,225,234)

Bacteria produced AMPs are referred to as bacteriocins, they have caused great interest among researchers because of their potent actions against MDR pathogens (usually quite specific and within the pico/nanomolar concentrations).(242,245) Bacteria use AMPs to kill or inhibit their competitor's growth, these molecules provide them with an advantage in their ecological niches allowing them to secure access to nutrients and generally aiding in their survival.(224,242)

Bacteria can synthesize a wide variety of bacteriocins with broad structural diversity, even including D amino acids and nonprotein residues, forming rings, and modifying structures through glycosylation and acylation. Polymyxin B and gramicidin S are some examples of these kind of peptides and which have also been developed as topical antibiotics, while vancomycin and daptomycin are antibiotics specifically active against Gram-positive bacteria.(232)

Bacteriocins are a heterogeneous group of peptides, in Gram-negative bacteria most have been identified in *Enterobacterales* and are classified as microcins or colicins (larger in size), while in Gram-positive bacteria they are classified into lanthionine containing (lantibiotics) and non-lanthionine containing peptides.(242) Lantibiotics are among the most characterized and studied bacteriocins, they have a lanthionine group and thioether-based intramolecular rings as a result of post-translational modifications of residues of serine or threonine and cysteine.(232,246) The lanthionine ring in lantibiotic's structure can serve as a binding motif for target recognition and so it is a key structure for its biological activity; furthermore, the ring provides resistance to proteases.(247) Some examples of lantibiotics are mersacidin and nisin,(232) the latter being the most well-known lantibiotic. Nisin was isolated from *Lactococcus lactis* and has been widely used in the food industry to preserve food thanks to its potent activity against Gram-positive bacteria.(224,225)

Aside from their use as therapeutic agents, bacteriocins have gained interest in the medical sector as bacteriocinogenic probiotics, having an immune-modulatory effect on intestinal mucosa and dendritic cells *in vitro*.(242)

4.5.1.2 Mechanisms of action: membrane disruption, internal targets and immune modulation

Regarding cationic AMPs modes of action, membrane interaction (as either membrane permeabilization, disruption or as non-receptor mediated lysis) is the most characteristic mechanism, while interference with intracellular functions and immunoregulatory functions of organisms have traditionally been regarded as secondary antimicrobial mechanisms (Figure 8). AMPs' structures and conformations are key to their antimicrobial activity, with amphipathic structures having a higher degree of interaction with the membrane of pathogens. Size, charge, secondary structure and amphiphilicity can influence the activity and specificity of AMPs. (218,222,248,249) This interaction can be explained as the electrostatic attraction of positively charged cationic AMPs and the polyanionic regions and structures present on the bacterial surfaces happening first on and through the outer bacterial layers, with teichoic and LTA in Gram-positives and LPS in Gram-negatives, and then with the inner cytoplasmic membranes rich in phospholipids (which have a negatively charged head group). A more specific mechanism has been suggested: the membrane is stabilized by the presence of the divalent cations Ca^{2+} and Mg^{2+} bound to the phosphate groups in the LPS core, AMPs have however a higher affinity for LPS than these cations and therefore, AMPs displace and bind to the LPS. AMPs take a larger space than the displaced cations, causing cracks and permeabilizing the outer membrane, allowing formation of pores and passage of the peptides themselves. The AMP(s)-membrane interaction is independent of the cationic peptide's primary and secondary conformation and facilitated by the amphiphilic nature of AMPs (hydrophobic amino acids mediate peptide absorption).(223,231,234,235,250–254)

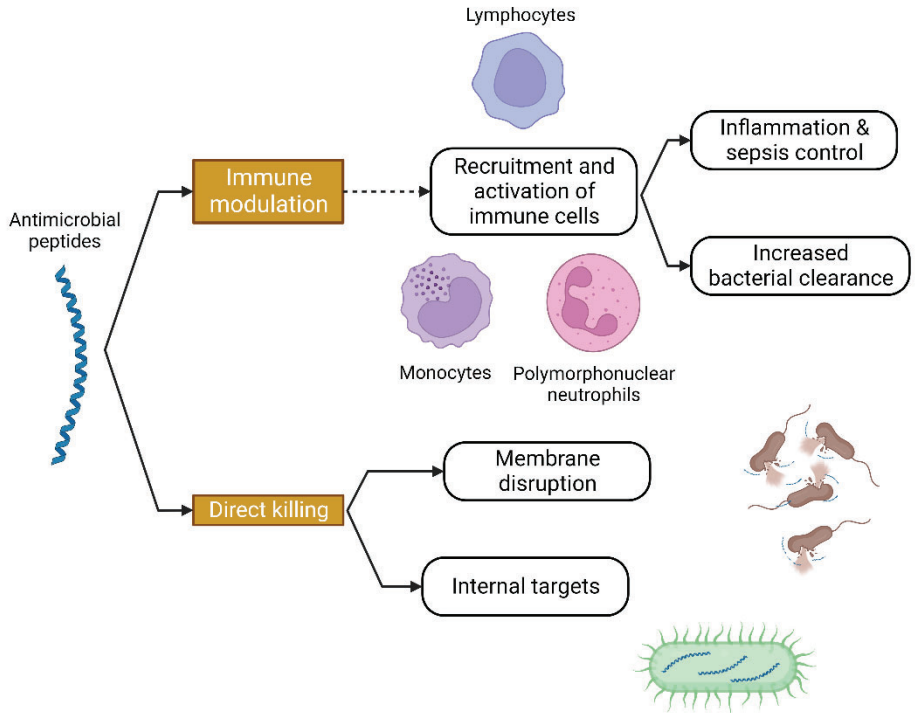

Figure 8. Mechanisms of action of AMPs through direct killing, or immune modulation. Adapted from (232), created with BioRender.com.

The models that explain AMPs interaction with cell membranes consider some degree of peptide conformation flexibility and that the membranes are fluid mosaics where proteins and phospholipids are intertwined and organized in bilayers, creating hydrophobic/philic regions.(222,234) Membrane fluidity and composition, peptide self-assembly ability, and other peptide characteristics such as charge, amphipathicity and hydrophobicity control the concentration threshold that guides AMP interaction with the membrane through any of the following models(255–257)(Figure 9):

- Toroidal pore model (e.g., arenicin, melittin, magainin 2 and lacticin Q): consecutive aggregation of AMPs thrusted into the lipid moieties through the hydrophilic regions causes the membrane to fold inward creating toroidal pores, channels composed of multiple peptides.(222,223,231,234,258)
- Barrer-stave model (e.g., alamethicin): the peptides are inserted parallel to the lipidic inner part of the membrane thanks to interactions between

AMPs hydrophobic regions and phospholipids, creating a transmembrane pore parallel to the membrane lipid residues.(222,223,231,234,259)

- Carpet model (e.g., cecropin P1): the membrane is lysed as a result to the detergent effect that accumulation of AMPs on top its surface has.(222,223,231,234,260,261)

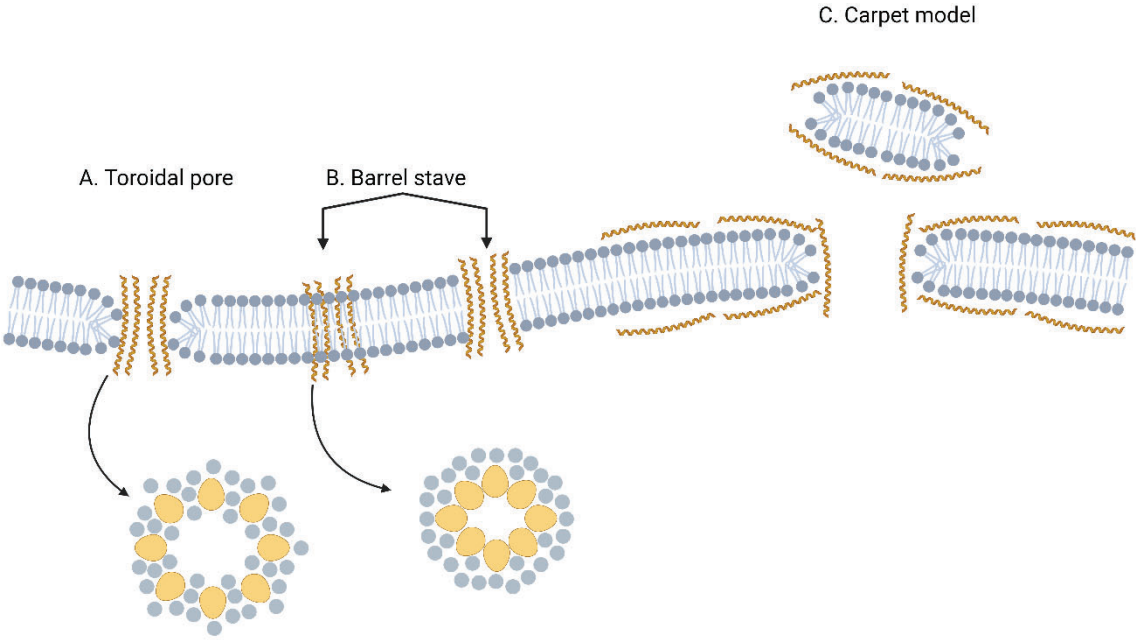

Figure 9. AMPs membrane lytic models with detailed top view (helical AMPs in yellow). Adapted from (222), created with BioRender.com.

Differences between bacterial and mammalian membranes must be considered when addressing AMPs activity upon the latter; ideally a therapeutical AMP would specifically target bacteria while sparing mammalian cells. First, the membranes of mammalian cells are composed of zwitterionic phospholipids (e.g., phosphatidylcholine, sphingomyelin or phosphatidylethanolamine), providing a neutral net charge, plus also of cholesterol, which can reduce AMPs activity since it stabilizes bilayers,(262) affects the fluidity and dipole potential of the phospholipids in the membrane and generally delays the binding of AMPs (Figure 10).(234,263–265) Moreover, eukaryotic membranes further impede binding of AMPs because of their higher transmembrane potential (factor that also regulates peptide binding to cellular envelopes, possibly via electrophoretic action on the polar residues of antibiotics which favours the initial stages of pore

formation)(266,267) when compared to that of pathogenic bacteria,(244,255) and due to the asymmetric distribution of its phospholipids.(227,234,263)

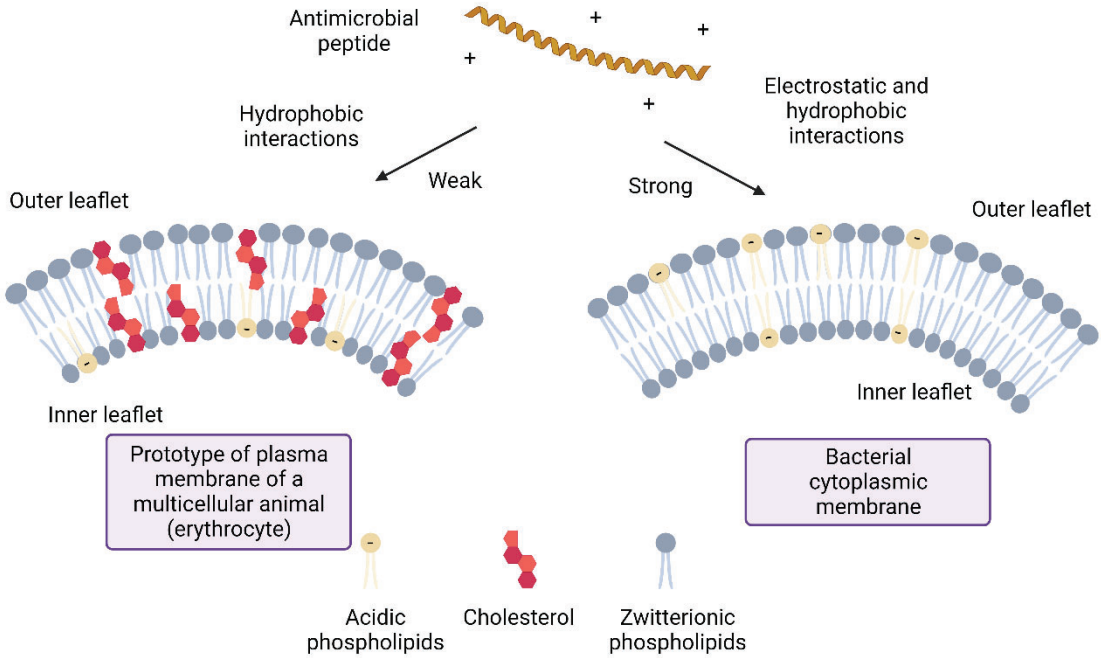

Figure 10. Bases of specificity of AMPs upon bacterial membranes compared to eukaryotic membranes. Adapted from (265), created with BioRender.com.

Coming back to AMPs' mechanisms of action, there is no doubt that at high concentrations the majority of known cationic AMPs interact with and affect the integrity of bacterial membranes, but there are numerous studies that describe the antimicrobial ability of some peptides by interacting with intracellular targets.(218,222,248) It is hypothesized that the antibacterial method, or mode of action, cationic AMPs undergo will depend on the peptide concentration, the growth phase of the affected bacterial cells, host infection localization, the bacterial species and the exact collaborative action of peptide units (which also considers the simultaneous or sequential action of AMPs). Moreover, it is possible that a single cationic AMP could have different bacterial targets. (222–224,231,268–270) But aside from these factors, it is evident that irrespectively of the intracellular target, the interaction between AMPs and membranes directly affects the mode of action.(224) Once AMPs have translocated through the membrane or passed via endocytosis,(222,271) they interfere with bacterial

essential pathways by binding to the molecules involved in these processes (Figure 11), inhibiting: the synthesis of DNA (e.g., CP10A) and proteins (e.g., pleurocidin), chaperone mediated protein folding (e.g., drosocin), formation of cytoplasmatic membrane septum (e.g., indolicidin) and the synthesis of cell wall (e.g., mersacidin).(218,222,248)

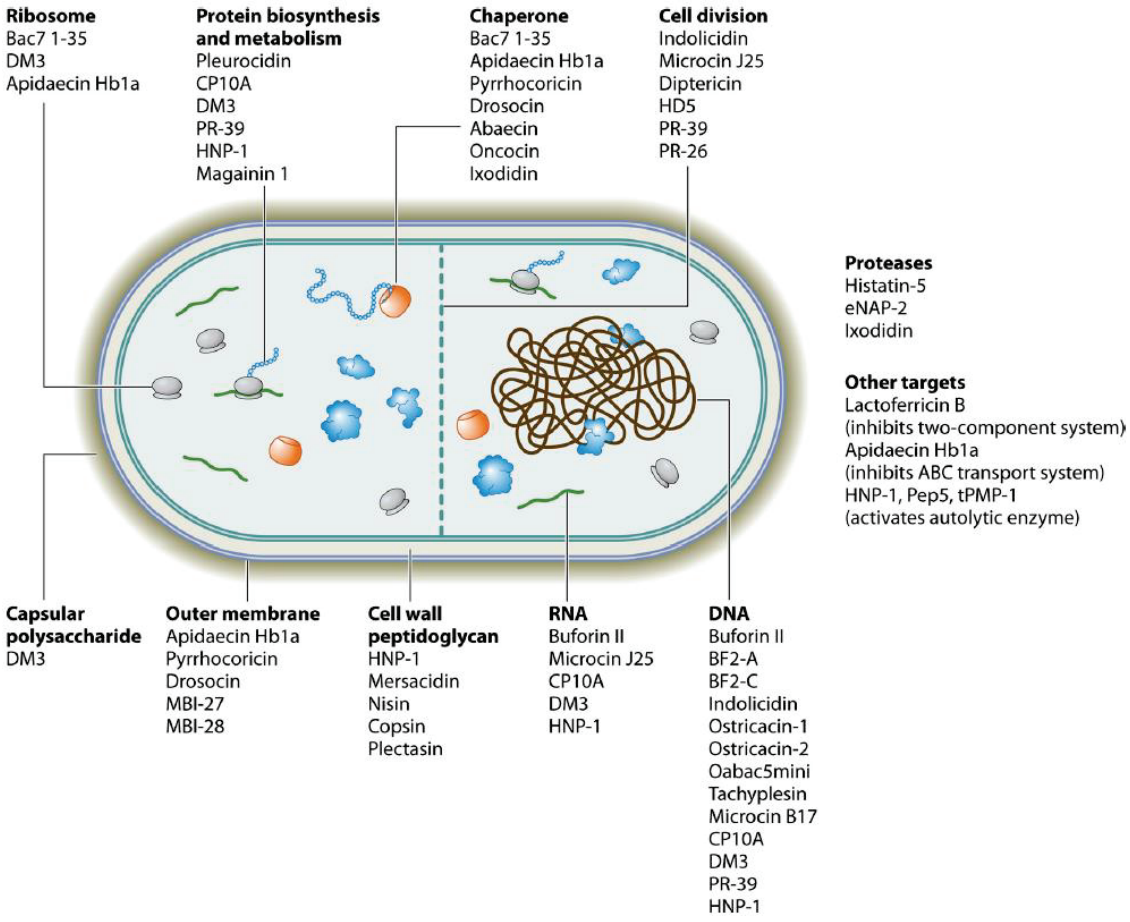

Figure 11. Metabolic pathways targeted by AMPs. Adapted from (222).

Finally, AMPs also affect the immunomodulatory response of the colonized hosts. The term ‘host defence peptides’ was classically used when referring to AMPs whose primary functions are associated with the immunomodulatory, chemoattractant or wound healing functions, (231) although in modern literature the term AMP seems to include all peptides and with more reason after having evidence that some peptides have different mechanisms of action.(99,223) AMPs’ immunomodulatory activity is exerted through the stimulation of

chemotaxis (peptides have been reported interacting with immune cells and promoting their response and recruitment),(272) the control of immune cell differentiation and start of adaptive immunity, and the regulation of production of proinflammatory cytokines (including suppression of toll-like receptors) so excessive inflammatory responses are avoided.(223,227,232,273) As described with the intracellular versus the membrane mechanisms of actions of AMPs, the immunomodulatory effect of an AMP can occur concomitantly with these two; moreover, peptides upregulating the innate immune response could be a novel approach to treating infections: there are descriptions of AMPs whose *in vivo* antibacterial activity is primarily attributed to their immunomodulatory effect.(232,274) Some examples of AMPs with immunomodulatory effects are: defensins upregulate the production of cytokines,(272), LL-37 inhibits the LPS and LTA induced production of the tumour necrosis factor α (TNF-α), IL-6 and IL-8 in human monocytes,(275) and the synthetic peptide IDR-1018 (an innate defence regulator derivative of bactenecin) controls macrophage differentiation towards a M1-M2 intermediate phenotype helping in supressing and regulating inflammatory functions needed to fight infection.(99,276,277)

4.5.1.3 AMPs applications and limitations.

AMPs antimicrobial activity is not limited to their use as single therapeutic agents. The action of AMPs in synergy with other antimicrobials and other AMPs or even in combination with many other AMPs in ‘cocktail’ (a common mechanism of the immune system of insects)(278) has been reported in literature. Different mammalian AMPs from diverse structural classes have been shown to synergize among each other and with human lysozyme,(279) and also double and triple combinations of AMPs from different organisms have synergy against Gram-negative pathogens *in vitro* suggesting this effect may be a common phenomenon in AMPs.(280) Combinatorial therapy provides the advantage of using lower doses of AMPs plus some peptides have been shown to act in synergy with the host’s own AMP arsenal.(280)

Moreover, applications of AMPs upon medical devices as gels, coatings or immobilized in their surfaces to prevent infections derived from implants, catheters and other medical equipment has gained the attention of researchers for its potential to be applied in healthcare settings.(218,281,282)

The use of AMPs as antimicrobials has historically been restricted due to their lack of stability and toxicity. Peptide drugs are generally affected by protease degradation and have poor penetration in intestinal mucosa, discouraging their oral administration; furthermore, rapid hepatic and renal clearance limits their therapeutic applications. All these shorten AMPs bioavailability and circulation time.(99,223,283–286) For this reason, many AMPs are limited to topical use, administered at the wound or site of surgery, or applied via nasal sprays. There are a number of techniques performed on AMPs aimed at reducing degradation and enhancing availability such as changes in formulation or encapsulation (e.g., using a polyethylene glycol hydrogel to encapsulate gold nanorods containing the peptide IK8),(287) cyclization, incorporation of non-natural or D-amino acids, end tagging with hydrophobic oligo amino acid stretches, blocking N- or C- terminus of the peptides with modifications (e.g., acetylation and amidation), the creation of prodrug molecules (e.g., colistimethate, a methane-sulfonated derivative of polymyxin E) or synthesis of peptide mimetics with a custom or non peptidic backbone.(232,286,288–293)

In terms of toxicity, although many AMPs have a more specific activity against bacterial membranes than eukaryotic cells, the varied mechanisms of action that characterise these peptides make them prone to being toxic against the latter,(232) some AMPs even having immunogenic effects.(286) Clinical development of AMPs would greatly benefit for thorough research of apoptosis induction and mast-cell degranulation as a result of systemic application of peptide therapies.(294) Structural modifications of AMPs have been proposed to reduce toxicity, also increasing bioavailability and half-lives. Formulations such as nanocarriers (adsorpting or encapsulating peptides) that allow for local AMP delivery to the infection site, paired with release over time, could be a way around

the toxicity AMPs show when used systemically.(223,295) Some modifications overcoming toxicity are: mastoparan-C analogues which a shorter length and obtained by changing some residues had less toxicity and more availability than the parent peptide,(296,297) nanogels of hyaluronic acid to encapsulate the peptide LLKKK18 (a LL-37 analogue) in an *in vivo* model of mycobacteria, to enhance delivery, protect from proteases,(298) or cyclisation of tachyplesin to create analogues with reduced haemolysis of while maintaining antibacterial activity.(299)

However, aside from toxicity and stability issues and excluding bacteriocins, which can be produced in bacteria by recombinant technology, the biggest challenge AMPs face as novel therapeutics is their high cost of manufacturing through conventional methods such as solid-phase synthesis. Peptides are usually very expensive drugs, which limits their testing and development, and that is not even considering that some of the modifications needed to increase their bioavailability or decrease toxicity further increase their cost of production. A cheaper platform for their production is needed and although attempts have been made (using recombinant DNA methods in bacteria, fungi, plants and animal models) none are feasible under current technology. (232,293)

It is worth mentioning that peptide therapeutics are generally regarded as advantageous compared to other small molecule drugs because after being processed they result into natural amino acids plus their short half-life translates in less tissue accumulation.(223)

4.5.1.4 Resistance mechanisms

Contrary to antibiotics, there is evidence that use of AMPs does not increase mutation rate or enhance recombination frequency in bacteria.(220,221,300,301) Evolution seems to have favoured a low potency and multiple targeted approach for AMPs rather the ‘traditional’ blockage of a specific target with high

affinity.(302,303) Although they might not be the sole reason, these characteristics might have extended the efficiency of AMPs.(232) However, this does not mean that there are no naturally AMP-resistant species of bacteria (for instance, *Serratia, Burkholderia* and *Proteus* spp which are resistant to polymyxins) or that resistance to these antimicrobials cannot develop (Table 3, e.g., through sequestration and export through EPs, modifications of LPS, formation of capsules or overexpression of the outer membrane protein OprH).(230,304)

Table 3. Summary of bacterial resistance mechanisms against antimicrobial peptides classified from Gram-negative and positive bacteria and detailing molecules and compounds involved in the mechanism; adapted from (230).

Mechanism	**Gram-negative bacteria**	**Gram-positive bacteria**
Extracellular proteins	Proteolytic degradation	Proteolytic degradation and sequestration
Exopolymers	Alginate and polysialic acid	Polysaccharide intercellular adhesin and poly-γ-glutamic acid
Surface modification	Repulsion through lipid A phosphate modification, increased outer membrane rigidity by lipid A acylation and the O-antigen of LPS	Repulsion through D-alanylation of teichoic acid, steric hindrance by L-rhamnosylation of wall teichoic acid and modification of lipid II
Efflux pumps	RND family export	ABC transporters export
Cytoplasmic membrane alteration	Increased membrane rigidity through phosphatidyl-glycerol acylation	Charge repulsion through phosphatidyl-glycerol amino-acylation

4.5.1.5 Clinical trials

Currently, there are four peptides in clinical trials, all in phase I and all studied for intravenous administration; three of these peptides are polymyxin derivatives (Table 4). SPR-206 is a new polymyxin derivative by Spero Therapeutics that has

low preclinical MIC values and has just completed phase I (also, a former promising potentiator from the same developer, SPR-741, was discontinued); SPR-206 is active against carbapenem resistant strains of *A. baumannii*, *P. aeruginosa* and *Enterobacterales*. MRX-8 is another polymyxin derivative developed by the company MicuRx, which has shown activity against *A. baumannii*, *P. aeruginosa* and *E. coli*, and that has undergone *in vivo* assays in a neutropenic mouse thigh and lung infection model of *K. pneumoniae* and *A. baumannii.* QPX90003 is a synthetic derivative of polymyxin whose structure has not been disclosed yet, that was developed in a partnership between Monash University (Australia) and the Biomedical Advanced Research and Development Authority for treatment of drug-resistant infections caused by *P. aeruginosa* and *A. baumannii* (phase I started in June 2021). And finally, RG60006 is an antibiotic-macrocyclic peptide compound that is being developed for *A. baumannii* infections by Roche currently in phase I.(80)

Table 4. Peptides currently in clinical trials. Adapted from (80)

Company name	Phase	Antibacterial class	Developer
SPR-206	1	Polymyxin	Spero Therapeutics
MRX-8	1	Polymyxin	MicuRx
QPX9003	1	Polymyxin	Qpex Biopharma
RG6006 (Abx MCP)	1	Macrocyclic peptide	Roche

Out of the few AMPs that have reached clinical trials many have failed. Data of why AMPs have been terminated in clinical trials provides relevant insights about issues of this strategy. For instance, friulimicin B had proven preclinical efficacy in murine infection models and similar properties to those of daptomycin,(305,306) but after transitioning into clinical studies was terminated in phase I due to having an adverse pharmacokinetic profile.(305) Another promising AMP, murepavadin, by Polyphor, was halted in phase III in treatment of patients with nosocomial pneumonia due to acute kidney injuries (after the merging of Polyphor and EnBiotix in 2021, they announced plans to develop this compound as an inhalation treatment for patients with cystic fibrosis with *P. aeruginosa* infections).(80,305)

Aside from safety, toxicity or pharmacokinetic issues, AMPs (and in a broader sense novel antimicrobials) may fail superiority clinical trials against an existing agent used in an indication.(305) Compared to clinical trials for non-infectious disease products, performing superiority trials for therapeutics targeting bacterial infections (being these AMPs or other novel/'non-traditional agents) carries some difficulties and concerns since these infections are associated with striking risks if not treated promptly: they have an acute onset and both high morbidity and mortality risk, moreover quick and precise diagnostics is rare, and patients' outcome usually depends on the speed at which (effective) antimicrobial chemotherapy is provided. Considering trials that compare new agents to a safe and or effective comparator, it is difficult for new agents to clear more effectively an infection (show superiority).(67) On the other hand, equivalence (non-inferiority) trials might represent a better exercise to assess new antimicrobials for bacterial infections as it will be further explained below. Some examples of AMPs that have not shown clear superiority in the past over conventional treatments are surotomycin, pexiganan and omiganan.(305,307)

Most antibiotics are currently approved through non-inferiority trials.(67,92,308) Due to the ever-evolving nature of AMR, the real value of a novel antibiotic is within its potential to effectively treat future patients, that is to say, its potential to provide a 'future superiority' rather than just a 'current non-inferiority'.(67)

Since a novel antibiotic for treating a bacterial infection in a non-inferiority trial is not hypothesized to provide patients a better outcome than they would have had outside of the trial (understood as, the trial's design contemplates not showing superiority), ethical concerns arise regarding if it is acceptable to carry out these trials. However, and as mentioned before, new antibiotics approved through these trials will potentially have great social value if or when they are able to tackle future resistant infections (which could also include the patients already included in the study). Moreover, considering a future scenario where resistance has spread, it would be way more unethical to wait until then to develop novel antimicrobials when we already have the tools to do so.(67,92)

After being studied for many decades, AMPs have not drastically changed the antibiotic pipeline but are nonetheless still promising and potent alternative agents, with great activity against resistant pathogens. The characteristics a AMP needs to be used clinically would include: high antimicrobial activity and specificity, low toxicity (specially against mammalian cellular membranes), proteolytic stability, low serum binding and low cost of manufacturing.(309,310) The limitations regarding stability and toxicity have impacted their development in clinical trials but strategies such as the use of hydrogels and other approaches to encapsulate AMPs and rational design of peptide analogues providing safer, less toxic, and more bioavailable peptides seem robust technologies that could help AMPs overcome these hurdles.(218)

4.5.2 Photoswitchable antimicrobial agents

There has been a rise in photopharmacology beginning around the 2010's. Research on this discipline is based on the conversion of stable isomers of a molecule by different light wavelengths, irreversibly or not depending on the approach. This last concept, a light controlled isomer conversion, is defined as photoswitching and the structures allowing for such conversion are called photoswitches. This concept is interesting since drug isomers will bind to different targets depending on their conformation since a molecule function and interactions are related to their shape.(311,312)

Compound selection to create photoswitchable antimicrobial agents is based in screening compounds that have room for insertion of a photoswitchable structure. So far, five photoswitches have been developed and functionally validated: azobenzene, spiropyran, diarylethene, iminothioindoxyl and acylhydrazone.(311,313,314) The general approach thought when using photoactive drugs is administering them in an inactive state with low toxicity (dark state), and only then use light to switch the compounds into their biologically active state so that only the desired site will be affected by the therapeutic

activity.(312) However, the opposite strategy (switching an antimicrobial to its inactive state when irradiated or generally exposed to light) might represent a much more interesting approach when considering the intricacies of the environmental dissemination of AMR.

Having selective control of antimicrobial activity through photoswitches provides advantages when considering the One Health dimension of AMR or even offering a site-selective control of antimicrobial activity, like limiting development of AMR if the photoswitchable antibiotics revert to inactive states after being secreted to the environment. Complementing technology such as photodynamic therapy, (312) that could help control site activation, are being studied.(311,315)

Modern efforts to build photoswitchable antimicrobial agents have been based on existing structures, developing modified structures of quinolones, gramicidins/tyrocidines and trimethoprims.(311) Production of photoswitchable antimicrobial agents is in early development and constitutes a promising strategy for the future of antimicrobial research and development.

5 WORK JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

In 2017, the WHO published a priority list of antibiotic resistant bacteria to guide global research, discovery and development of antibiotics, and *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were classified in the first tier as critical priority pathogens. Different strategies can be followed to discover and develop new antibiotics, one of them being the design and development of peptides with activity against multidrug-resistant pathogens such as the above-mentioned.

Hypothesis:

Our hypothesis is that two kinds of peptides, lineal and cyclic, could show good *in vitro* antibacterial activity and low toxicity. The structure of these peptides could be optimized by changing their structure, producing derivatives and improving their activity and toxicity. We hypothesize that the reason why the peptides will present a good bactericidal activity is because they would act upon the bacterial membrane.

And finally, there is the issue of environmental antibiotic release and its ecological impact, which enables the emergence of resistant bacteria. Regarding this problem, we hypothesize that producing a photoswitchable antibiotic, that becomes inactive in the presence of sunlight, would be helpful in controlling the spread and increase of resistance in the environment.

Objective:

The main objective was to characterize and optimize the peptides CAP-18, lineal peptide, and PLP-3, bicyclic peptide, in terms of their antimicrobial activity and toxicity, and to develop a photoswitchable molecule with antimicrobial activity.

Specific objectives

1. Optimize CAP-18 derived peptides through antimicrobial activity screening against a collection of multidrug-resistant strains of *A. baumannii* and *P. aeruginosa*.

2. Test if the antimicrobial activity of CAP-18 derived peptides is bactericidal or bacteriostatic against *A. baumannii* and *P. aeruginosa*.

3. Define the *in vitro* biological profile of CAP-18 derived peptides against eukaryotic cell lines and human erythrocytes to obtain toxicity data.

4. Visualize the effects of CAP-18 derived peptides upon the integrity of bacterial cells of *A. baumannii* and *P. aeruginosa* strains.

5. Assess the antimicrobial activity of PLP-3 against a panel of multidrug-resistant strains of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*.

6. Define the *in vitro* toxicity of PLP-3 against human cells and erythrocytes.

7. Investigate PLP-3's membrane permeabilization on *A. baumannii* and *P. aeruginosa* cells.

8. Analyse the antimicrobial activity of photoswitchable tyrocidine A analogues against a panel of Gram-positive and Gram-negative multidrug-resistant strains.

9. Obtain the *in vitro* toxicity values of photoswitchable tyrocidine A analogues against human erythrocytes.

6 RESULTS

Manuscript 1: Novel cathelicidin-based peptides with antibacterial activity against MDR Gram-negative pathogens.

Authors

Javier Moreno-Morales, Daniela Kalafatovic, Marko Babic, Salvador Guardiola, Clara Ballesté-Delpierre, Ernest Giralt and Jordi Vila.

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Abstract

Antimicrobial resistance (AMR) is a modern global health threat. Infections caused by resistant organisms might only be treated with novel antimicrobials, thus the discovery and development of new and effective agents is of importance. Gram-negative MDR bacteria are one of the most concerning menaces related to AMR; finding new and effective antibiotics against these pathogens is of dire need. Antimicrobial peptides (AMPs) are a heterogeneous group of innate defence system peptides with broad antimicrobial activity. In this study, we describe the optimisation of the antimicrobial peptide CAP-18 through the synthesis and characterization of derivatives based on its structure. Derivatives

were selected based on their activity: CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} presented MIC_{90} values between 0.5 and 16 µg/mL against a collection of MDR and colistin resistant strains of *A. baumannii* and *P. aeruginosa*; killing kinetic studies of the peptides on strains of these species showed a strong bactericidal effect particularly against *A. baumannii* strains. These derivatives were less toxic against HeLa than A549 cells, presented low haemolysis against human erythrocytes and a wide therapeutic window: D-CAP-18 was the most haemolytic derivative with an IC_{50} of 274 µg/mL but still was 550 times more selective against *A. baumannii* cells than human erythrocytes. TEM visualization of peptide-treated bacterial samples provided insight into the derivatives mechanism of action, which seems to be unrelated to colistin resistance due to their potent activity on colistin resistant strains, suggesting bacterial membrane damage happened on *A. baumannii* and *P. aeruginosa* strains. CAP-18 derived peptides D-CAP-18, CAP-18_{31} and D-CAP-18_{31} are promising candidates with potent activity against MDR *A. baumannii* and *P. aeruginosa* strains.

Article title: Novel cathelicidin-based peptides with antibacterial activity against MDR Gram-negative pathogens.

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Running title: Cathelicidin-based antimicrobials against Gram negative MDR

Highlights:

- Novel antimicrobial peptides rationally designed from CAP-18 were synthetised.
- CAP-18 derived peptides are potent against *A. baumannii* and *P. aeruginosa* strains.
- Peptides are bactericidal against *A. baumannii* and *P. aeruginosa* strains.
- Derivatives are 300X more selective against bacteria than human erythrocytes.

Abstract:

Antimicrobial resistance (AMR) is a modern global health threat. Infections caused by resistant organisms might only be treated with novel antimicrobials, thus the discovery and development of new and effective agents is of importance. Gram-negative MDR bacteria are one of the most concerning menaces related to AMR; finding new and effective antibiotics against these pathogens is of dire need. Antimicrobial peptides (AMPs) are a heterogeneous group of innate defence system peptides with broad antimicrobial activity. In this study, we describe the optimisation of the antimicrobial peptide CAP-18 through the synthesis and characterization of derivatives based on its structure. Derivatives were selected based on their activity: CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} presented MIC_{90} values between 0.5 and 16 µg/mL against a collection of MDR and colistin resistant strains of *A. baumannii* and *P. aeruginosa*; killing kinetic studies of the peptides on strains of these species showed a strong bactericidal effect particularly against *A. baumannii* strains. These derivatives were less toxic against HeLa than A549 cells, presented low haemolysis against human erythrocytes and a wide therapeutic window: D-CAP-18 was the most haemolytic derivative with an IC_{50} of 274 µg/mL but still was 550 times more selective against *A. baumannii* cells than human erythrocytes. TEM visualization of peptide-treated bacterial samples provided insight into the derivatives mechanism of action, which seems to be unrelated to colistin resistance due to their potent activity on colistin resistant strains, suggesting bacterial membrane damage happened on *A. baumannii* and *P. aeruginosa* strains. CAP-18 derived peptides D-CAP-18, CAP-18_{31} and D-CAP-18_{31} are promising candidates with potent activity against MDR *A. baumannii* and *P. aeruginosa* strains.

Keywords: antimicrobial peptides; AMR; novel antibiotic; bactericidal; cathelicidin

Abbreviations:

AMR Antimicrobial Resistance
AMPs Antimicrobial Peptides
LPS Lipopolysaccharide
MDR Multidrug Resistant
NMR Nuclear Magnetic Resonance
TEM Transmission Electron Microscopy

1. Introduction:

The fact that AMR is a current global health threat is proven by recent estimates: 1.27 million deaths worldwide and 133.000 in the WHO European region are attributable to bacterial AMR only in 2019. [1,2]

Since antibiotics are principal in modern medicine, [3] WHO has produced reports and information to increase public awareness and guide discovery and development. WHO created the 'AWaRe' classification to help conserve the antibiotics that we currently have focusing on One-Health, [4] a pathogen priority list to guide antibiotic development [5] and an annual review of the clinical and preclinical antibacterial pipelines. [6] Both *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are classified into the critical priority category in WHO's pathogen priority list. These two species are usually responsible for healthcare-associated infections; nosocomial infections by multidrug resistant (MDR) bacteria turn into longer hospital stays, increased treatment costs and higher mortality. [7]

A. baumannii's MDR nature and propensity for clonal spread turn it into a dangerous nosocomial pathogen. This species was identified almost 4 decades ago and after developing resistance to most first-line antibiotics, only few and more toxic antibiotics such as colistin remain as a last resort for treating MDR infections of *A. baumannii*. [8–10]

Similarly, *P. aeruginosa* is a common agent in healthcare associated infections with a prevalence of 7.1 to 7.4% of nosocomial infections, being especially

prevalent in intensive care units. [11–13] Although the most common site of infection is pneumonia, *P. aeruginosa* also causes surgical site infections, urinary tract infections and bacteraemia. Infections by MDR strains are devastating in paediatric burn intensive care units, being one of the most prevalent etiological agents. [14,15]

There is a dire need for novel antibiotics and a number of alternative approaches are studied in antibacterial discovery. These modern strategies include monoclonal antibodies to neutralize bacterial toxins, the use of phage therapy, direct targeting of virulence factors, nanoparticles, antisense oligonucleotides that inhibit gene expression, vaccines and research in antimicrobial peptides (AMPs). [16–18]

AMPs are ubiquitous immune effectors used by multicellular organisms to kill and destroy microbes. [19,20] They have a wide antimicrobial action and act against viruses, fungi, yeast and bacteria. [21,22] AMPs are likely to produce resistance in bacteria in a slower or limited pace due to their non-specific and rapid action. [23,24]

AMPs are usually 10 to 50 residues long and are composed of hydrophobic, hydrophilic and cationic residues, the latter aiding in a total positive net charge that is key for their antimicrobial action. The positive charge establishes initial binding of AMPs to membranes through electrostatic interactions. However, one key aspect of their action upon bacteria is the amphipathic effect that hydrophobic and hydrophilic amino acids produce allowing their structural insertion into bacterial membranes. [24,25]

AMPs not only produce a fast disruption on the bacterial membrane structure through non-specific interactions but after crossing into the cell, they might reach intracellular targets that control protein folding, cell division, synthesis and metabolism of nucleic acids, cell wall and protein. [24,26–28]

Cathelicidins are a group of peptides with different structure, size and amino acid sequence. They are stored in neutrophils, macrophages and epithelial cells, and

released upon leukocyte activation from within secretory granules. Immune system peptides were classified into the cathelicidin family based on the presence of a conserved cathelin domain at their N-terminus. The C-terminus domain (about 12 to 80 residues in length) is inter and intraspecies diverse and usually has α-helical or β-hairpin conformations and can be rich in Proline and Arginine. [29–32] Cathelicidins are stored as prepropeptides (inactive precursors) in neutrophil granules and cleaved by elastases before being released as mature peptides. [33]

CAP-18 is an 18 kDa cationic peptide isolated from rabbit granulocytes. It is 37 amino acids in length (GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLAPRTDY) and has potent antimicrobial properties: it has been shown to bind and neutralize lipopolysaccharide (LPS), interact with inflammation pathways and protect mice from LPS at high concentrations. [34–36] CAP-18's secondary structure is a rigid α-helix with patches of cationic and hydrophobic groups that could facilitate interaction with bacterial structures thus promoting its antimicrobial activity. [37]

This study aims to analyse and optimize the antimicrobial activity of CAP-18 through the synthesis of peptide analogues derived from its structure, against a collection of *A. baumannii* and *P. aeruginosa*. In addition, the aim is to define the *in vitro* biological profile against human cell lines and erythrocytes as well as to infer the potential mechanism of action from Transmission Electron Microscopy (TEM) of the peptides with the most potent activity.

2. Materials and methods:

Peptide synthesis:

The original CAP-18 peptide was synthesized at the Institute for Research in Biomedicine (IRB, Barcelona, Spain). Peptides CAP-18_{31}, CAP-18_{21}, CAP-18_{23}, CAP-18_{19}, CAP-18_{18} and CAP-18_{14} were synthesized at Iproteos (Barcelona, Spain). Finally, peptides D-CAP-18, D-CAP-18_{31}, RETROE CAP-18, RETROE CAP-18_{31} (Table 1) were synthesized by GenicBio (Shanghai, China).

Table 1. Sequences of peptides used in the study.

Peptide	Sequence
CAP-18	GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLAPRTDY
CAP-18_{31}	GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKL
CAP-18_{21}	GLRKRLRKFRNKIKEKLKKIG
CAP-18_{23}	FRNKIKEKLKKIGQKIQGLLPKL
CAP-18_{19}	IKEKLKKIGQKIQGLLPKL
CAP-18_{18}	FRNKIKEKLKKIGQKIQG
CAP-18_{14}	IKEKLKKIGQKIQG
D-CAP-18	glrkrlrkfrnkikeklkkigqkiqgllpklaprtdy
D-CAP-18_{31}	glrkrlrkfrnkikeklkkigqkiqgllpkl
R-CAP-18	ydtrpalkpllgqikqgikklkekiknrfkrlrkrlg
R-CAP-18_{31}	lkpllgqikqgikklkekiknrfkrlrkrlg

CAP-18 (Mw=4433,48) was synthesized at IRB through solid phase synthesis (Table 2) on the CEM Liberty Blue™ Automated Microwave-assisted Peptide Synthesizer at 0.10 mmol scale using Wang resin preloaded with Tyr (1.03 mmol/g substitution). The resin pre-loading was performed manually on the Wang resin through the ester bond formation. 0.516 mmol of resin (1.03 mmol/g substitution) was swelled in DCM (45 min). The coupling was performed in DCM

using 4 eq Fmoc-Tyr(tBu)-OH, 4 eq DIC and 0.4 eq DMAP for 30 min and repeated three times. The new loading was determined (1.03 mmol/g). [38]

To allow the chain growth, a series of deprotection, coupling and washing steps was performed. 20% w/v piperidine in DMF with 0,1M Oxyma was used for Fmoc deprotection. Post-deprotection washing with DMF (3 x 2 mL) was followed by coupling using a 5-fold excess of reagents: Fmoc-AA-OH (0.2 M in DMF, 2.0 mL), DIC (0.5 M in DMF, 1.0 mL) and Oxyma (1.0 M in DMF with 0.1M DIPEA, 0.5 mL). After each coupling, the resin was washed with DMF (5 x 3 ml). Several amino acids were double coupled (in bold): **G**-**L**-**R**-K-**R**-**L**-**R**-**K**-**F**-**R**-N-**K**-**I**-**K**-E-**K**-**L**-K-**K**-**I**-G-Q-**K**-**I**-Q-**G**-**L**-**L**-P-**K**-**L**-A-**P**-**R**-T-D-Y.

After synthesis, the resin was rinsed with DMF (3 x 5 ml), DCM (3 x 5 ml) and diethyl ether (5 x 5 ml). The peptide was cleaved from the resin (4 h) with TFA/TIS/H2O (95/2.5/2.5) and precipitated in cold ether. The obtained crude material was analysed without any purification (92% pure). Following, the peptide was purified using preparative HPLC.

Table 2. CAP-18 synthesis characterization.

Peptide	Molecular formula	Mw (g/mol)	$[M+H]^+$	Rt (min), UPLC	Purity
CAP-18	$C_{202}H_{356}N_{64}O_{47}$	4433,48	4434,48	1.35	>95%

Crude peptides were analysed on a Waters UPLC H-class coupled to an electrospray ion source ESI-MS Micro mass ZQ, using acetonitrile/water with 0.1 % TFA (gradient: 0-100% acetonitrile) as the solvent system on a BEH C18-column (internal diameter, 2.1 mm; length, 50 mm; particle size, 1.7 µm). Detection was at 214 nm. Purification was performed using preparative HPLC (gradient: 10-45% acetonitrile). Purity was checked by analytical reverse-phase

UPLC (supplementary figure 1). LC-MS was used to confirm the identity of the obtained peptides (supplementary figures 2 and 3).

Bacterial strains:

The antimicrobial activity of the peptides was tested against a collection of Gram-negative bacilli, *A. baumannii* and *P. aeruginosa*, selected by their resistance or susceptibility to colistin and/or classified as MDR strains. The origin of the strains was either clinical or commercial strains belonging to the American Type Culture Collection (ATCC), that complemented the collection for comparison and control (Supplementary Tables 1 and 2).

Supplementary Table 1. MIC results for *A. baumannii* strains. * Marks MDR strains.

A. baumannii strains	MIC (µg/mL)				
	Colistin	CAP-18	CAP-18_{31}	D-CAP-18	D-CAP-18_{31}
ATCC 17978	≤0,125	0.25	0.5	0.25	0.5
8*	≤0,125	≤0.125	0.5	0.25	0.25
19*	≤0,125	0.25	0.5	0.5	0.5
80*	≤0,125	0.25	0.25	0.5	0.25
174	≤0,125	0.25	0.25	0.25	0.25
180	≤0,125	0.25	0.25	0.25	0.5
226*	≤0,125	0.25	0.25	0.25	0.25
253*	≤0,125	≤0.125	≤0.125	≤0.125	≤0.125
451*	≤0,125	0.25	0.5	0.5	0.25
CR17*	>64	0.5	1	0.5	0.5
CR49*	64	≤0.125	0.5	0.25	0.5
CR86*	>64	0.25	0.5	0.25	0.5
210*	32	≤0.125	0.5	0.5	4
42*	0,5	0.25	0.25	0.25	0.25
CS01	1	0.5	0.5	0.5	0.5

Supplementary Table 2. MIC results for *P. aeruginosa* strains.

P. aeruginosa strains	MIC (µg/mL)				
	Colistin	CAP-18	CAP-18_{31}	D-CAP-18	D-CAP-18_{31}
S1	≤0,125	≤0,125	0,5	0,25	0,25
S2*	≤0,125	≤0,125	0,5	0,25	0,5
R2	64	2	8	2	4
S3	≤0,125	0,5	2	0,25	4
S4	≤0,125	≤0,125	0,5	≤0,125	0,5
S5	≤0,125	0,25	1	0,5	0,5
R3*	64	1	8	2	4
36a*	0,25	0,5	1	1	2
38a*	≤0,125	1	4	1	4
ATCC 27853	≤0,125	1	4	2	4
C1	0,25	4	16	2	16
C2	0,5	0,25	16	1	16
C3	≤0,125	0,25	2	2	8
C4	0,25	4	4	1	8
C5	≤0,125	1	2	1	4

Antimicrobial susceptibility testing:

Minimum Inhibitory Concentration (MIC) of the antimicrobial agents against bacterial strains was obtained through broth microdilution assays following a protocol based on the Clinical & Laboratory Standards Institute's guidelines. [39] Modifications regarding microplate material and broth were included when testing the peptides' activity and are detailed below. Colistin MICs were obtained following recommendations of the joint CLSI-EUCAST Polymyxin Breakpoints Working Group using Cation Adjusted Mueller-Hinton Broth (90922, 500g Sigma-Aldrich®, MO, USA) and strains *Escherichia coli* 13846 (*mcr*-1 positive) and *P. aeruginosa* ATCC 27853 were used as quality controls in all assays. [40] Bacteria were cultured in Columbia Agar plates (Columbia Agar with 5 % Sheep Blood 90

mm Stacker plates, Becton Dickinson, NJ, USA) and incubated overnight at 37°C. Polypropylene round bottomed 96-well plates (96-well Polypropylene Storage Microplates, 267334, Thermo Fischer Scientific, MA, USA) were used to prevent peptide binding to plate walls. AST broth (BD Phoenix™ AST broth, 8 mL, 246003, Becton Dickinson, NJ, USA) was used when testing the peptides' activity. MIC values were determined after 18 to 22h incubation at 37° C. Three biological and technical replicates were performed per strain.

When testing MICs at physiological albumin concentrations, AST supplemented with 40 mg/mL of human albumin (A1653-5G, Merck, MA, USA) was used following broth microdilution protocol detailed above to test antimicrobial activity of peptides. Non supplemented AST broth was used as a control of each peptide against the selected bacterial strains. Strains tested were *A. baumannii* CR17 and ATCC 17978 and *P. aeruginosa* R3 and ATCC 27853, a colistin-resistant and colistin-susceptible strain of each species respectively. Peptide range tested included serial dilutions from 16 to 0.03 µg/mL. Three biological and technical replicates were included per strain.

Haemolysis Assays:

Commercially available human blood (IWB1K2E10ML, Innovative Research Inc, MI, USA) was used to produce a 50% haematocrit solution by mixing at 1:1 with PBS 1X (P5493, 1L, Sigma, MO, USA). Three consecutive PBS 1X washes plus centrifugation at 2,600 rpm for 10 minutes steps were performed. Finally, a 2% erythrocyte solution was prepared in PBS 1X and chilled on ice until used.

Polypropylene plates were filled with 50 µL PBS 1X per well for the samples and 50 µL 2% Triton X-100 (TX-100, 9002-93-1, 100mL, Sigma, MO, USA) for the haemolysis controls. A solution at 4X final concentration of the assay of each peptide was prepared in PBS and 50 µL were added to the first column of each plate, followed by serial dilutions in the microplate. Then, 50 µL of the 2% erythrocyte solution was added to each plate. Positive controls consisted in a 1:1 mix of PBS 1X and erythrocytes at 2%.

Plates were incubated at 37 ºC for 4h and then centrifuged at 1,500 rpm for 5 minutes. Eighty µL were extracted from the supernatant of each well carefully and transferred to a polystyrene flat bottom plate for reading and read at 450 nm in an Epoch microplate spectrophotometer (Epoch™, BioTek, VT, USA).

Cytotoxicity Assays:

Cell viability assays were performed against HeLa and A549 cells. Cells were seeded in Corning 96-well microplates at 2 x 10^3 cells/well and 5 x 10^3 cells/well and incubated 24h at 37 ºC. Then, cells were treated with peptides dissolved in Dulbecco's Modified Eagle's Medium (high glucose, 10% FBS) for 24h; peptide concentration range 0.1-100 µM. Peptide solution was then removed, and fresh medium was added to the cells. Finally, 50 µL of activated-XTT solution was added and incubated for 4h at 37 ºC. A PowerWave X reader (BioTek, VT, USA) was used to measure absorbance at 450 nm. Cell viability was calculated through the ratio of the absorbance of peptide treated cells over absorbance of untreated cells. Triplicates were run for each experiment.

Time-Kill kinetic assays:

Time killing curves were designed to test MIC, 2xMIC, 4xMIC and 8xMIC based on MIC data from peptides CAP-18, CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} against the strains *A. baumannii* CR17, ATCC 17978, *P. aeruginosa* R2 and 121110 (Supplementary Tables 1 and 2). Timepoints to withdraw aliquots of liquid cultures for each peptide, concentration and strain were established at: 0, 2, 4, 8 and 24 h after inoculation.

McFarland 0.5 solutions (~1.5 · 10^8 CFU/mL) were prepared from single picked colonies grown O/N at 37 ºC in Columbia Agar plates.

Thirteen mL tubes were used to prepare solutions containing 5 mL of total volume by adding: 1 mL of peptide solution at 5 times chosen assay concentration to test, 3.75 mL AST and 0.25 mL of a 1:10 McFarland dilution in AST. Positive controls

contained 4.75 mL AST and 0.25 mL of strain inoculum diluted in AST; negative controls only contained 5 mL of AST.

After adding the inoculum into each tube and vortexing, a 150 µL aliquot was taken from each tube at timepoint 0h. All tubes were then placed in a 37 ºC shaker -incubator at 180 rpm.

Likewise, for timepoints 2, 4, 8 and 24h, a 150 µL aliquot was withdrawn at each time for each tube. Each of these aliquots was serially diluted in 1:10 ratio in PBS 1X and serial dilutions spread in LB agar plates and incubated O/N at 37º C.O/N. Colonies grown in LB agar plates were counted.

Bacterial concentration (CFU/mL) for each strain, timepoint and peptide concentration tested were calculated. Bactericidal effect was considered when there was a decrease of at least 3 in log CFU/mL for each peptide concentration at a given timepoint against each bacterial strain studied. Three replicates were run per peptide and strain.

Transmission Electron Microscopy sample preparation and visualization:

Bacterial strains *A. baumannii* CR17 and *P. aeruginosa* R2 and 121110 under treatment with CAP-18, CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} were chosen for TEM visualization.

Five mL AST broth cultures were prepared in 50 mL Falcon tubes and inoculated with single colonies grown in Columbia Agar incubated at 37ºC. Liquid cultures were then diluted 1:100 in fresh AST broth and incubated at 37 ºC until OD 0.6 was reached. Cultures were then centrifuged 10 min at 3000 g at 4 ºC, and supernatant was discarded and replaced by fresh AST.

Fifty mL Falcon tubes were filled up to 5 mL with 4 mL of 0.6 OD inoculum and 1 mL of peptide treatment at 4x or 8xMIC and incubated at 37 ºC for either 2 or 4h, depending on the strain and peptide. Results from TKC assays were used as a

guidance to choose peptide concentrations and incubation times for TEM sample visualization.

After incubation, tubes were centrifuged thrice for 15' at 5000 g and 4 ºC substituting supernatant after each centrifugation by adding 10 mL PBS 1X to the pellet and resuspending. Supernatant was discarded after the final centrifugation step.

Pellet was fixed by adding 1 mL of 2.5% glutaraldehyde + 2% paraformaldehyde in PBS 0,1M at pH 7,4, and centrifuged 10' at 5000 g and 4 ºC. Supernatant was discarded 30 min after centrifugation and pellet was washed twice with 1 mL of fresh fixing solution. Samples were stored 4 ºC O/N and processed by Electronic Microscopy Unit, Medicine Faculty, University of Barcelona. Images were obtained in a JEOL 1010 transmission electron microscope using a CCD Orius camera (Gatan).

3. Results:

Antimicrobial susceptibility testing:

Antimicrobial activity screening of CAP-18 and 7 derived peptides was performed against a colistin-resistant and colistin-susceptible strain of each *A. baumannii* and *P. aeruginosa* (Table 3). This screening aimed to select peptides with most potency against representative strains of these pathogens.

Table 3. Initial antimicrobial activity screening of CAP-18 and 7 derived peptides against a colistin-resistant and susceptible strain *A. baumannii* and *P. aeruginosa*. MIC results in µg/mL.

		MIC (µg/mL)							
Species	Strain	CAP-18	CAP-18_{31}	CAP-18_{23}	CAP-18_{21}	D-CAP-18	D-CAP-18_{31}	R-CAP-18	R-CAP-18_{31}
A. baumannii	CR17	0.5	1	32	32	0.5	0.5	2	1
	CS01	0.5	0.5	16	2	0.5	0.5	2	1
P. aeruginosa	121007	1	8	>64	32	2	2	2	0.5
	121110	≤0,125	0,5	>32	32	0,25	0,25	32	4

CAP-18, CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} were selected for further antimicrobial activity characterization due to the potency shown in initial screening results against selected strains. Next, broth microdilution assays of these 4 peptides were done against a panel of 30 bacterial strains from *A. baumannii* and *P. aeruginosa* (15 strains per species) to obtain MIC_{50} and MIC_{90} values (Table 4). Colistin was included as control to allow the comparison of the tested peptides' activities against the panel not only due to its peptidic nature but also since it is the last resource antibiotic used in the clinic.

Table 4. MIC_{50} and MIC_{90} (in µg/mL) values of the bacterial strain panel against CAP-18, CAP-18_{31}, D-CAP-18, D-CAP-18_{31} and colistin.

	A. baumannii		*P. aeruginosa*	
Antimicrobial	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
CAP-18	0.25	0.5	0.5	4
CAP-18_{31}	0.5	0.5	2	16
D-CAP-18	0.25	0.5	1	2
D-CAP-18_{31}	0.5	0.5	4	16
Colistin	≤0,125	>64	≤0,125	64

Colistin MIC_{90} values were at 64 µg/mL for both species tested since the strain collection was selected deliberately. However, all peptides MIC_{90} values were below the established threshold against these species, showing great activity against a heterogeneous and resistant panel of strains.

Due to the potency shown against both species, all four peptides were deemed interesting for further research regarding toxicity, killing kinetics and microscopy characterization against representative strains of both species.

Antimicrobial activity analysis in human albumin supplemented media:

Overall, MIC values in albumin supplemented media for all 4 peptides tested were over MICs in not-supplemented media against all strains tested (Table 5).

Table 5. MIC in μg/mL of CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} against *A. baumannii* and *P. aeruginosa* strains. Albumin 40 mg/mL supplemented media is indicated as 'AST + Albumin' and non-supplemented media as 'AST'. MIC fold changes obtained by subtracting non-supplemented to albumin supplemented media. *Indicates that MIC fold differences might be larger since albumin supplemented MIC is over the assay range (from 16 to 0.03 μg/mL).

		MIC (μg/mL)											
		CAP-18			CAP-18_{31}			D-CAP-18			D-CAP-18_{31}		
Species	Strain	AST + Albumin	AST	MIC fold change	AST + Albumin	AST	MIC fold change	AST + Albumin	AST	MIC fold change	AST + Albumin	AST	MIC fold change
A. baumannii	CR17	4	0.5	3	4	1	2	4	0.5	3	4	0.5	3
	ATCC 17978	8	0.25	5	8	0.5	4	8	0.25	5	8	0.5	4
P. aeruginosa	R3	16	1	4	16	8	1	16	2	3	16	4	2
	ATCC 27853	8	1	3	16	4	2	8	2	2	16	4	2

Peptide MICs in albumin supplemented media against *A. baumannii* strains were still within the tested peptide range (16 to 0.03 µg/mL). However, there were large MIC fold changes when comparing MICs in both media. In albumin supplemented media, both CAP-18 and D-CAP-18 had an MIC of 8 µg/mL against strain 17978 while in not-supplemented media both peptides had an MIC of 0.25 µg/mL. This translates to a 5-fold change / difference in their MIC value against the strain 17978 between both media conditions for these peptides.

Regarding *P. aeruginosa*, only CAP-18_{31} against strain R3 kept a single MIC fold change (from 8 µg/mL to 16 µg/mL in supplemented media) while the MICs of the other 3 peptides against this strain attained larger fold differences (4-fold for CAP-18, 3-fold for D-CAP-18 and 2-fold for D-CAP-18_{31}); strain 27853 also registered higher MIC values in albumin supplemented media for all peptides, up to 16 µg/mL for CAP-18_{31} and its enantiomer D-CAP-18_{31}, but still within the tested peptide range.

Haemolysis and cytotoxicity assays:

Human erythrocyte haemolysis by CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} was analysed. Peptide absorbance is expressed as a ratio to total haemolysis with TX-100 incubation (Supplementary Figure 4).

All peptides had haemolysis under 10% for concentrations <32 µg/mL (that concentration being 7.2 µM for CAP-18 and its D-isomer and 8.5 µM for CAP-18_{31} and its respective D-isomer), suggesting low toxicity against erythrocytes at clinically relevant concentrations especially when comparing haemolysis IC_{50} values (Table 6) to MIC_{90} values per pathogenic species (Table 4) or when considering MIC values against pathogenic strains in albumin supplemented medium (Table 5).

Table 6. Peptide haemolysis and cell viability IC_{50} values.

Peptide	Haemolysis IC_{50} (μg/mL)	A549 IC_{50} (μM)	HeLa IC_{50} (μM)
CAP-18	158.41	0.87	9.12
CAP-18_{31}	690.97	1.31	13.1
D-CAP-18	274.76	2.09	2.11
D-CAP-18_{31}	404.48	12.32	6.16

A preliminary therapeutic window of each peptide against each pathogen tested was obtained by dividing haemolysis IC_{50} values over each peptide and species MIC_{90} value [41] (Table 7).

Table 7. Ratio between haemolysis IC_{50} and MIC_{90} of each peptide against the pathogen species tested.

Peptide	Therapeutic window (IC_{50}/MIC_{90})	
	A. baumannii	*P. aeruginosa*
CAP-18	316.8	39.6
CAP-18_{31}	1381.9	43.2
D-CAP-18	549.5	137.4
D-CAP-18_{31}	809.0	25.3

As expected, the widest therapeutic window was found against *A. baumannii* since the peptides' activity against this species was the most potent; CAP-18_{31}'s ratio against this *A. baumannii* was quite remarkable since it had the highest IC_{50} at 690.97 μg/mL, meaning CAP-18_{31} was 1381-fold more selective against *A. baumannii* cells compared to human erythrocytes.

Peptide therapeutic ratios against *P. aeruginosa* were significantly smaller when compared to those found against *A. baumannii*. D-CAP-18 was the least haemolytic peptide, being 137-fold more selective against *P. aeruginosa* than for human erythrocytes while the peptide with the narrowest therapeutic window was also the D-isomer D-CAP-18_{31} with just 25-fold improvement.

Regarding cell viability assays, high toxicity values were recorded for peptides tested against A549 cells (Table 6). D-isomers had lower toxicity against this cell type than their enantiomers. CAP-18 had an IC_{50} value of 0.87 μM (3.8 μg/mL), while D-CAP-18 at 2.09 μM (9.2 μg/mL). The least toxic peptide was D-CAP-18_{31} at 12.32 μM (45.9 μg/mL).

Toxicity values were lower against HeLa cells than A549 for all peptides. The least toxic peptides were CAP-18_{31} at an IC_{50} of 13.1 μM (48.8 μg/mL) and CAP-18 at 9.12 μM (40.4 μg/mL). Contrary to the results seen in A549 cells, D- isomers fared worse, D-CAP-18 being the most toxic peptide against HeLa cells at an IC_{50} of 2.11 μM (9.35 μg/mL) which was however close to its IC_{50} value against A549 cells.

Time-Kill kinetic assays:

In vitro antibacterial activity of compounds CAP-18, D-CAP-18, CAP-18_{31} and D-CAP18_{31} against *A. baumannii* and *P. aeruginosa* strains were studied through time killing kinetics.

For *A. baumannii* CR17 (Figure 1) CAP-18 had fast and potent antimicrobial activity since it reached cidal concentrations at all concentrations tested at 2h incubation timepoint; bacterial concentration decayed at 2xMIC to 0 CFU/mL at 8h although growth was observed after 24h, but for 4xMIC and 8xMIC bactericidal effect was seen since the first timepoint at 2h (Figure 1 a). D-CAP-18 also had potent antimicrobial activity against this strain, bactericidal effect was seen at 2xMIC, 4xMIC and 8xMIC at 2h; however, there was regrowth at 2xMIC and only 4xMIC and 8xMIC maintained 0 CFU/mL up until 24h (Figure 1 c). For CAP-18_{31}, bactericidal effect was recorded at 2h for 2xMIC, 4xMIC and 8xMIC and even for 1MIC at 4h; the same effect was observed as with the previous peptides, where 4xMIC and 8xMIC remained at 0 CFU/mL until the last timepoint (Figure 1 b). The shorter peptide enantiomer D-CAP-18_{31} had cidality at 2xMIC after 2h, and at 4h for both 4xMIC and 8xMIC; there was regrowth at all concentrations but the latter, where 0 CFU/mL remained constant until 24h incubation (Figure1 d).

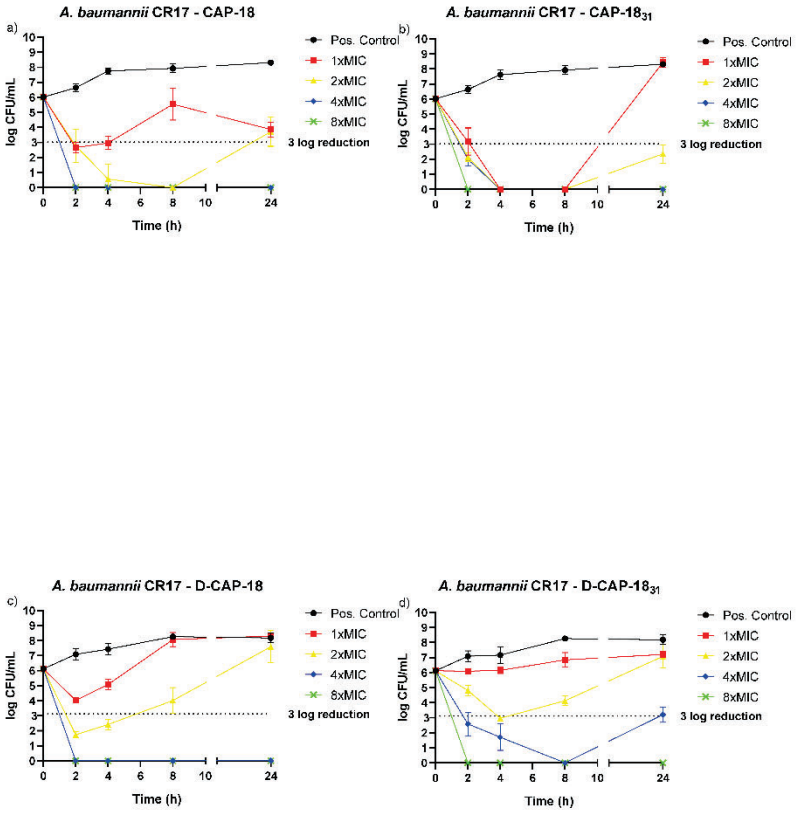

Figure 1. *A. baumannii* CR17 TKCs against a) CAP-18; b) CAP-18$_{31}$; c) D-CAP-18; d) D-CAP-18$_{31}$.

For *A. baumannii* 17978 (Figure 2), CAP-18 presented bactericidal effect at 4xMIC after 8h incubation whilst this effect was seen as soon as 4h at 8xMIC, although there was regrowth for all concentrations tested at 24h (Figure 2 a); the enantiomer D-CAP-18 was bactericidal on this strain after 4h incubation at 8xMIC by reaching the lowest CFU/mL value for this curve although growth recovered over time until 24h (Figure2 c). CAP-18$_{31}$ provided bactericidal effect both at 4xMIC and 8xMIC after 4 and 2h incubation respectively; bacterial concentration at 8xMIC and 8h had its lowest value for this peptide's curve but it also grew back afterwards (Figure2 b). D-CAP-18$_{31}$ behaved similarly to its enantiomer and was bactericidal at 4xMIC and 8xMIC at 4 and 2h respectively; it is noteworthy that

bacterial concentration stayed at 0 CFU/mL at 8xMIC from 4 to 8h incubation even if bacteria concentration increased up to 24h after that (Figure2 d).

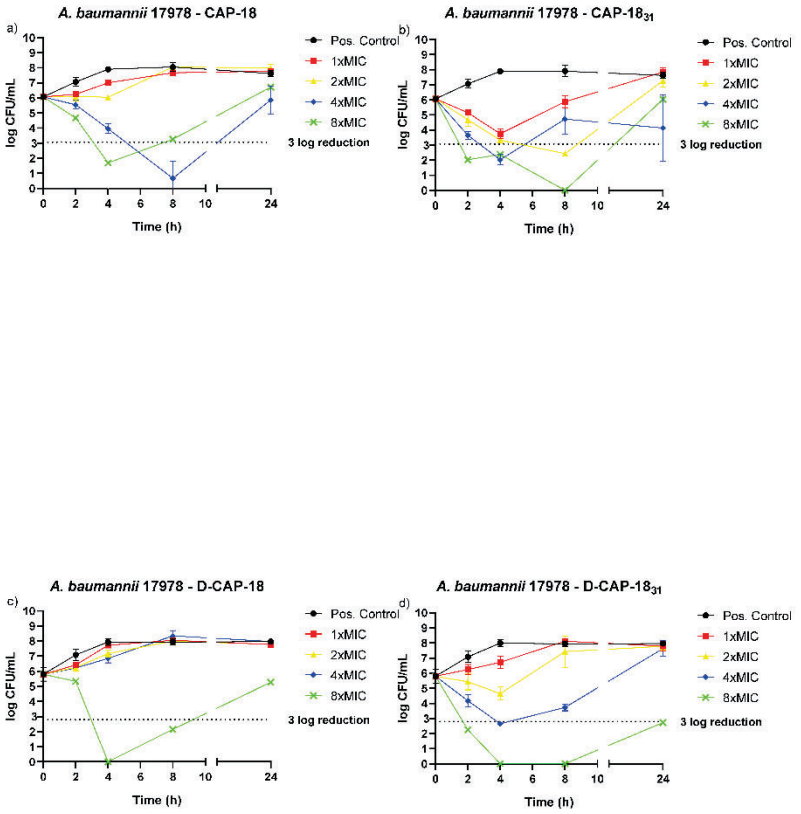

Figure 2. *A. baumannii* 17978 TKCs against a) CAP-18; b) CAP-18$_{31}$; c) D-CAP-18; d) D-CAP-18$_{31}$.

Regarding *P. aeruginosa* R2 (Figure 3), CAP-18 was bactericidal at 4xMIC after 8h incubation and at 8xMIC at 4h; both these concentrations were completely bactericidal for strain R2 which stayed at 0 CFU/mL even after 24h incubation at 37 ºC (Figure 3 a). The enantiomer D-CAP-18 had a longer incubation time (8h) to reach bactericidal activity at 4xMIC but stronger effect than CAP-18 at 8xMIC since at this same concentration cidal effect was recorded as early as 2h and CFU/mL, which remained at 0 between 4 and 8h incubation time (Figure 3 c). The shorter peptide, CAP-18$_{31}$ showed a strong cidal effect over time: this peptide

was bactericidal at 4h incubation for both 4xMIC and 8xMIC, but also at 2xMIC at 8h, reaching 0 CFU/mL at this timepoint for all three concentrations; however, bacterial regrowth was recorded at all concentrations of the peptide back to 24h incubation but for 8xMIC (Figure 3 b). The enantiomer D-CAP-18$_{31}$ had similar activity for 4xMIC and 8xMIC, however it was bactericidal at 8xMIC sooner at 4h than at 4xMIC which was seen at 8h incubation (Figure 3 d).

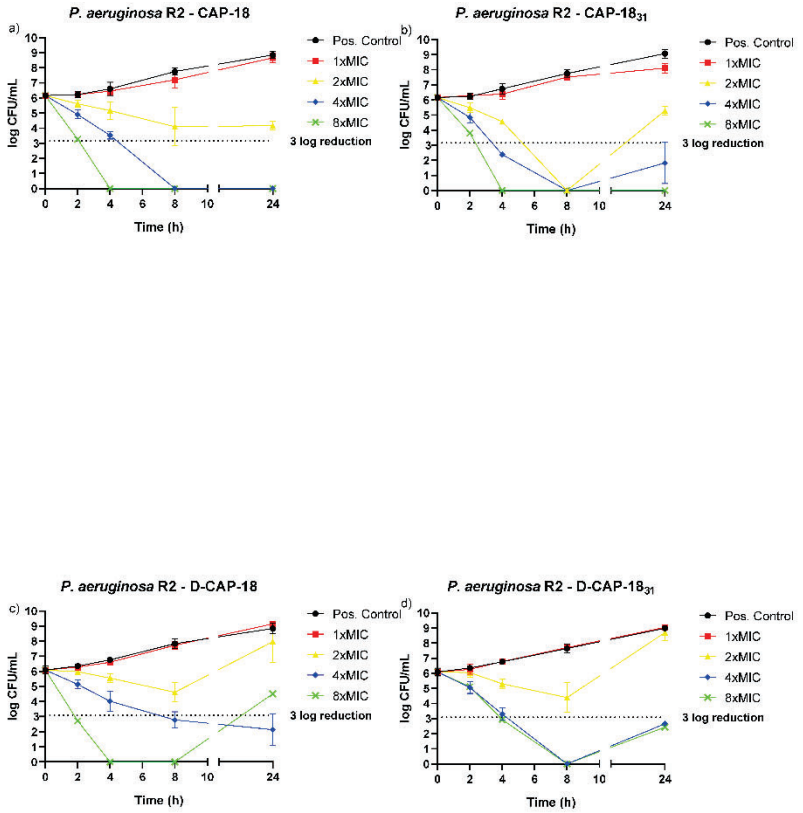

Figure 3. *P. aeruginosa* R2 TKCs against a) CAP-18; b) CAP-18$_{31}$; c) D-CAP-18; d) D-CAP-18$_{31}$.

For the last strain, *P. aeruginosa* 121110 (Figure 4), CAP-18 was bactericidal at 4xMIC at 4h and 8xMIC at 2h incubation maintaining CFU/mL levels up to 8h at this last concentration (Figure 4 a). For D-CAP-18 there was just bactericidal effect at 2h and 8xMIC, maintaining close bacterial concentration until 4h which

then grew over time. For CAP-18$_{31}$ there was no bactericidal effect at any concentration tested although MIC for this strain was 0.5 µg/mL. Lastly, D-CAP-18$_{31}$ acted similarly to its L-enantiomer and did not yield bactericidal effect at any concentration even if the MIC was 0.25 µg/mL. It was noted that for all four peptides, final bacterial concentrations reached the same value as for the untreated control curve at 24h.

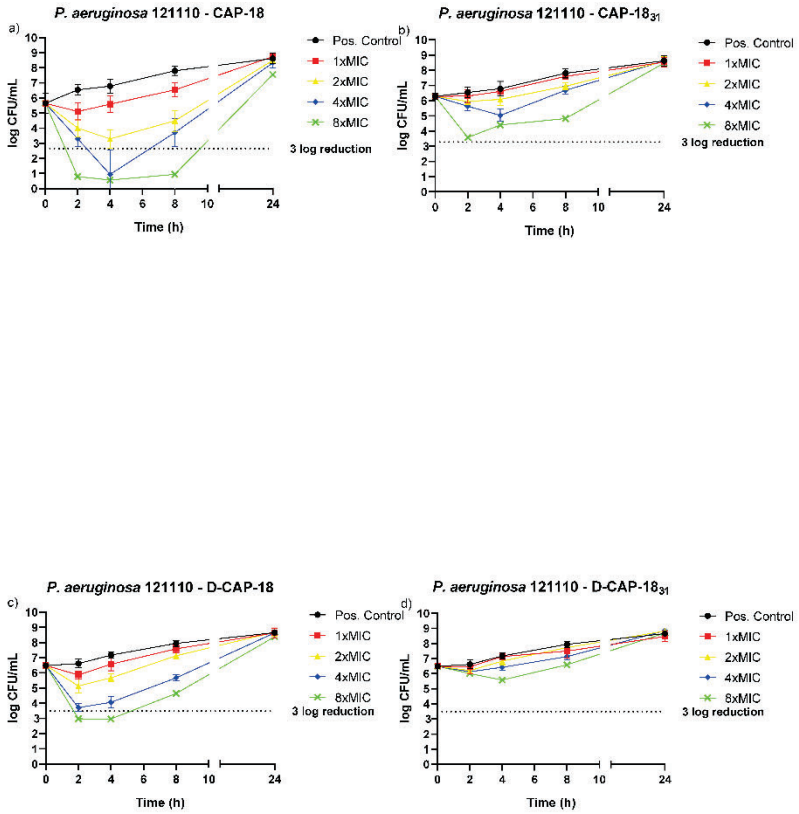

Figure 4. *P. aeruginosa* 121110 TKCs against a) CAP-18; b) CAP-18$_{31}$; c) D-CAP-18; d) D-CAP-18$_{31}$.

Transmission Electron Microscopy sample visualization:

The effect of CAP-18, CAP-18_{31}, D-CAP-18 and D-CAP-18_{31} on cell morphology of *A. baumannii* CR17 and ATCC 17978 and *P. aeruginosa* R2 and 121110 strains was studied through TEM (Figure 5).

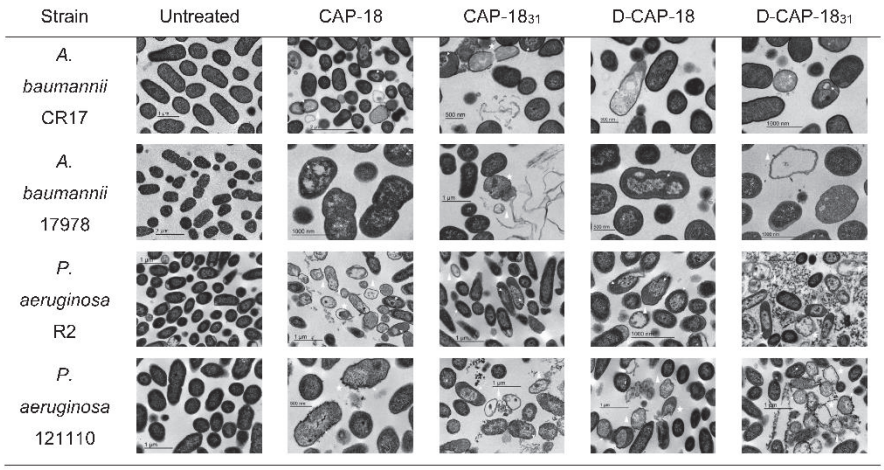

Figure 5. TEM images of *A. baumannii* CR17 and 17978 and *P. aeruginosa* R2 and 121110. White arrows indicate cytoplasm aggregation; white stars indicate ruptured membranes; white triangles indicate empty membranes. *P. aeruginosa* untreated cells show wrinkly membranes due to sample chemical fixation and dehydration steps.

4. Discussion:

Peptide design and antimicrobial susceptibility profile:

Rational design and activity screening is a commonly reported process for AMPs in literature and has also been described for shorter fragments of CAP-18. [42,43] Larrick et al., 1993 analysed shorter CAP-18 derivatives with truncated N-terminus or C-terminus based on their antimicrobial profile against both Gram-positive and negative bacteria; none of the peptides from this study being similar to our collection. [34,42,43] This work provided the hypothesis that the activity of CAP-18 derived peptides relied not only on sequence but also on peptide structure and that truncation of the N-terminus might result into loss of activity.

In the present study we designed, synthetised and analysed CAP-18_{23}, CAP-18_{19}, CAP-18_{18} and CAP-18_{14} (Table 1) with the aim of testing if truncation of N-terminus while keeping Q residues (polar amino acids of neutral charge whose presence might increase the peptides' activity) and different length motives at the C-terminus, could result in short and active derivatives. In an initial screening of these peptides against 4 *A. baumannii* strains (data not included), the only peptide with an MIC under 64 µg/mL was CAP-18_{23}; it was proposed that a longer sequence of this peptide might have helped keep an active structure on this derivative compared to its shorter relatives CAP-18_{19}, CAP-18_{18} and CAP-18_{14}.

Two shorter derivatives were obtained by truncating the C-terminus end of CAP-18: CAP-18_{31} and CAP-18_{21}. Finally, after screening CAP-18 and CAP-18_{31}, enantiomers and retro-enantiomers of these peptides were synthetised to check how antimicrobial activity and toxicity would be affected by using D-amino acids and reversing their sequences: D-CAP-18, D-CAP-18_{31}, R-CAP-18 and R-CAP-18_{31} (Table 1).

Regarding the screening results of the 7 peptides (Table 3), the parent peptide CAP-18 together with its enantiomer D-CAP-18 were peptides with most potent antimicrobial activity throughout the panel; their MIC values ranged from 0.5 to 2 µg/mL. Close to them activity-wise, CAP-18_{31} and its D-isomer D-CAP-18_{31} also

compare in similar fashion, the only difference being the latter peptide showed a better activity against both *P. aeruginosa* strains (2 µg/mL for D-CAP-18_{31} *versus* 8 µg/mL for CAP-18_{31}).

CAP-18_{23} and CAP-18_{21}, shorter derivatives of CAP-18, both presented loss of antimicrobial activity against the strains tested when compared to a longer derivative CAP-18_{31}. Their MIC values were > 16 µg/mL for all strains but for CAP-18_{21}, which had an MIC of 2 µg/mL against *A. baumannii* CS01. These high MIC values deterred further research using shorter peptides. We hypothesized that both regions of CAP-18 excluded in these shorter peptides play an important role in the peptides' structural conformation thus affecting their antimicrobial activity. Moreover, it is confirmed that derivatives produced based on a truncation of 5 or more amino acids in the N-terminus of CAP-18 (8 amino acids for CAP-18_{23}) do translate into peptides with lower antimicrobial activity [34,42]. This is illustrated when comparing CAP-18_{23}'s MIC values to those of CAP-18_{31}.

Retro-enantiomers R-CAP-18 and R-CAP-18_{31} show lower activity compared to the original peptides and the D-isomers with MICs at 2 and 1 µg/mL respectively against *A. baumannii* strains. Also, higher MIC values of 2 and 32 µg/mL for R-CAP-18 and of 0.5 and 4 µg/mL of R-CAP-18_{31} versus those of CAP-18 and CAP-18_{31} against *P. aeruginosa* strains discouraged further screening of the retro-enantiomer derivatives.

Tested against a panel of 30 strains of *A. baumannii* and *P. aeruginosa*, the activity of CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} was maintained against MDR and colistin resistant strains of *A. baumannii*. MIC_{90} values of 0.5 µg/mL were obtained for all peptides tested against *A. baumannii*. Regarding MIC_{90} of CAP-18 and its enantiomer D-CAP-18 for *P. aeruginosa*, 4 and 2 µg/mL respectively, these values were higher when compared to MIC_{90} of each peptide for *A. baumannii*. CAP-18_{31} and its enantiomer D-CAP-18_{31} also showed higher MIC_{90} values of 16 µg/mL each, although MIC_{50} values were kept at 2 and 4 µg/mL respectively.

Antimicrobial activity of AMPs developed against *A. baumannii* and/or *P. aeruginosa* has been reported in recent literature. LL-37 is one of the most studied peptides from the cathelicidin family [24,44–46], its activity against *A. baumannii* and *P. aeruginosa* has been widely reported [47,48]. Linear fragments of LL-37 were also analysed for antimicrobial activity against clinical MDR and ATCC *A. baumannii* strains in a similar fashion to our present study. A fragment peptide of LL-37 named KS-30 (KSKEKIGKEFKRIVQRIKDFLRNLVPRTES) presented the best activity against the *A. baumannii* isolates with MICs between 8-16 µg/mL[49], at least 4 folds over the MIC_{90} of all three CAP-18 derived peptides we produced and analysed against MDR *A. baumannii* strains (Supplementary Table 1 and Table 4).

Further comparison with other AMPs against *A. baumannii,* OMN6, a 40-residue cyclic peptide derived from cecropin A structure, presented MICs ranging between 4-8 µg/mL against susceptible and MDR *A. baumannii* strains [50]. Mastoparan, a peptide isolated from wasp venom [51], showed an MIC_{90} of 8 µg/mL against a collection of colistin resistant and susceptible strains of *A. baumannii*. [52] Altogether, the above-mentioned peptides (mastoparan and OMN6) [52,53] were 4 folds over the MIC_{90} of CAP-18 and the other 3 derivatives against *A. baumannii,* highlighting the interest for their further development.

AS-hepc3$_{(41-71)}$ and AS-hepc3$_{(48-56)}$, two truncated AMPs based on *Acanthopagrus schlegelii*'s hepcidin, exhibited antibacterial activity against *A. baumannii* 19606 at 8 and 16 µM respectively and against *P. aeruginosa* strains with both peptides MICs for all three tested strains at 8 µM.[54] rSparanegtin, a recently described recombinant antimicrobial peptide from the mud crab *Scylla paramamosain*, was screened for activity against *P. aeruginosa* with MICs ranging from 12 to 24 µM. [55] In comparison, the present study shows MIC_{90} values of CAP-18 and the three derivatives against *P. aeruginosa* ranged between 0.45 and 4.29 µM and against *A. baumannii* between 0.11 and 0.13 µM (Supplementary Table 2 and Table 4), still lower activity values against this species.

The effect of albumin on the peptides' antimicrobial activity:

Human serum albumin is present with a negative net charge at high physiological concentrations and has powerful binding ability. It can bind to positively charged peptides such as AMPs. Therefore, assessing how novel peptides bind to albumin is important for understanding and translating their behaviour into clinical settings.[56,57]

Results suggest that the polycationic nature of these peptides encourages electrostatic binding with human albumin which caused an increase in the MIC observed. Differences in activity could be explained by competitive binding of the peptides between albumin and bacterial surfaces. This effect would limit the total free concentration of these peptides *in vitro* and could potentially translate in higher doses needed to achieve the antimicrobial effect *in vivo*. It is still noticeable however, that even if MIC values are still higher for the four peptides against *A. baumannii* in albumin supplemented media, they fall well within the tested range in attainable concentrations and could indicate favourable *in vivo* testing concentrations against this pathogen. Moreover, *in vivo* pharmacokinetic properties of these peptides could result in a different outcome than the one described *in vitro*, so that high protein binding might not compromise their activity.

Haemolytic and cytotoxic effect in human cells:

One of the main hurdles for the clinical development of AMPs is their potential unspecific cytotoxicity.[24,58] Studies of haemolysis and cell viability assays in early preclinical stages allow for the selection of agents with the lowest toxicity from those that presented the most potent antimicrobial activity prior to *in vivo* assays.

Comparing our results to that of AMPs found in literature, peptide OMN6 showed no haemolytic effect in suspensions of 10% mouse erythrocytes in the range of concentrations between 27 to 868 μg/mL (6.25–200 μM) of peptide.[50] Another recently described peptide, AS-hepc3$_{(48-56)}$'s haemolytic activity was described by

incubating with 2 % mouse erythrocytes. 98% of erythrocytes were intact after 1h incubation with the peptide AS-hepc3$_{(48-56)}$ at 512 μM.[54] In both cases these peptides show haemolysis values over CAP-18 and its derived peptides, meaning less haemolysis than our peptides. However, it is worth noting existing methodological differences when comparing these results to our work. We studied haemolysis on human erythrocytes while OMN6 and AS-hepc3$_{(48-56)}$ haemolysis was described on mouse erythrocytes. Erythrocyte membrane characteristics of these species, such as abundance of sialic acid (which in human erythrocytes has been suggested to affect binding of positively charged peptides), could in part explain differences in haemolysis results.[59,60] It is difficult to provide a direct comparison between the peptides in these studies and ours due to methodological heterogeneity (e.g., differences in wavelength measurements or assay incubation times).

Mastoparan was also less haemolytic than CAP-18 and D- CAP-18$_{31}$ with a haemolysis value around 6.6% at 64 μg/mL while both CAP-18$_{31}$ and D-CAP-18 (4.4% and 3.7% haemolysis at 64 μg/mL respectively) were less haemolytic than mastoparan at 64 μg/mL.[61]

Regarding cytotoxicity, the toxicity of mastoparan and 3 analogues was analysed against HeLa cells in a study by Vila-Farrés et al., 2015 [61] showing an IC_{50} of 32 μM, less toxic than CAP-18 (at 9.12 μM). Comparing mastoparan analogues cytotoxicity to CAP-18 analogues, the guanidilated mastoparan version Gu-INLKALAALAKKIL-NH_2 toxicity was close to that of CAP-18$_{31}$, their IC_{50} being 13 and 13.1 μM respectively. Regarding enantiomers, mastoparan enantiomer H-inlkalaalakkil-NH_2 fared better toxicity-wise at an IC_{50} of 10 μM than either D-CAP-18 (2.11 μM) or D-CAP-18$_{31}$ (6.16 μM), the latter being comparable to the most toxic mastoparan analogue, H-INLKALAALAKKIL-$CH_2CH_2NH_2$ at an IC_{50} of 5 μM.

Bactericidal activity analysis:

There were bactericidal concentrations of all peptides against both *A. baumannii* strains between 2 and 4h of incubation. Peptide incubations against *P. aeruginosa* R2 generally took longer to reach bactericidal effect when compared to *A. baumannii* strains. In a similar fashion as for R2, for *P. aeruginosa* 121110 only CAP-18 and D-CAP-18 showed cidality while both CAP-18_{31} and D-CAP-18_{31} did not present any bactericidal effect.

Comparing our results to those of other AMPs found in recent literature we can find many examples of AMPs with a bactericidal effect on either *A. baumannii* or *P. aeruginosa*. Both OMN6 and mastoparan were tested in time killing assays against colistin resistant and susceptible *A. baumannii* strains. OMN6 presents bactericidal effect at the higher concentrations tested (8, 16 and 32 µg/mL)[50]. Mastoparan also showed bactericidal activity against both strains tested at the highest concentration used (8 µg/mL for the colistin resistant strain and 32 µg/mL for the susceptible) [52].

AS-$hepc3_{(41-71)}$ and AS-$hepc3_{(48-56)}$ at 16 µM each had a powerful bactericidal effect at just 30 and 60 minutes respectively against *P. aeruginosa* PAO1. [54] Although a shorter time point than two hours was not studied for CAP-18 and its derived peptides against any of the species tested, we can hypothesize the same is true for our peptides at high concentrations such as 4xMIC and 8xMIC as for AS- $hepc3_{(41-71)}$ and AS-$hepc3_{(48-56)}$ at 16 µM due to the usual mechanism of action of AMPs. Other than time kill assays at shorter periods, membrane permeability studies at up to 1h incubation (with 5 to 10 minutes readings) should provide enough information as to how fast the action of these peptide is exerted upon the membranes of these bacterial strains, which most probably would be one of their mechanisms of action.

Bacterial membrane interaction assessment through Transmission Electron Microscopy (TEM):

Untreated *A. baumannii* CR17 cells grown in AST media showed a normal coccobacillus morphology with no structural damage of the inner membrane and a smooth well defined outer membrane. Under peptide treatment, cells lost outer membrane definition (D-CAP-18), presented inner empty vesicles (D-CAP-18_{31}) and clear spots in the cytoplasm (present in all peptide-treated samples), suggesting possible aggregation of the bacterial cell contents and even membrane rupture (CAP-18_{31}) maybe due to outer membrane peptide location resulting in full cell leakage.

When comparing *A. baumannii* 17978 untreated cell images with peptide treated images, the latter presented intracellular aggregation and seemingly polar concentration of inner contents (in CAP-18 and D-CAP-18 images) with clear cytoplasm spots, burst (CAP-18_{31}) and even completely empty membranes (D-CAP-18_{31}) that could suggest a process in which peptide action on the outer membrane is followed by complete cell leakage, leaving wobbly and distorted empty membranes.

Micrographs of peptide treated *P. aeruginosa* R2 most notably show different stages of cellular damage. Elongated outer membranes with empty inner spaces and inner content aggregation (on CAP-18_{31} and D-CAP-18 images), disintegrating bacterial cells with compromised membranes in different lysis states (CAP-18 and D-CAP-18_{31}).

In peptide treated samples of *P. aeruginosa* 121110, cytoplasm-empty membranes filled with contorted vesicles were recorded (D-CAP-18), different grades of cell lysis were observed (images of CAP-18, D-CAP-18 and D-CAP-18_{31}) and bubbles protruding from cell outer membrane were seen (CAP-18) possibly as a secondary step after aggregation of cellular content and previous to leakage.

Even though TEM imaging is an insightful technique, further characterization of the peptides is still needed to understand their mechanism of action.

Nonetheless, it can be hypothesized by combining information obtained through these TEM studies and previous reports on the mechanism of action of cathelicidins, CAP-18 and synthetic peptides derived from their structure, [42,62] that CAP-18 and derived peptides possibly bind to LPS and that the disruption of the bacterial membrane might be the mechanism through which these peptides kill bacteria.

5. **Conclusions:**

AMPs are a promising alternative strategy for treatment of resistant bacteria. Derivatives of CAP-18, CAP-18_{31}, D-CAP-18 and D-CAP-18_{31}, were the most active compounds against MDR *A. baumannii* and *P. aeruginosa* strains tested.

MICs in albumin supplemented media were higher for all peptides, suggesting there is interaction with human albumin due to electrostatic interactions. In view of these results, we can foresee peptide concentration to be adjusted for *in vivo* experiments due to unspecific albumin binding.

Regarding toxicity against human erythrocytes: CAP-18_{31} and D-CAP-18_{31} were the least toxic peptides with IC_{50} values of 690.97 and 404.48 µg/mL respectively. An estimation of the peptide's biological profile resulted in the widest therapeutic windows for the four peptides against *A. baumannii*, ratios against this species range from 300 to 1300 folds, meaning they are at least 300 times more selective against bacterial cells than human erythrocytes.

Toxicity of the peptides upon human cell lines was assessed. IC_{50} values pointed at higher toxicity against A549 than HeLa cells, with D-isomers being less toxic than their counterparts against A549. For HeLa cells, L-isomers CAP-18_{31} and CAP-18 showed the highest IC_{50} values between 40 and 48 µg/mL. These results in HeLa cells are in the range of those of mastoparan analogues. [61]

Time killing kinetics of CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} were studied against a colistin-resistant and colistin-susceptible strain of *A. baumannii* and *P. aeruginosa*. All peptides showed bactericidal concentrations at short

incubation times (2 to 4h) against *A. baumannii* while longer incubation times were needed against *P. aeruginosa* R2 and 121110 (between 2 to 8h). The bactericidal effect shown in our peptides agrees with time killing kinetic assays of other AMPs found in literature. CAP-18_{31} and D-CAP-18_{31} did not have any bactericidal concentration at the timepoints tested against *P. aeruginosa* 121110 despite having MICs of 0.5 and 0.25 µg/mL respectively.

TEM micrograph visualizations of CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} against colistin-resistant and colistin-susceptible strains of *A. baumannii* and *P. aeruginosa* seem consistent with disruption of bacterial membrane. This hypothesis agrees with previous literature regarding CAP-18 and derived peptides showing binding to LPS and bacterial membrane permeabilization. [36,42,43,63] Additional experimental work such as characterization of resistant mutants is needed to elucidate these derivatives precise mechanisms of action.

In view of the screening and results, we consider peptides CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} promising candidates for the treatment of *A. baumannii* and *P. aeruginosa* infections caused by MDR bacteria, especially against colistin resistant strains since their mechanism of action seems to be unrelated to colistin resistance. Further studies focused on the optimization of the molecules to decrease human cell toxicity will be carried out as well as preliminary *in vivo* studies to assess toxicity and effectiveness of the peptides' treatment in infection models.

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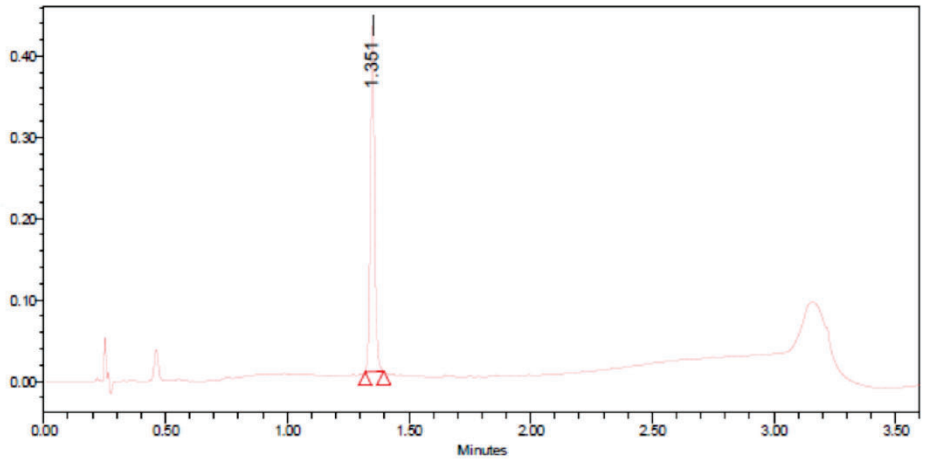
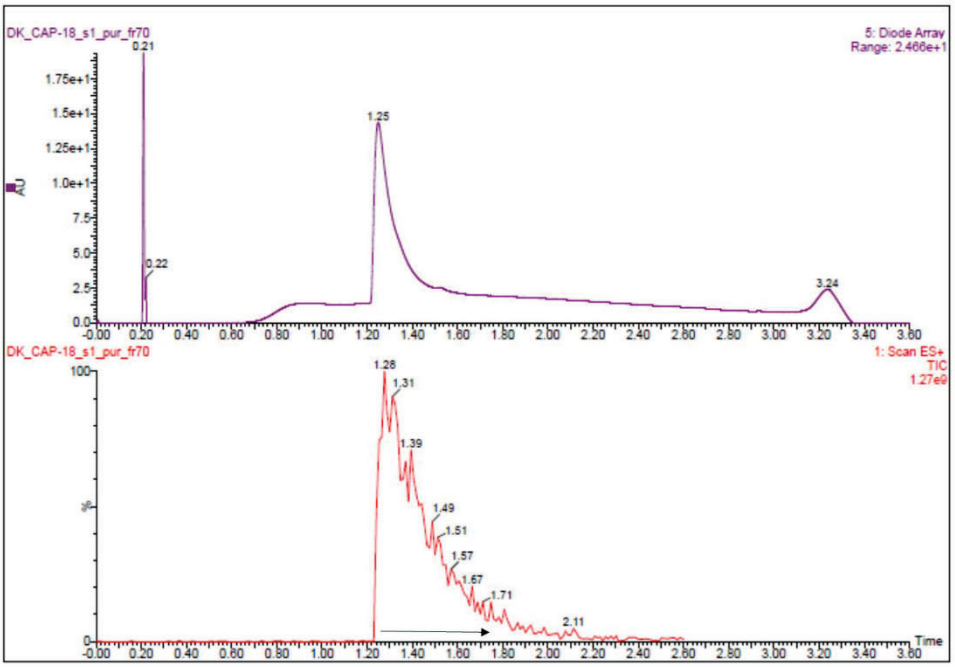
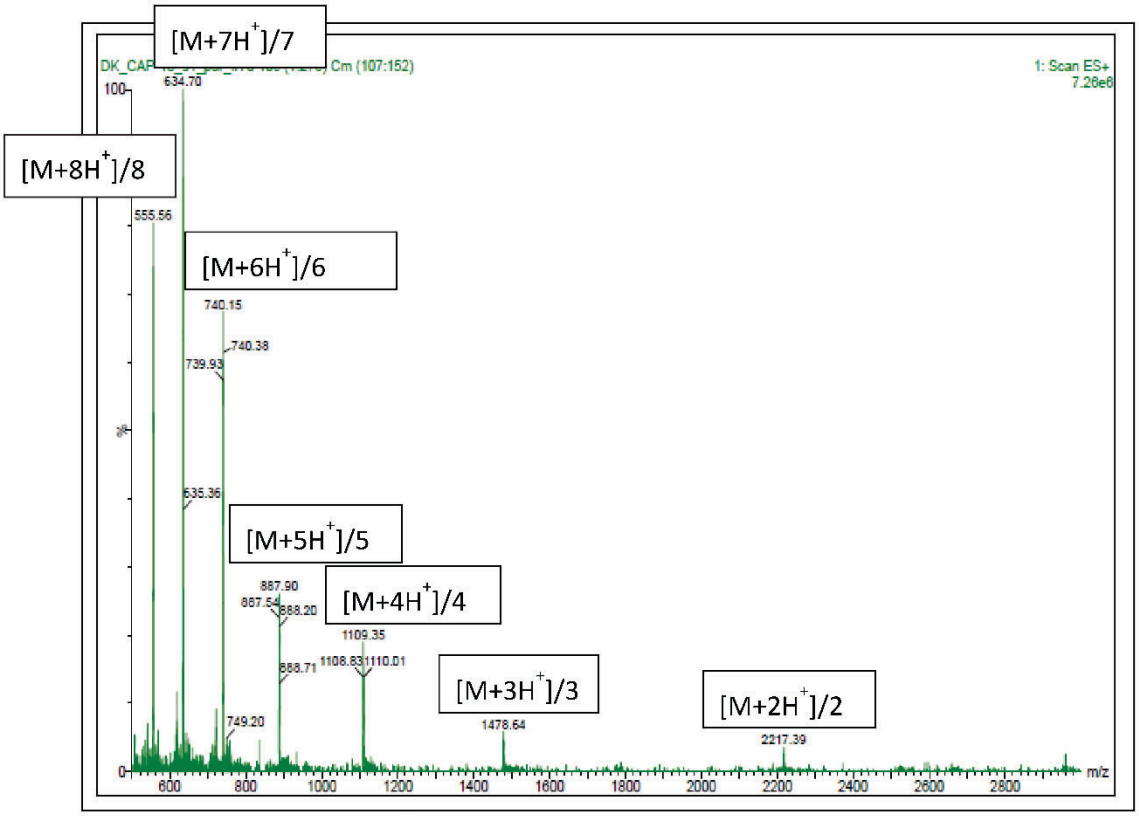
Tables and Figures:



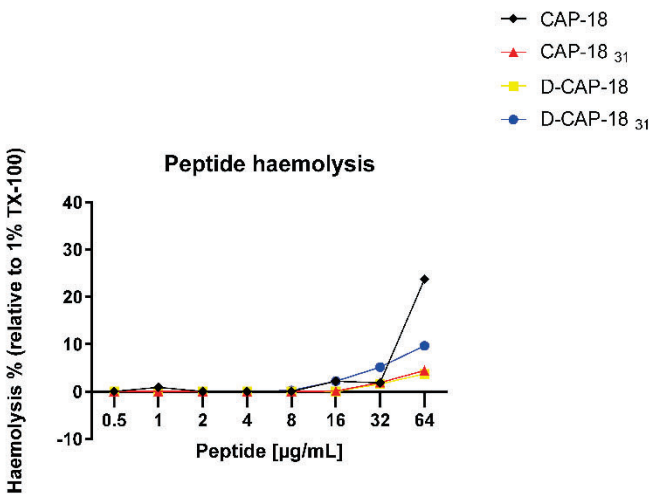
Supplementary Figure 1. CAP-18 UPLC after purification.



Supplementary Figure 2. CAP-18 UPLC-MS after purification.



Supplementary Figure 3. CAP-18 detected mass fragments.



Supplementary Figure 4. Peptide erythrocyte haemolysis % (relative to 1% TX-100 treatment) versus peptide concentration for CAP-18, CAP-18$_{31}$, D-CAP-18 and D-CAP-18$_{31}$

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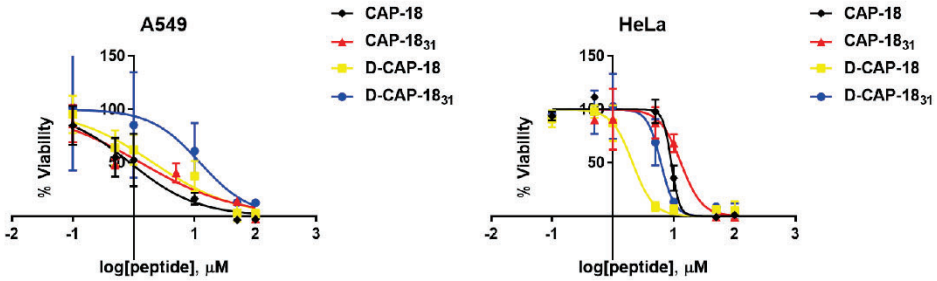

Supplementary Figure 5. Viability of A549 and HeLa human cells treated for 24h with increasing concentrations of CAP-18, CAP-18$_{31}$, D-CAP-18, D-CAP-18$_{31}$ and colistin (for A549) as measured with the XTT viability marker.

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Paper 1: A new synthetic protegrin as a promising peptide with antibacterial activity against MDR Gram-negative pathogens.

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Abstract

Protegrins are a family of natural peptides from the innate immune system of vertebrates with broad-spectrum antimicrobial activity. However, Protegrin-1's toxicity and haemolysis at low concentrations renders it useless for therapeutic application. We rationally de-signed PLP-3, a novel synthetic PG-1-like peptide, comprising key activity features of protegrins in a constrained bicyclic structure. Our main objective was to investigate PLP-3's activity against MDR strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and to analyse its haemolysis and cytotoxicity. Peptide synthesis was performed via solid phase and intramolecular ligation in solution, and the correct folding of the peptide was verified by circular dichroism. Antimicrobial activity was performed through broth microdilution. Bacterial strains test panel contained 45 strains belonging to *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* (15 strains per species) comprising, colistin resistant and MDR strains. Cytotoxicity was assessed by XTT cell viability assays using HeLa and A549 cells and haemolysis on human erythrocytes. PLP-3, a synthetic bicycle-constrained analogue of PG-1, was synthesized. Antimicrobial activity screening showed MIC_{90} values of 2 mg/L for *A. baumannii*, 16 mg/L for *K. pneumoniae* and 8 for *P. aeruginosa*. Haemolysis IC_{50} value is 48.53 mg/L. Cytotoxicity against human HeLa and A549 cells showed values of ca. 200 mg/L in both cell lines resulting in a 100-fold selectivity window for bacterial over human cells. PLP-3 has potent antimicrobial

activity specially against *A. baumannii* while maintaining low haemolysis and toxicity against human cell lines at antimicrobial concentrations. These characteristics make PLP-3 a promising peptide with an interesting therapeutic window.

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A new synthetic protegrin as a promising peptide with antibacterial activity against MDR Gram-negative pathogens

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Objectives: Protegrins are a family of natural peptides from the innate immune system of vertebrates, with broad-spectrum antimicrobial activity. However, the toxicity and haemolysis of protegrin-1 (PG-1) at low concentrations renders it useless for therapeutic application. We rationally designed PLP-3, a novel synthetic PG-1-like peptide, comprising key activity features of protegrins in a constrained bicyclic structure. Our main objective was to investigate PLP-3's activity against MDR strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and to analyse its haemolysis and cytotoxicity.

Methods: Peptide synthesis was performed via solid phase and intramolecular ligation in solution, and the correct folding of the peptide was verified by circular dichroism. Antimicrobial activity was performed through broth microdilution. The test panel contained 45 bacterial strains belonging to *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* (15 strains per species) comprising colistin-resistant and MDR strains. Cytotoxicity was assessed by XTT cell viability assays using HeLa and A549 cells and haemolysis of human erythrocytes.

Results: PLP-3 was successfully synthesized, and its antiparallel β-sheet conformation was confirmed. Antimicrobial activity screening showed MIC_{90} values of 2 mg/L for *A. baumannii*, 16 mg/L for *K. pneumoniae* and 8 mg/L for *P. aeruginosa*. The haemolysis IC_{50} value was 48.53 mg/L. Cytotoxicity against human HeLa and A549 cells showed values of ca. 200 mg/L in both cell lines resulting in a 100-fold selectivity window for bacterial over human cells.

Conclusions: PLP-3 has potent antimicrobial activity, especially against *A. baumannii*, while maintaining low haemolysis and toxicity against human cell lines at antimicrobial concentrations. These characteristics make PLP-3 a promising peptide with an interesting therapeutic window.

Introduction

Antimicrobial resistance (AMR) is a global health threat.[1–3] Globally, it has been recently estimated that 1.27 million deaths were directly attributable to MDR bacteria in 2019.[4] There is an urgent need to find novel antibacterial agents; however, traditional drug discovery methodologies have not produced a single new antibiotic in over 30 years.[5,6] The WHO has published a list of priority pathogens for which development of novel and effective antimicrobials is of utmost importance. Among these, the 'critical priority' category includes Gram-negative pathogens such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.[7]

Among the species of the *Acinetobacter* genus, *A. baumannii* is responsible for most clinical infections and nosocomial outbreaks due to its MDR nature and propensity for clonal spread.[8] Unfortunately, only more toxic antibiotics (e.g. colistin) remain as the last resort for treating infections caused by MDR *A. baumannii*.[9] Over the past years, a steady increase in the number of *K. pneumoniae* isolates resistant to both carbapenems and fluoroquinolones has been reported.[10] Around 32 600 cases of MDR *P. aeruginosa* were estimated only in USA hospitalized patients during 2017.[11,12] Infections caused by *P. aeruginosa* are especially damaging in patients who receive artificial ventilation and cystic fibrosis patients.[13,14]

The design of novel molecules with antimicrobial activity and better pharmaceutical properties has been historically inspired by host-defence natural peptides. Protegrins are arginine-rich antimicrobial peptides (AMPs) that belong to the cathelicidin family of components of the innate immune system.[15,16] First

discovered in porcine leucocytes,[16] protegrins have antimicrobial activity against bacteria, fungi and some enveloped viruses.[17] They typically display an antiparallel β-hairpin structure, which is stabilized by one or more disulphide bonds.[18] Among the different family members, protegrin-1 (PG-1) is the most abundant and well-studied member of the protegrins. PG-1 is an 18-residue cationic peptide that preferentially folds into an antiparallel β-hairpin structure, which is stabilized by two disulphide bonds, while the N- and C-terminal tails remain more flexible.[18,19] PG-1 exhibits broad-spectrum antimicrobial activity, with a mechanism consistent with membrane disruption via pore formation.[20] However, PG-1 has unfavourable drug properties and significant haemolysis and toxicity at low concentrations against human cells, limiting its therapeutic application.[21,22]

To address these issues, researchers have developed several synthetic PG-1 analogues with enhanced bactericidal activity and reduced toxicity.[21,23–26] Among these, arguably the most notable example is murepavadin (also known as POL7080), developed by Polyphor.[22] This cyclic β-hairpin peptidomimetic has specific and potent bactericidal activity against *P. aeruginosa*[27] and successfully completed Phase II clinical trials in hospital patients suffering from life-threatening *Pseudomonas* lung infections. Unfortunately, Phase III clinical trials were discontinued due to the onset of kidney damage in over half of the patients.[5,27]

Given the unique physicochemical properties of protegrins and their potent activity against Gram-negative bacteria, we sought to rationally design a new PG-1-like peptidomimetic (named PLP-3), condensing the key activity features of protegrins in a highly constrained peptide bicyclic structure. The activity of PLP-3 was tested against MDR strains of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* and its selectivity profile was assessed via haemolysis and cytotoxicity studies.

Materials and methods

Solid-phase peptide synthesis and intramolecular chemical ligation

PLP-3 was synthesized on Dawson Dbz AM resin (Merck Millipore, MA, USA) with a substitution of 0.4–0.5 mmol/g. The first amino acid (Val) was manually coupled by preactivating *N*-Fmoc-protected amino acid (3 equivalents) with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 3 equivalents) and *N*, *N*-diisopropylethylamine (DIPEA, 3 equivalents) in the minimal volume of *N*,*N*-dimethylformamide (DMF).[28] The mixture was added to the resin and allowed to react in an orbital shaker for 30 min. Peptide chains were then elongated on a CEM Liberty Blue microwave peptide synthesizer. Fmoc deprotection was carried out using 10% (w/v) piperazine and 0.1 M OxymaPure in a 9:1 mixture of *N*-methylpyrrolidone and ethanol. The *N*-Fmoc-protected amino acids (5 equivalents, 0.2 M in DMF) were activated with OxymaPure (5 equivalents, 1 M in DMF) and *N*, *N*′-diisopropylcarbodiimide (DIC) (5 equivalents, 0.5 M in DMF). Coupling reactions were performed for 3 min at 90°C, except for Cys, His and Arg residues, which were coupled at 50°C for 10 min. Last, the N-terminal Cys residue was introduced as an *N*-Boc-protected amino acid. After chain elongation, the resin was washed extensively with DMF and 1,2-dichloroethane. 4-Nitrophenyl chloroformate was dissolved in the least amount of 1,2-dichloroethane, added to the resin and left to gently agitate for 1 h at room temperature (RT). The resin was washed with methylene chloride and DMF and reacted for 30 min with a 0.5 M solution of DIPEA in anhydrous DMF. Finally, the linear C-terminally activated peptide was cleaved with concomitant removal of the side-chain protecting groups, using a 92.5:2.5:2.5:2.5 mixture of trifluoroacetic acid (TFA)/water/triisopropyl silane (TIS)/dioxa-1,8-octane-dithiol (DODT) for 2 h at RT.

To cyclize the peptide backbone, a ligation buffer [6 M guanidinium hydrochloride, 200 mM sodium phosphate, 20 mM tris(carboxyethyl) phosphine (TCEP), 100 mM 4-mercaptophenol; pH 7] was freshly prepared and bubbled with nitrogen. The peptide was dissolved at a 2–3 mM concentration and the solution was stirred at RT for 4 h. The reaction was then acidified, extracted with *t*-butyl methyl ether (2 × 50 mL) and loaded on a Porapak C18 Cartridge for desalting. The guanidinium salts were washed away with buffer, while the peptide was eluted in water/acetonitrile (ACN) (1:1) and freeze-dried. To yield the bicyclic peptide, intramolecular disulphide bonds were formed under highly diluted conditions (20–40 μM) by stirring an aqueous solution of the peptides (pH 8) under air atmosphere for 24 h. Peptides were purified by semi-preparative HPLC on a Waters 2700 sample manager equipped with a Waters 2487 dual-wavelength absorbance detector, a Waters 600 controller, a Waters fraction collector and MassLynx software by using a Sunfire C18 column (150 × 10 mm × 3.5 μm, 100 Å, Waters, MA, USA) with a flow rate of 6.6 mL/min, solvent A = 0.1% TFA in water and solvent B = 0.1% TFA in ACN. Peptide purity and identity was assessed by UPLC and UPLC-MS, respectively (see Figure S1, available as Supplementary data at *JAC* Online).

Circular dichroism (CD) assay

CD spectra were recorded using a Jasco 810 UV-Vis spectropolarimeter, equipped with a CDF 426S/426L Peltier. Peptide samples (reduced and oxidized) were dissolved in PBS buffer and spectra were recorded at concentrations of 100 and 20 μM. TCEP (5 mM) was added to the reduced peptide sample to prevent disulphide bond formation. A third reading was done after adding 20% trifluoroethanol (TFE) to the samples. The following parameters were used: sensitivity (standard, 100 mdeg), start (260 nm), end (190 nm), data pitch (0.5 nm), scanning mode (continuous), scanning speed (200 nm/min), response (1 s), band width (1.0 nm) and accumulations (3). A blank spectrum of the buffer was subtracted from all recordings, and the resulting spectra were smoothed using the Savitzky–Golay method (convolution width = 21) and taken to zero at the far-UV region (λ = 260 nm).

Antimicrobial activity testing

MICs were obtained via broth microdilution following CLSI guidelines.[29] PLP-3 MICs were obtained using Mueller–Hinton Broth (MHB; CM0405, 500 g, Thermo Fisher Scientific, MA, USA) while colistin MICs were obtained following the procedure established by the joint CLSI/EUCAST Polymyxin Breakpoints Working Group using CAMHB (90922, 500 g Sigma–Aldrich®, MO, USA).[30] *Escherichia coli* 13846 (*mcr*-1 positive) and *P. aeruginosa* ATCC 27853 were used as quality controls for colistin in all broth microdilution assays. Three biological and technical replicates were performed per strain.

MICs in the presence of albumin

MHB was supplemented with 40 mg/mL human albumin (A1653-5G, Merck, MA, USA) and the broth microdilution protocol was performed. MHB without albumin supplementation was used for control. Strains tested were *A. baumannii* CR17 (MDR and colistin resistant) and ATCC 17978. Three biological and technical replicates were performed per strain.

Erythrocyte haemolysis assay

A 50% haematocrit solution [1:1 PBS 1× (prepared from 10×, P5493, 1 L, Sigma, MO, USA)] was obtained from a commercially available human blood sample (IWB1K2E10ML, Innovative Research Inc., MI, USA) by

performing three consecutive washes with PBS 1× and centrifuging (2600 rpm for 10 min) between washes. A final 4% erythrocyte solution was prepared with PBS 1× and chilled on ice until used. Colistin and PLP-3 stocks were prepared at 256 mg/L in MHB and left at 4°C until used. Ninety-six-well, round-bottom plates were filled with 50 μL PBS 1×, except for the Triton X-100 (TX-100, 9002-93-1, 100 mL, Sigma, MO, USA) haemolysis controls, which were left empty. Fifty microlitres of each antimicrobial was added to the first column of each plate and serial dilution was performed, then 50 μL of erythrocyte solution was added to all wells. Plates were incubated for 4 h at 37°C and then centrifuged at 1500 rpm for 5 min. From each well, 80 μL of the supernatant was transferred to a new plate and read in a spectrophotometer at 450 nm (Epoch™, BioTek, VT, USA).

Cytotoxicity in human cells

Viability assays using XTT were performed using HeLa and A549 human cells. Cells were seeded in 96-well Corning microplates at 2×10^3 cells/well and 5×10^3 cells/well. After 24 h at 37°C, cells were treated with PLP-3 dissolved in DMEM (high glucose, 10% FBS) for 48 or 72 h (range 0.1–100 μM). The peptide solution was then removed and substituted with fresh medium. Fifty microlitres of activated XTT solution was added and incubated for 4 h at 37°C. Absorbance at 450 nm was measured in a PowerWave X reader (BioTek, VT, USA). Each experiment was performed in triplicate.

Bacterial membrane permeabilization

Membrane permeabilization effect of PLP-3 upon *A. baumannii* 19606 and *P. aeruginosa* 27853 was assessed using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, L13152, Illkirch, France) following instructions provided by manufacturer. Bacterial cultures were grown to OD 0.5, then centrifuged twice at 3000 g for 10 min by resuspending first in saline solution and then in the fluorophore solution (incubation with inoculum for 30 min at RT in the dark). PLP-3 broth microdilution was prepared in microplates for a range of 2–16 mg/L. Stained bacterial inoculum was added to the plate and read using a TECAN microplate reader (Tecan Infinite M2000 PRO, Zurich, Switzerland) at intervals of 5 min for a total of 60 min (SYTO9 read at 485/530 nm and propidium iodide 485/630 nm excitation/emission respectively). Three replicates were performed for each strain and PLP-3 concentration tested.

Results and discussion

PLP-3 design

As the main member of the protegrin family of peptides, the structural requirements of PG-1 for antibacterial activity have been thoroughly inspected.[26,31–33] In summary, the key features relating to its Gram-negative antibacterial activity are: (i) a well-defined β-hairpin conformation with tight intramolecular packing, which is typically stabilized by disulphide bonds and intrachain hydrogen bonds; (ii) a polycationic nature consisting of guanidinium groups at the edges of the hairpin, which form electrostatic interactions with the phosphate groups of the bacterial membrane; and (iii) amphipathic character, with hydrophobic side chains located at the centre of the β-hairpin, which interact favourably with the lipid bilayer hydrophobic core. These structural features, as a whole, enable the peptide (often in oligomers)[34,35] to insert into the lipid bilayer like a wedge, with the non-polar core facing the lipid acyl chains, and trigger the formation of pores that cause membrane permeabilization and cell rupture.[33]

To condense these features into an 'idealized' PG-1 analogue, we sought to eliminate the flexible tails present in natural protegrins and link the antiparallel β-strands of the peptide in a head-to-tail fashion, resulting in a highly constrained macrocyclic structure. In this regard, structural rigidity has been linked to antimicrobial potency and lower toxicity,[21,36] while it also prevents metabolic degradation by exopeptidases *in vivo*. To induce the β-hairpin folding, we introduced a D-Pro residue at the i+1 position of each β-turn, a motif that strongly stabilizes β-turn formation even in short peptide sequences.[37] Finally, to narrow down the vast chemical space of peptide sequences that display PG-1-like features, we took advantage of *in silico* state-of-the-art structural tools. In particular, we used the generalized kinematic loop closure method,[38] available in the Rosetta software suite, to sample cyclic backbone conformations that mimic the antiparallel β-hairpin geometry of PG-1. We then used the Rosetta FastDesign protocol to introduce amphipathic side chains (mostly arginines and hydrophobic residues) at the desired sites of the molecule, and to find the optimal disulphide bonding sites in the molecule, which would further rigidify the structure. PG-1-like structures were inspected and subjected to energy landscape calculations to identify those designs that would preferentially fold in a PG-1-like β-hairpin conformation. From these, PLP-3 emerged as the lead candidate, adopting a slightly twisted β-hairpin conformation and a distinct low-energy minimum (Figure 1). The bioactive structure is stabilized by six evenly spaced intramolecular hydrogen bonds and is further pinned at its centre by a disulphide bond. Remarkably, the structure of PLP-3 was nearly identical [backbone root-mean-square deviation (RMSD) = 0.8 Å] to the bioactive structure of PG-1 inserted in a lipid bilayer,[34] which is considered as most relevant for its antimicrobial activity. In addition, the side-chain orientations and electrostatic charges of PG-1 were closely mimicked in our designed structure, while further pushing its amphiphilic character by increasing the number of both positive charges and hydrophobic residues (by one and two, respectively, despite the smaller size of PLP-3 compared with the natural molecule).

To study the activity of our new protegrin analogue, PLP-3 was chemically synthesized using the intramolecular native chemical ligation strategy, which enables the production of Cys-containing cyclic peptides in a rapid and straightforward manner (Figure S1). This approach first consists of the Fmoc-based solid-phase peptide synthesis (SPPS) of the linear precursor with a C-terminal *N*-acylurea moiety, which acts as thioester precursor during the ligation. Treatment of the unprotected peptide in solution with a thiol additive (4-mercaptophenylacetic acid) then catalyses the chemical ligation, which triggers the fast formation of the head-to-tail cyclic peptide at neutral pH. Importantly, the formation of the intramolecular product is favoured over the competing intermolecular reaction, thus avoiding the formation of undesired dimers. Finally, the thiol groups were oxidized under mild basic conditions to yield the final bicyclic peptide PLP-3.

CD assay

To experimentally validate our structural *in silico* design method, we investigated the conformational propensity of

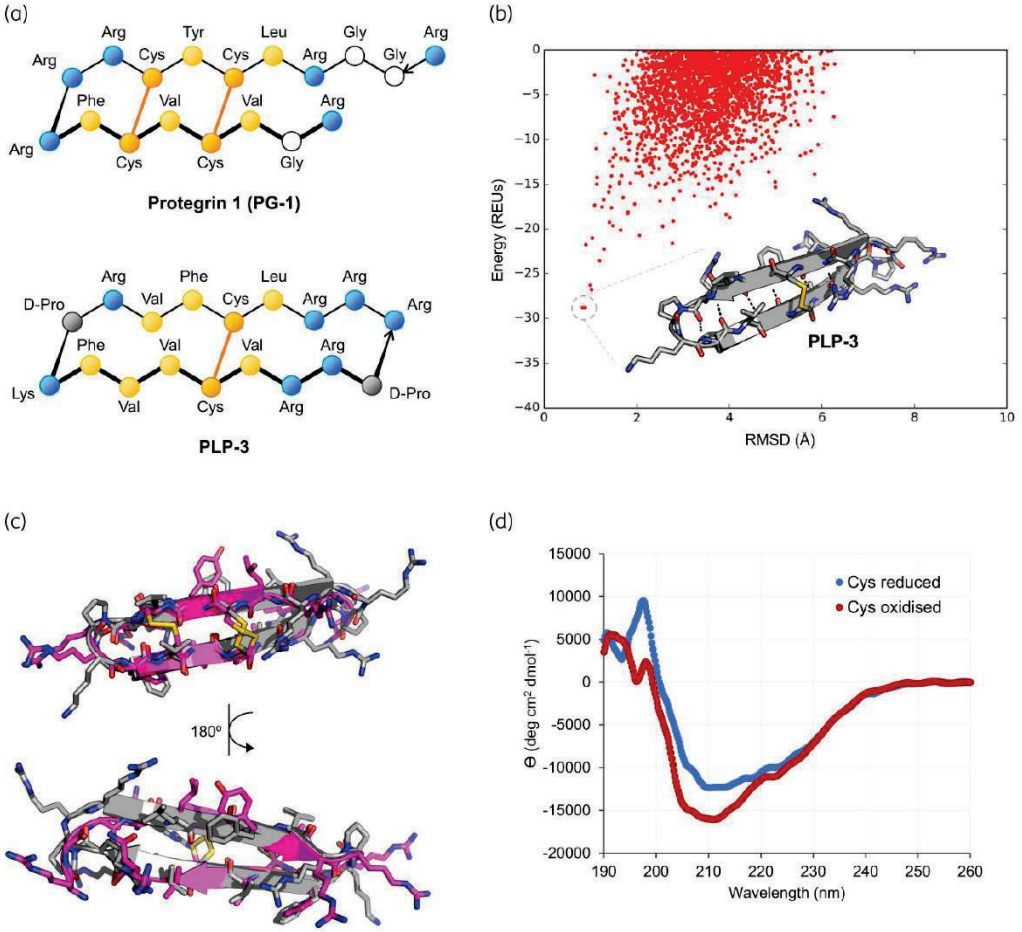

Figure 1. (a) Structure representation of PG-1 versus PLP-3. (b) Energy landscape calculations showing convergence towards low-energy structures close to PG-1 architecture. (c) Superposition of the lowest-energy energy structure of PLP-3 (in grey) with the bioactive structure of PG-1 in the lipid membrane (in magenta, PDB ID: 1ZY6). Both structures align with a backbone RMSD of only 0.8 Å. (d) CD spectra of PLP-3 and Cys-reduced PLP-3 at pH 7.4 and 25°C. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

PLP-3 in solution by CD spectroscopy. The spectrum of PLP-3 at physiological pH and 25°C showed a minimum at ca. 212 nm, a negative broad band at 220 nm and a maximum at 201 nm, which altogether represent the typical signature of a β-hairpin-stabilized structure.[37,39] Reduction of the disulphide bond to yield the monocyclic peptide resulted in a small decrease in CD signal intensity, thus suggesting that although the cyclic peptide is already well folded into an antiparallel β-sheet structure, the disulphide staple further contributes to stabilizing the bioactive conformation of the bicyclic peptide.

Antimicrobial activity

Initial PLP-3 antimicrobial activity screening was performed against a colistin-susceptible and a colistin-resistant strain of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* (data not shown). These assays confirmed potent antimicrobial activity across these species and prompted us to test a larger panel of strains of each species, comprising MDR, colistin-susceptible and colistin-resistant bacterial strains. Broth microdilution testing against 15 strains of each species provided PLP-3 MIC_{50} and MIC_{90} values (Table 1). Strains included were either of clinical

Table 1. MICs of PLP-3 and colistin against a panel of Gram-negative strains

Species	Strain	Resistance	MIC (mg/L)	
			CST	PLP-3
A. baumannii	8[a]	CIP, GEN, IPM, NET	1	1
	19[a]	CIP, GEN, IPM, NET, TOB	1	2
	80[a]	CIP, GEN, IPM, NET	1	2
	174[b]	AMK, CIP, CST, GEN, IPM, MEM, NET, TOB	1	2
	180	CIP, GEN	1	1
	226[a]	AMK, CIP, GEN, IPM, MEM, NET, TOB	1	1
	253[a]	AMK, CIP, GEN, IPM, MEM, NET, TOB	1	2
	317[b]	AMK, CIP, CST, GEN, IPM, MEM, NET, TOB	1	1
	383	CIP, IPM, MEM	1	2
	ATCC 19606		1	2
	CR17[a]	CST, CAZ, CTX, FEP, IPM, MEM, PIP, TIC, RIF, CIP	>64	1
	CR49[a]	MEM, CIP, CST, TOB, IPM	64	1
	CR86[a]	CST, IPM, MEM, CIP	>64	1
	210[a]	AMK, CIP, CST, GEN, IPM, MEM, NET, TOB	32	2
	ATCC 17978		1	1
P. aeruginosa	R1[a]	CAZ, FEP, IPM, CIP, CST	>64	8
	R2	TOB, CST	>64	16
	R3[a]	CAZ, FEP, IPM, MEM, CIP, TOB, ATM, CST	>64	4
	S1	FEP, CIP	0.5	16
	S2[a]	CAZ, FEP, CIP, TOB	1	4
	S3[b]	CAZ, IPM, MEM, CIP, TOB, ATM, CST	1	4
	S4[b]	CAZ, IPM, MEM, CIP, TOB, ATM, CST	1	16
	S5[b]	CAZ, IPM, MEM, CIP, TOB, ATM, CST	1	4
	36a[a]	CAZ, TZP, CIP, IPM	1	2
	38a[a]	CAZ, TZP, CIP, IPM	2	2
	ATCC 27853		1	2
	C1	SAM, LVX, FOF, TGC, GEN, CIP, CST	4	32
	C2	SAM, CAZ, TOB, LVX, FOF, TIC, FEP, AMK, TGC, TZP, ATM, GEN, CIP, CST	4	16
	C3	SAM, FOF, TGC, CST	1	16
	C4	SAM, FOF, TGC, TZP, CST	2	2
K. pneumoniae	31[a]	CAZ, FEP, CTX, CST, CIP, LVX	64	1
	32[a]	IPM, MEM, CAZ, FEP, CTX, AMK, CST, CIP, LVX	>64	4
	33[a]	IPM, MEM, CAZ, FEP, CTX, AMK, CIP, LVX	0.5	4
	34[a]	IPM, MEM, CAZ, FEP, CTX, AMK, GEN, CST, CIP, LVX	>64	2
	35[a]	IPM, MEM, CAZ, FEP, CTX, AMK, CIP, LVX	0.5	2
	36[a]	IPM, MEM, CAZ, CTX, AMK, CIP, LVX	0.5	4
	37[a]	IPM, MEM, CAZ, FEP, CTX, AMK, CST, TGC, CIP, LVX	64	1
	38[a]	MEM, AMK, GEN, ATM, CIP, TZP, AMC, TGC, TOB, CTX, CAZ, IPM, ETP	0.5	4
	ATCC 13883		0.5	2
	40[a]	MEM, AMK, ATM, CIP, TZP, AMC, C/T, TGC, TOB, CTX, CAZ, IPM, ETP	0.5	2
	41[a]	IPM, MEM, CAZ, FEP, CTX, AMK, TGC, CIP, LVX	1	2
	42[a]	AMK, GEN, CST, CIP, LVX	>64	8
	43[a]	IPM, MEM, CAZ, FEP, CTX, AMK, GEN, CST, TGC, CIP, LVX	>64	8
	44[a]	MEM, AMK, ATM, CIP, TZP, AMC, C/T, CST, TGC, TOB, CTX, CAZ, IPM, ETP	>64	4
	45[a]	IPM, MEM, CAZ, FEP, CTX, AMK, CIP, LVX	0.5	4

CIP, ciprofloxacin; GEN, gentamicin; IPM, imipenem; NET, netilmicin; TOB, tobramycin; AMK, amikacin; CST, colistin; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; PIP, piperacillin; TIC, ticarcillin; RIF, rifampicin; ATM, aztreonam; SAM, ampicillin/sulbactam; LVX, levofloxacin; FOF, fosfomycin; TGC, tigecycline; TZP, piperacillin/tazobactam; C/T, ceftolozane/tazobactam.

[a]MDR strains (not susceptible to at least one agent in at least three different antimicrobial categories used for the effective treatment of infections caused by such a pathogen).

[b]Strain is susceptible to all antimicrobials listed.

origin, relevant to the current clinical situation, or commercially available strains belonging to ATCC (the latter used for comparison and control).

Colistin-resistant strains were included in these assays for comparison since this antibiotic is a cyclic peptide and constitutes a last-resort clinical agent. PLP-3 showed potent antibiotic activity against the collection of *A. baumannii* strains. The range of PLP-3 MICs is the narrowest for the species tested, from 1 to 2 mg/L (Table 1). This translates into an MIC_{50} value of 1 mg/L and MIC_{90} of 2 mg/L (Table 2). Remarkably, PLP-3's activity is preserved even against colistin-resistant *A. baumannii* strains. Regarding *P. aeruginosa* strains (Table 1), the PLP-3 MIC range was between 1 and 8 mg/L. Although showing slightly higher MIC_{50} and MIC_{90} values (4 and 8 mg/L, respectively) compared with *A. baumannii*, PLP-3 had consistent antibiotic activity throughout colistin-resistant and -susceptible strains. The highest variability in MIC values was found for the *K. pneumoniae* collection, ranging from 2 to 32 mg/L. The MIC_{50} of this collection was 4 mg/L and the MIC_{90} was 16 mg/L (Table 2).

PLP-3 presents low MIC values for Gram-negative pathogens, even MDR strains, when compared with recently developed linear and cyclic AMPs through *ab initio* computational design.[40] Antimicrobial activity of arenicin-3 structure-based derivatives against Gram-negative bacteria and MDR/XDR strains is close to that of PLP-3, specifically when comparing PLP-3 against linear compound AA139; although not testing against the same cell lines, PLP-3 (data shown below) was slightly under AA139 toxicity-wise.[41] From another recent preclinical study, activity of linear peptide WW307 against resistant Gram-negative bacteria is described, with MIC values close to or slightly higher against these resistant strains than our PLP-3. However, WW307 shows potent synergy with Gram-positive antibiotics against Gram-negative bacteria and biofilm inhibition and eradication, which has not yet been tested for PLP-3.[42]

Antimicrobial activity in human albumin-supplemented media

To assess the antimicrobial activity of PLP-3 in a physiologically relevant environment, MIC assays via broth microdilution were performed at physiological concentrations of human albumin (40 mg/mL). To this end, two *A. baumannii* strains, MDR strain CR17 and colistin-susceptible ATCC 17978, were selected. MHB was supplemented with 40 mg/mL human albumin. As a control for both strains, PLP-3's activity was also tested in non-supplemented broth, and colistin was used as control antibiotic. In albumin-supplemented broth, PLP-3 achieved MIC values of 2 mg/L for CR17 and 4 mg/L for ATCC 17978 (compared with MIC values of 1 mg/L for both strains in unsupplemented broth). The activity observed for colistin was as expected: >64 mg/L for CR17 and 0.25 mg/L for ATCC 17978. Thus, even if the polycationic nature of PLP-3 might induce some electrostatic binding to human albumin, these results show that the free concentration of PLP-3 is sufficiently high to effectively inhibit bacterial growth.

Table 2. PLP-3 MIC_{50} and MIC_{90} values for each species tested

	PLP-3	
Species	MIC_{50} (mg/L)	MIC_{90} (mg/L)
A. baumannii	1	2
K. pneumoniae	4	16
P. aeruginosa	4	8

Selectivity assays against human cells

Unspecific and potentially toxic side effects, like haemolysis, are one of the main reasons hampering the clinical development of AMPs. To address these issues at an early stage of development, cytotoxicity and haemolysis studies underlie the basis for compound selection towards *in vivo* studies. We assessed the potential haemolysis of human erythrocytes caused by PLP-3, using 1% Triton X-100 as a control for total haemolysis. Absorbance values of PLP-3 were represented in relation to total haemolysis (TX-100 values). As shown in Figure 2(a), very low erythrocyte haemolysis was observed at concentrations below 16 mg/L (the maximum MIC_{90} value for the three species tested). The haemolysis IC_{50} value was 48.5 mg/L, which suggests that PLP-3 has relatively low toxicity against erythrocytes at clinically relevant concentrations.

To further evaluate the selectivity profile of PLP-3, we performed cytotoxicity assays on two types of human cell lines that are commonly employed as reference cell lines: human cervical carcinoma HeLa cells, and human adenocarcinoma epithelial A549 cells. Both cell lines are recommended for infectious diseases and toxicology research by ATCC. Cell viability and proliferation were unaffected at low and mid PLP-3 concentrations, and only significant inhibition was detected at the highest concentration tested (225 mg/L), as shown in Figure 2(b and c). In conclusion, these results reveal a wide therapeutic window of 2 log units (compared with the MIC_{90} values on the tested pathogen species) that is well suited to a potential therapeutic application in humans.

Bacterial membrane permeabilization

Fluorescence intensity measures of SYTO9 and propidium iodide allowed the measurement of membrane permeabilization by different PLP-3 concentrations against *A. baumannii* 19606 and *P. aeruginosa* 27853. After just 5 min incubation with PLP-3, the percentage of permeabilized *A. baumannii* 19606 cells ranged from 12% at 2 mg/L to 55% at 16 mg/L. The effect on *P. aeruginosa* 27853 was similar, producing 17% permeabilized cells at 2 mg/L and up to 58% at 16 mg/L. These initial assays suggest rapid dose-dependent bacterial membrane permeabilization induced by PLP-3.

Although PLP-3's mechanism of action is yet to be described through further experimental research, it is reasonable to hypothesize that it might resemble that of PG-1 since they are closely related molecules. PG-1's mechanism of action seems to be consistent with membrane disruption via pore formation.[20] First, solid-state NMR studies have suggested that when a high enough concentration of PG-1 is reached, PG-1 octamers are formed, which participate in the formation of stable pores in bacterial membranes.[43] Further experimental and model studies based on these pore-spanning octamers on bacterial cell membranes propose three concomitant processes as the drivers of

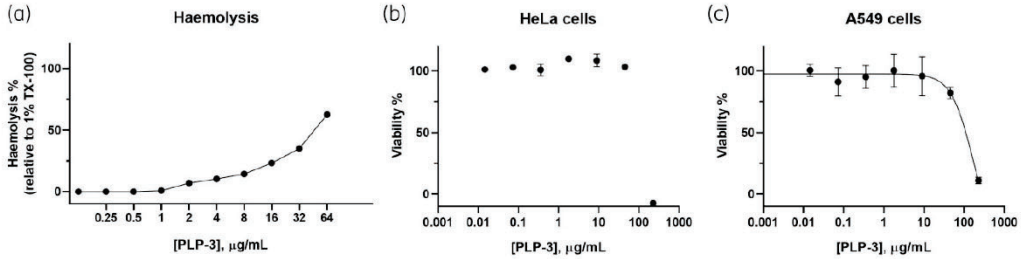

Figure 2. (a) PLP-3 haemolysis % relative to 1% TX-100. Viability of HeLa (b) and A549 (c) human cells treated for 48 h with increasing concentrations of PLP-3, as measured with the XTT viability marker.

cell death: (i) a decay in transmembrane potential; (ii) a release of potassium ions; and (iii) an influx of sodium into the cell. Data suggest that influx/efflux of osmolytes other than sodium and potassium does happen in this process. The influx and efflux of osmolytes results in water rushing inside the bacterial cell, with consequent swelling and final irreversible disruption of the structural integrity of the cell.[44] Moreover, murepavadin, another PG-1-based AMP, has a distinct mechanism of action; it binds to an outer membrane protein, the LPS transport protein D (LptD), which takes part in LPS synthesis pathways in Gram-negative bacteria.[27] Due to AMPs' prototypical mode of action on the bacterial cell membrane, resistance to these molecules is hypothesized to arise more slowly.[45]

In conclusion, our *de novo*-designed peptidomimetic PLP-3 is a novel antimicrobial agent with potent activity against colistin-resistant, -susceptible and/or MDR strains of *P. aeruginosa*, *K. pneumoniae* and especially *A. baumannii*. In addition, based on our data, resistance to colistin does not appear to affect the activity of PLP-3 against *P. aeruginosa* and *A. baumannii*. Due to its low haemolytic activity at antimicrobial concentrations and its low toxicity against human cell lines, PLP-3 shows a therapeutic window that could be exploited in further *in vivo* experiments, especially against *A. baumannii*.

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Transparency declarations

None to declare. Patent applications have been submitted by the authors in relation to this work.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online.

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Paper 2: Controlling Antibacterial Activity Exclusively with Visible Light: Introducing a Tetra-ortho-Chloro-Azobenzene Amino Acid.

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Abstract

The introduction of a novel tetra-ortho-chloro-azobenzene amino acid (CEBA) has enabled photoswitching of the antimicrobial activity of tyrocidine A analogues by using exclusively visible light, granting spatiotemporal control under benign conditions. Compounds bearing this photoswitchable amino acid become active upon irradiation with red light, but quickly turn-off upon exposure to other visible light wavelengths. Critically, sunlight quickly triggers isomerisation of the red light-activated compounds into their original *trans* form, offering an ideal platform for self-deactivation upon release into the environment. Linear analogues of tyrocidine A were found to provide the best photocontrol of their antimicrobial activity, leading to compounds active against *Acinetobacter baumannii* upon isomerisation. Exploration of their N- and C-termini has provided insights into key elements of their structure and has allowed obtaining new antimicrobials displaying excellent strain selectivity and photocontrol.

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Controlling Antibacterial Activity Exclusively with Visible Light: Introducing a Tetra-*ortho*-Chloro-Azobenzene Amino Acid

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Dedicated to Prof. Mercedes Álvarez on her retirement

Abstract: The introduction of a novel tetra-*ortho*-chloroazobenzene amino acid (CEBA) has enabled photoswitching of the antimicrobial activity of tyrocidine A analogues by using exclusively visible light, granting spatiotemporal control under benign conditions. Compounds bearing this photoswitchable amino acid become active upon irradiation with red light, but quickly turn-off upon exposure to other visible light wavelengths. Critically, sunlight quickly triggers isomerisation of the red light-activated compounds into their original *trans* form, offering an ideal platform for self-deactivation upon release into the environment. Linear analogues of tyrocidine A were found to provide the best photocontrol of their antimicrobial activity, leading to compounds active against *Acinetobacter baumannii* upon isomerisation. Exploration of their *N*- and *C*-termini has provided insights into key elements of their structure and has allowed obtaining new antimicrobials displaying excellent strain selectivity and photocontrol.

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Antibiotic resistance is an increasing concern worldwide. Accumulation of antibiotics in the environment increases the evolutionary pressure on microorganisms, which evolve and adapt at a great pace. Moreover, the number of new antibiotic classes entering the pipeline has been alarmingly low during the last decades.[1] Although new antimicrobials are still needed, a change of paradigm on how we fight bacterial infections is on high demand as new drugs are bound to suffer from the emergence of resistances as well.[2] On this regard, gaining the ability to switch drugs on and off offers a unique opportunity to avoid the release of active drugs into the environment, thus reducing the chances of resistance arising and disseminating. In particular, visible light can be used as a clean and harmless stimulus to modulate the structure of a molecule and thus, its biological profile.[3]

Natural antimicrobial peptides play a crucial role as first line of defence against pathogenic bacteria.[4] These are produced by a plethora of microorganisms, but also by complex animals, including humans.[5]

Previous works have shown how the introduction of photoswitches into small molecules can allow control over the activity of antimicrobial compounds, but only recently has the control of antibacterial activity with visible light been reported.[6] In this context, a photoswitchable amino acid fully operated under visible light is a coveted goal in order to gain control over the structure of peptides under harmless conditions.[7,8] Tetra-*ortho*-chloro-azobenzenes can be isomerised efficiently under red or green light into their *cis* form and will quickly return to their *trans* isomer under other visible light sources unless kept in the dark. Importantly, the use of harmful UV light is avoided. This feature offers a unique platform for safe in situ activation of drugs, also through living tissue, and subsequent deactivation upon release into the environment after their therapeutic use, once exposed to sunlight.

The pioneering work of Feringa and co-workers showed that photocontrol of antibacterial activity to fight the appearance of resistances is possible.[6a] Short after, Ulrich and Komarov reported the use of visible light to trigger the antimicrobial activity of a gramicidin S photoswitchable peptidomimetic, which could be deactivated upon irradiation with UV light thanks to a diaryl-ethane photoswitch (Figure 1A).[6b,f,i] Other works have also used gramicidin S as a platform to study the photocontrol of antibacterial activity in

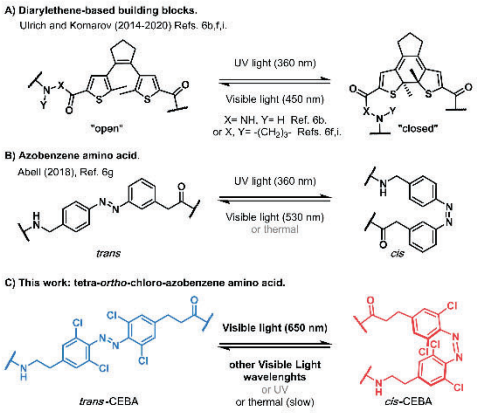


Figure 1. Photoswitchable building blocks used to photocontrol the antibacterial activity of tyrocidines' and gramicidins' analogues.

cyclic peptidomimetics (Figure 1B).[6g] However, all reports on the photocontrol of peptidomimetic's antibacterial activity require the use of UV light for one of the isomerisations. In this context, we embarked on the design of a photoswitchable amino acid building block that could be operated with visible light for both *trans*→*cis* and *cis*→*trans* isomerisations, resulting in CEBA (tetra-*ortho*-chloro-diethylelene-azobenzene Amino acid) (Figure 1C). Importantly, it was crucial to achieve fast *cis*→*trans* upon exposure to sunlight and other white light sources in order to achieve self-deactivation of the compounds upon release into the environment.

Tyrocidine A is a cyclic decapeptide with antimicrobial activity originally found along gramicidin in tyrothricin, a mixture of peptide antibiotics isolated from *Brevibacillus brevis*. Like gramicidin, tyrocidine is primarily active against Gram-positive bacteria. Modification of its structure has granted access to analogues with improved biological profiles such as an expanded panel of susceptible bacterial strains and higher therapeutic indexes.[9] However, its precise mechanism of action has remained elusive.[10]

We hypothesized that modification of tyrocidine A's structure by introducing our tetra-*ortho*-chloro-azobenzene amino acid (CEBA) in substitution of . Gln6 and Tyr7 would distort the natural peptide's β-hairpin secondary structure while in its *trans* configuration. However, upon isomerisation under red light, the *cis* form would become the major isomer and re-establish the biologically active secondary structure of the natural product (Figure 2).

Azobenzene amino acid **3** possesses the required features and its synthesis proved to be reliable and easily scalable, offering a useful route to this building block (Scheme 1). First, 3-(4-nitrophenyl)propanoic acid **4** was turned into the corresponding nitroso derivative **5** and upon condensation with aniline **6**, the azobenzene scaffold was set, yielding amino acid **7**. Subsequent palladium-catalysed chlorination gave the desired amino acid photoswitch **3** in very good yield.[11–13] Overall, Tfa-CEBA-OH (**3**) was obtained in 46% yield in just three steps, needing only one chromatographic purification at the end of the synthesis.

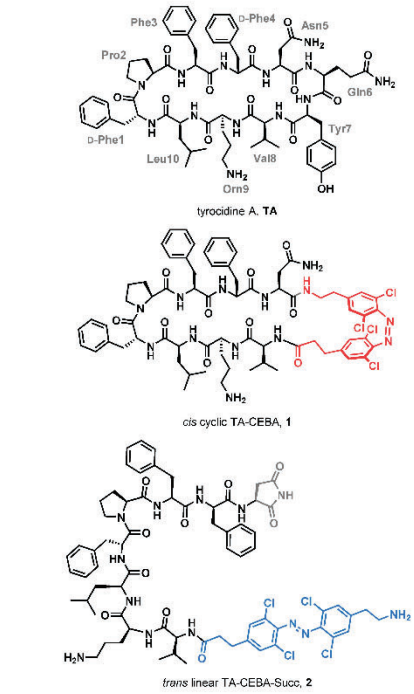
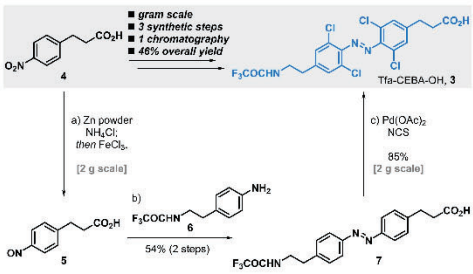


Figure 2. Tyrocidine A and examples of cyclic and linear visible light active photoswitchable analogues described in this work.



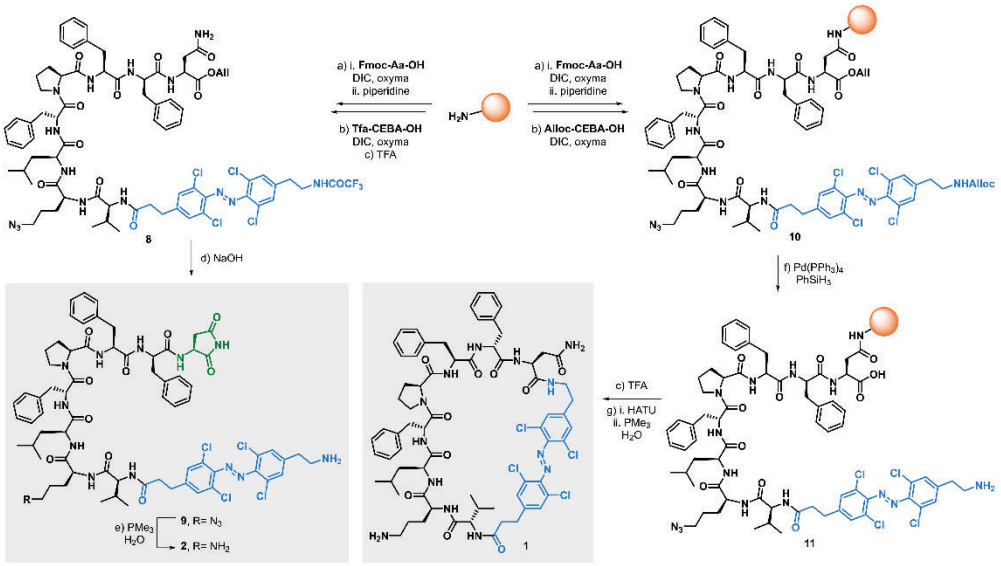
Scheme 1. Synthesis of tetra-*ortho*-chloro-azobenzene amino acid **3** (Tfa-CEBA-OH). Reaction conditions: a) Zn powder (2.5 equiv), NH_4Cl (1.5 equiv), H_2O, 2-methoxyethanol, 0 °C to rt, 17 h; *then* $FeCl_3$ (3.0 equiv), EtOH, H_2O, 0 °C, 1.5 h. b) **6** (1 equiv), AcOH, rt, 18 h, 54% (2 steps). c) $Pd(OAc)_2$, NCS (6 equiv), AcOH, 140 °C, 19 h, 85%. Tfa = trifluoroacetyl; NCS = *N*-chlorosuccinimide.

At this stage, we were ready to synthesize a photoswitchable analogue of tyrocidine A using solid phase peptide

synthesis (SPPS) starting from Gln5 anchored from its amide side chain to the resin (Scheme 2). Elongation of the peptide was performed uneventfully and TFA-CEBA-OH (**3**) was introduced last, prior to acidic cleavage to produce **8**. Subsequent basic hydrolysis in solution did not yield the expected *N*- and *C*-termini unprotected peptide, but a product with the same *m/z* as the target photoswitchable cyclic tyrocidine A analogue. Further analysis and mechanistic studies revealed that basic conditions had triggered cyclisation of the Asn residue side chain onto the allyl ester to form succinimide **9**, whose unanticipated structure prompted us to study it further and carry on with the remaining deprotection step.[14] Thus, the azide group in **9** was reduced to yield the corresponding amine **2** via a mild Staudinger reduction.[15] Alternatively, and in order to obtain the desired cyclic analogue **1**, The photoswitch was introduced as its allyl carbamate analogue Alloc-CEBA-OH, which was obtained quantitatively from **3** in a two-step, one-pot operation.[14] Once peptide **10** was ready, both termini were deprotected under Pd catalysis to yield **11**. At this stage, conditions were screened to optimise cyclisation of the peptide, which proved to be very reagent and solvent dependent.[14] In the end, the cyclised product was most efficiently obtained in solution, and a one-pot Staudinger reduction was performed last to reveal the free amine in **1**.

Photoisomerisation of the compounds was studied using **1** and **2** as models for cyclic and linear compounds, respectively. A kinetic profile of *trans* to *cis* isomerisation under red light (650 nm) was recorded and in both cases a virtually identical behaviour was observed.[14] Upon exposure to red light, the amount of *cis* isomer reaches a plateau after ca. 40 min, indicating the minimum time required to achieve the highest ratio of *cis* isomer under these conditions. The reverse process was also studied using daylight to irradiate the samples, as it would be the ideal source of light to trigger deactivation of the compounds.[14] To our delight, less than three minutes were required for the opposite isomerisation, highlighting the viability of the proposed idea of self-deactivation upon release into the environment (Figure 3). Importantly, the same behaviour was observed for all compounds described in this work.

With **1** and **2** in our hands, we carried out some preliminary antibacterial activity assays, which showed a more interesting biological profile for **2** (see below). This finding prompted us to investigate further the role of the *N*- and *C*-termini moieties present in this linear analogue. Thus, using the same synthetic approach, we prepared a small library of linear peptides with modifications on both termini. These included substitution of the photoswitchable amino acid CEBA for a photoswitchable carboxylic acid analogue without the 2-aminoethyl tail (**12**) and substitution of the Gln-derived succinimide moiety for the corresponding glutamine carboxylic acid or glutamine amide (Figure 4).



Scheme 2. Synthesis of linear and cyclic Visible Light photoswitchable tyrocidine A analogues **1** and **2**. Reaction conditions: a) see Supporting Information for details on manual and microwave-assisted SPPS methods. b) Alloc-CEBA-OH or Tfa-CEBA-OH (2.3 equiv), DIC (2.5 equiv), oxyma (2.5 equiv), DMF, rt, 16 h. c) TFA/CH_2Cl_2 (95:5), rt, 5×45 min. d) NaOH (3.5 equiv), THF, MeOH, H_2O, rt. e) PMe_3 (3 equiv), H_2O, THF, 0 °C to rt. f) $Pd(PPh_3)_4$ (10 mol%), $PhSiH_3$ (20 equiv), CH_2Cl_2, rt, 15 min. g) HATU (2 equiv), DIPEA (6 equiv), DMF, rt, 16 h; *then* PMe_3 (5 equiv), H_2O, 0 °C to rt. Alloc = Allyloxycarbonyl; DIC = *N,N'*-diisopropylcarbodiimide; oxyma = ethyl cyanohydroxyiminoacetate; DMF = dimethylformamide; TFA = trifluoroacetic acid; THF = tetrahydrofuran HATU = 1-[bis (dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; DIPEA = diisopropylethylamine.

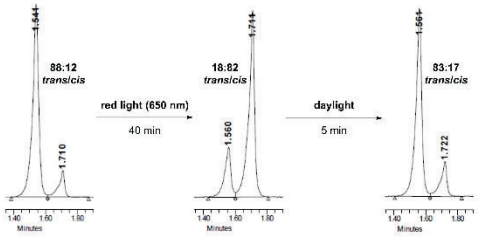


Figure 3. Photoisomerisation of 2. UPLC analysis of sequential *trans*→*cis* and *cis*→*trans* isomerisation of 2.

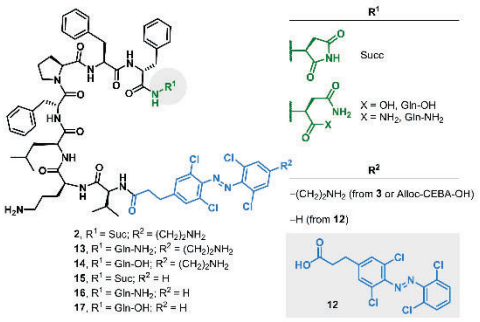


Figure 4. Library of linear photoswitchable analogues of tyrocidine A.

With all the analogues in hand, we next tested them against a panel of Gram-positive and Gram-negative bacterial strains (see the complete table in the Supporting Information). To our surprise, cyclic analogue **1** displayed a lack of photocontrol against all strains tested and its biological profile was very similar to **TA**, with the exception of *A. baumannii* (Table 1). Very interestingly, linear analogue **2** also had a similar profile, although photoswitchability against *A. baumannii* (both a susceptible -ATCC 19606- and a colistin-resistance strain -CR17-) was now observed and at least a 3-fold decrease in the MIC of the compound was observed upon activation with red light. Substitution of the Gln-derived succinimide for the corresponding glutamine amide (**13**) or carboxylic acid (**14**) had completely opposite effects on the compounds' activity against *A. baumannii*, showing lower MICs for analogue **13** (8–16 μg mL^{-1}) than analogue **14** (>64 μg mL^{-1}) in either photostationary state, whereas they behaved very similarly against the rest of the panel. Removal of the 2-aminoethyl tail from CEBA (analogues **15**–**17**) drastically changed the biological profile of the peptides, as they lost activity against most strains, including Gram-positive bacteria. However, upon irradiation at 650 nm, an increased susceptibility was observed for compounds **16** and **17** against *S. pyogenes*. In particular, **17** displayed great selectivity against this strain, whereas it remained inactive against all the other ones tested during this study.

Depending on the compound, differences were also observed in terms of their haemolytic effect.[14] **TA**, **2**, **13** and **15** were less haemolytic in their non-irradiated state compared to the irradiated one, whereas compounds **1**, **14**, **16** and **17** showed the opposite effect. In the case of compound **17**, which showed good antibacterial activity against *S. pyogenes* in the irradiated state, a high IC50 value (173 μg mL^{-1}) was also observed (low haemolytic effect), which can likely be attributed to its lack of a net positive charge under the assay conditions and is an optimistic starting point for therapeutic purposes.

In conclusion, we have developed an efficient synthesis of the novel tetra-*ortho*-chloro-photoswitchable azobenzene amino acid (CEBA) that can be incorporated into peptidic sequences to grant photocontrol using exclusively visible light. This has led to the development of **2**, a photoswitchable tyrocidine A linear analogue with a *C*-terminal succinimide moiety that can be activated under red light to become active against a clinically relevant Gram-negative strain such as *A. baumannii*. Importantly, the compound quickly becomes inactive against this pathogenic strain upon exposure to sunlight, enabling self-immolation upon release to the environment. Taking into consideration the available data for the linear analogue **17**, selectivity and photocontrol have been observed against *S. pyogenes*, achieving the highest IC50 value in both photostationary states out of all the analogues tested. To confirm their antimicrobial effect against this species, further studies increasing the number of *S. pyogenes* strains should be conducted. In addition, the decreased toxicity (haemolytic assay) observed in some of the non-irradiated compounds is an important added value of this strategy that may also contribute to preserving the ecosystem and should be considered in further studies. We hope that these findings will help advancing in the development of more efficient strain selective and self-

Table 1. Selected in vitro antibacterial activities of **TA** and its photoswitchable analogues.[a]

	TA		**1**		**2**		**13**		**14**		**15**		**16**		**17**	
	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]	NoIrr	Irr[b]
Bacillus subtilis[c]	4	4	4	8	2	2	1	2	4	16	>64	>64	64	64	>64	>64
Streptococcus pyogenes 016[c]	**4**	**4**	**4**	4	4	**4**	4	4	8	**4**	>64	**64**	**>64**	**16**	**>64**	**16**
Escherichia coli ATCC 25922[d]	>64	>64	>64	>64	>64	64	>64	32	>64	>64	>64	>64	>64	>64	>64	>64
Acinetobacter baumannii ATCC 19606[d]	16	16	>64	>64	**64**	**8**	8	8	>64	>64	>64	>64	>64	>64	>64	>64
Acinetobacter baumannii CR17[d]	32	16	>64	>64	>64	32	16	8	>64	>64	>64	>64	>64	>64	>64	>64

[a] Measured as minimum inhibitory concentration (MIC) in μg mL^{-1}. [b] Plates were irradiated at 650 nm for 40 min before incubation in the dark. [c] Gram-positive strain. [d] Gram-negative strain.

deactivating photoswitchable antimicrobials that help diminish the increasing threat of antibiotic resistance.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: antibiotics · azobenes · peptidomimetics · photochemistry · tyrocidine A

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7 DISCUSSION

AMR is already a global health threat, and it demands immediate action and effective measures such as antimicrobial stewardship and infection prevention measures.(1,80) These interventions to prevent the emergence and spread of multidrug resistant bacteria should be taken from a local to global level, in this sense four different scenarios have been established: i) at health Centers, mainly hospitals and long-term care facilities; ii) At the community, in which four players are involved, which are: people in the community, pharmacists, primary care physicians and veterinarians; iii) at a national level, with the implementation of strategic plans to combat AMR and iv) at the international level, meaning that in order to have a successful international organization and results, all national plans should be integrated, and in this case, WHO should play the role as an integrative institution.(316) The magnitude of AMR is better understood through a One Health perspective and its impact on human health can turn what nowadays we deem common bacterial infections and routine medical interventions into high risk if there are no effective antimicrobial treatments.(10,16) Aside from the aforementioned measures, discovery and development of novel and effective treatments against drug resistant bacteria is essential for filling the clinical pipelines with promising candidates and thus, for securing public health for current and future generations.(72,80,83,317)

The papers and manuscript included in this thesis present different approaches for design and development of novel antimicrobial agents effective against multidrug-resistant bacteria.

Manuscript 1 marks the first of the antimicrobial discovery and development strategies included in this Ph.D. project. This article analyses the approach of optimizing a known and described linear peptide through production of analogues or derivatives from its structure and further analysing them antimicrobial activity-wise. The process of rational designing peptides and further screening of the derivatives to obtain those with the most potent antimicrobial activity is a common practice when studying and producing novel AMPs(318,319) and compared to novel approaches to design AMPs (e.g., *in silico* machine-learning motif

identification through physiochemical properties(320), *ab initio*(321) or molecular dynamic plus *de novo* design(322)), has the advantage of being based in a determined and characterized single structure upon which powerful tools for rational design, used and developed in novel approaches, can also be used and without the nuisance of needing quality cured AMP databases (which can have mismatching and outdated data). Our hypothesis when producing CAP-18 derived peptides was that these analogues would have higher antimicrobial activity against *A. baumannii* and *P. aeruginosa* strains than their parent peptide, and that it would be related both to the specific sequence designed but also to their conformation. To test this hypothesis, we produced peptides with different lengths which included different motives from the parent peptide CAP-18. Therefore, reasoning behind the derivatives' design was as follows. First, four peptides (CAP-18_{14}, CAP-18_{18}, CAP-18_{19} and CAP-18_{23}) were designed and synthesized to test whether truncating the N-terminal end of CAP-18 would result analogues having better antimicrobial activity than that of the parent peptide against *A. baumannii* and *P. aeruginosa* strains. These peptides were designed keeping all Q residues from CAP-18 in their structure since these polar amino acids of neutral charge might have an effect on the peptides' antimicrobial activity. Peptide design was thought so that the same N-terminal part was kept for peptides in couples, while each member of the couple included different motive lengths in their C-terminus to allow for further analysis. These couples were CAP-18_{14} and CAP-18_{18}, and CAP-18_{19} and CAP-18_{23}. Then, truncation of C-terminus of CAP-18 was to follow. Peptides CAP-18_{21} and CAP-18_{31} were designed and synthesized for this reason, the latter peptide including the aforementioned Q motive to check for its relevance in the peptide's antimicrobial activity against the selected strains. Finally, enantiomers and retroenantiomers of CAP-18 and CAP-18_{31} (since these latter two peptides arguably showed the most powerful activity against selected strains in an initial screening) were synthesized with the purpose of having analogues with potentially different toxicity and stability properties as has been reported in literature that peptides containing D amino acids are more resistant to proteolytic activities.(323–325) Four peptides were designed and produced, namely, D-CAP-18, D-CAP-18_{31}, R-CAP-18 and R-CAP-18_{31}.

As with CAP-18, rational design was used in Paper 1 to produce PLP-3's structure. At the beginning of PLP-3's project, the structure of the template peptide, PG-1, was thoroughly analysed to identify the motives and structural conformations principal to its antimicrobial activity: the β-hairpin conformation, polycationic nature with guanidium groups at the hairpin's edges and having amphipathic character (which allows for interaction with the bacterial membrane's lipids).(326–329) The objective followed when designing PG-1 derived peptides was the structural optimization of PG-1's features; then, our objective moved towards assessing the activity of a single selected peptide derivative against a panel of MDR Gram-negative strains. At first, the design of PG-1 derivatives sought to produce rigid structures; this was prioritized because rigidity could provide more stability *in vivo* by turning the peptide derivatives resistant against exopeptidase actions, and moreover, rigid structures could also result in both better antimicrobial activity and less toxicity.(330,331) It was considered discarding flexible tails from PG-1 and linking the antiparallel β-strands of the peptide from head to tail to obtain macrocycle and constrained structures that had more rigidity. Finally, *in silico* structural tools (the generalized kinematic loop closure method in Rosetta software) were used to narrow down the possible chemical structures and peptide sequences displaying PG-1 features selecting analogues with cyclic backbone conformations and protocols to introduce amphipathic side chains and disulphide bonds(332). All the *in silico* generated peptides that fell under the previously described conditions were inspected to check for those which would fold in a PG-1 like β-hairpin conformation. PLP-3 was selected because it adopted a slightly twisted β-hairpin structure while having the lowest energy structure from all the candidates produced. Upon later analysis, PLP-3's structure was noticeably similar to that of PG-1 inserted in a lipid bilayer,(333) despite PLP-3 having a smaller size than PG-1, which might be relevant for both these peptides' antimicrobial activity.

In contrast with the structural optimization approaches developed in Manuscript 1 and Paper 1 upon antimicrobial peptide templates, the strategy followed for the design of photoswitchable antibiotics in Paper 2 is specifically focused on the modification of the tyrocidine A molecule to harbour a tetra-ortho-chloro-

azobenzene amino acid (CEBA), an essential building block for photocontrol of antimicrobial activity. Regarding the characteristics of the analogues' two components, briefly, tyrocidine A is a known cyclic decapeptide with β-hairpin structure, that has antimicrobial activity primarily against Gram-positive bacteria and that is produced with other bacteriocins, such as gramicidin and tyrothricin, by *Brevibacillus brevis*; and then, regarding the photoswitchable building block characteristics: tetra-ortho-chloro-azobenzenes are capable of isomerising to their *cis* form by being exposed to red or green light (which is maintained if kept in the dark) and then return into their *trans* isomer upon exposure to visible light. Photoswitchable analogues were produced thanks to the design of variations of a tetra-ortho-chloro-azobenzene amino acid (CEBA) and their introduction into the antimicrobial peptide's structure. The design of tyrocidine A analogues was based on the substitution of the residues Gln6 and Tyr7 from the original tyrocidine A structure by CEBA, so that original tyrocidine A structure would stay distorted while under visible light exposure (*trans* configuration) and would revert to the biologically active β-hairpin conformation when exposed to red light (*cis* configuration). Up to thirteen photoswitchable linear and cyclic analogues of tyrocidine A were synthesized by including modifications upon their CEBA building blocks, which were then classified and analysed by their antimicrobial activity before and after red light activation against a panel of multidrug resistant pathogens. Finally, the seven analogues with the most potent activity against the pathogen panel were included in the results from Paper 2.

The strategies for peptide design vary between these projects depending on the objectives we tried to accomplish. In Manuscript 1, linear peptides of different lengths were designed by including motives from the parent peptide CAP-18 that could be of interest due to their possible relation with the parent peptide's antimicrobial activity. However, in Paper 1, when designing peptides derived from PG-1, we did not only consider the specific motives or structural features that relate to PG-1's antimicrobial activity, but we also had in mind designing a peptide that kept a bicyclic structure because of the stability advantages these structures could provide. Regarding Paper 2, design of tyrocidine A analogues was directly aimed towards adding the photoswitchable building block CEBA to their structure,

so that different CEBA modifications were included and also producing linear or cyclic analogues; in this project, even if producing analogues with an attractive antimicrobial activity was pursued, having effective photocontrol of that activity was prioritized.

There has been an evolution in how we have faced developing novel antimicrobials through the projects in this thesis. Our initial research involved working with linear peptides derived from CAP-18, which is a straightforward approach when compared to the rational design process of PG-1's derivative, PLP-3. However, linear peptides such as the ones in CAP-18 project, are often susceptible to proteolysis and non-specific binding to human serum or plasma proteins. Therefore, in our next studies in Paper 1 we opted to focus on a PG-1 candidate with a bicyclic structure that could overcome this proteolysis hurdle.(329,334) Later, in Paper 2, we moved forward from just producing AMPs with improved properties, in order to address the issue of antibiotic resistance spread through the release of antibiotics into the environment. Drawing from our experience and knowledge on AMPs, we focused on pursuing a strategy that included a One Health perspective. That is why we aimed to create photocontrollable analogues of tyrocidine A.

Antimicrobial activity screening results of these peptides against a panel of bacterial strains allowed for comparison. In Manuscript 1, the most potent peptides were CAP-18, CAP-18_{31} and their enantiomers D-CAP-18 and D-CAP-18_{31}. Retroenantiomers had a slight lower antimicrobial activity compared to D-enantiomers and thus it was decided to leave them aside. Shorter peptide derivatives truncating either end of the parent peptide CAP-18 resulted in analogues with higher MIC values (a loss in antimicrobial activity). In view of these assay results, we hypothesize that those regions excluded in the shorter peptides played an important role in the antimicrobial activity of the peptides, maybe because shorter length and missing motives translated into a structural conformation with less activity. As reported in literature, a truncation of 5 or more

residues in the N-terminal end of CAP-18 resulted into peptides with worse antimicrobial activity in our screening.(318,335)

Regarding future steps of CAP-18 derivative project, aliquots of CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} were synthesized for *in vivo* safety and efficacy assays in a murine skin infection model. To such end, lyophilised peptides were provided to the 'Servei de Desenvolupament del Medicament' at the Facultat de Farmàcia I Ciències de l'Alimentació at the University of Barcelona and in order to produce hydrogels from each peptide (based on water, propylene glycol, carbopol and trometamol) as well as a placebo hydrogel without peptide. A Contrary to *in vitro* assays, lyophilised peptides were synthesized in hydrochloride form instead of the usual trifluoroacetic (used in peptide synthesis to cleave the peptide product from the resin) since the presence of trifluoroacetic salts in peptides for *in vivo* testing has been reported to affect biological and physio-chemical properties of peptides.(336–338) After careful *in vitro* analysis to check that the antimicrobial activity of the peptide hydrogels was kept as well as that the placebo formulation did not inhibit bacterial growth, these hydrogels were sent to the Biomedical Institute of Sevilla for *in vivo* assays on the aforementioned murine skin infection model.

All CAP-18 derived peptides have MIC_{90} values of 0.5 mg/L against *A. baumannii* strains while PLP-3's MIC_{90} sits 2 folds higher against the same species at 2 mg/L. Tyrocidine A linear analogue 2 is the only comparable photoswitchable peptide to these values, with MIC values between 8 and 32 mg/L against both *A. baumannii* and *A. nosocomialis* strains tested in Paper 2. Antimicrobial activity comparison against *P. aeruginosa* is more complex: PLP-3's MIC_{90} is at 8 mg/L, 2 folds higher than D-CAP-18 at 2 mg/L and a single fold higher than its enantiomer CAP-18 at 4 mg/L; both CAP-18_{31} and D-CAP-18_{31} MIC_{90} are at 16 mg/L which is just a fold higher than PLP-3. No photoswitchable tyrocidine A analogue had a comparable antimicrobial activity against the *P. aeruginosa* strain tested in Paper 2.

Moving now into haemolysis assays, although they were performed at an erythrocyte concentration of 2% for PLP-3 and tyrocidine A photoswitchable derivatives versus a 1% for CAP-18 derived peptides, haemolysis values for CAP-18 derived peptides remain lower than those of PLP-3 at the same concentrations, while all photoswitchable tyrocidine A analogues (but analogue 17) stand as the most toxic of these three projects. Comparing results from Manuscript 1 and Paper 1, haemolysis at a peptide concentration of 16 mg/L is lower than 2.2% for all CAP-18 derived peptides while around 23.3% for PLP-3. These lower results for CAP-18 derivatives are also clearly seen when comparing haemolysis IC_{50} values: while PLP-3 has an IC_{50} of 48.5 mg/L, D-CAP-18 (the peptide with the lowest IC_{50} value) is at 274.76 mg/L. Irradiated photoswitchable derivatives (active in *cis* conformation) present the lowest haemolysis IC_{50} values ranging from as low as 13 mg/L for analogue 13 to 33 mg/L for both analogue 14 and 15 (both under PLP-3's IC_{50}) and the only analogue with a higher IC_{50} value is analogue 17 at 173 mg/L; taking as a group, irradiated photoswitchable derivatives IC_{50} values are the lowest and point that these AMPs are the most toxic of all the projects in this thesis. CAP-18 derived peptides haemolysis results suggest low toxicity against human erythrocytes at clinically relevant concentrations, as is seen when comparing IC_{50} values to MIC_{90} per each pathogenic species described. Cytotoxicity assays of CAP-18 derived peptides and PLP-3 upon human cell lines A549 and HeLa (commonly employed as reference cell lines and recommended for infectious diseases and toxicology research) revealed that cell proliferation and viability were not as affected by PLP-3 than for CAP-18 derived peptides, in contrast to results from haemolysis assays. In the case of HeLa cells, PLP-3 did not significantly inhibit cell viability and proliferation until the highest concentration tested at 225 mg/L, while CAP-18 derived peptides had lower IC_{50} values: 48.9 mg/L for CAP-18_{31}, 23 mg/L for D-CAP-18_{31} or as low as 9.3 mg/L for D-CAP-18 (CAP-18 was at 40.4 mg/L). Finally, for A549 cells PLP-3's IC_{50} was around 100 mg/L, and again lower values were registered for CAP-18 derived peptides: 4.9 mg/L for CAP-18_{31}, 46 mg/L for D-CAP-18_{31} and 9.3 mg/L for D-CAP-18 (noticeably, CAP-18 was at 3.7 mg/L).

Finally, results from TEM plus time kill kinetic assays for CAP-18 derived peptides and the membrane permeability assays for PLP-3 hint into the importance of peptide and bacterial membrane interaction (and possibly disruption) as a mechanism of action for all these peptides. Incubation of CAP-18 derived peptides with strains of *A. baumannii* and *P. aeruginosa* produced damages upon their bacterial membranes, intracellular component aggregation and cell leakage; these phenomena recorded in TEM micrographs is consistent with bacterial membrane disruption. PLP-3's membrane permeability assays showed that this peptide had a rapid and dose-dependent permeabilization of the bacterial membrane in both *P. aeruginosa* and *A. baumannii* cells (having a percentage of permeabilized cells of both of these species of over 50% at a concentration of 16 mg/L just after 5 minutes of incubation). Even though PLP-3's mechanism of action is yet to be described with further assays, these results point to the importance of its interaction with the bacterial membrane which would not be farfetched considering PG-1's mechanism of action has been reported to be related to membrane disruption through formation of pores.(339) Moreover, regarding time kill kinetic assays, CAP-18 derived peptides have bactericidal activity against *A. baumannii* strains within 2 to 4 hours of incubation, while for *P. aeruginosa* only the peptides CAP-18 and D-CAP-18 produced this effect between 2 and 8 hours of incubation on both strains tested. CAP-18_{31} and D-CAP-18_{31} achieved bactericidal effect upon *P. aeruginosa* R2 after 4 to 8 hours of incubation but did not show bactericidal effect upon strain *P. aeruginosa* 121110 even at 8 times its MIC.

This section would not be complete without reflecting on the possible shortcomings that using AMPs could have as antimicrobial therapies. AMPs have been proven to be versatile immune modulators, which makes them powerful drugs to control and fight infections(232). Activating the innate immune response has by itself the characteristics of an ideal antimicrobial therapy: it is rapid, it is related with the action of a group of mechanisms (which arguably could lower the resistance emergence rate) and it is relatively nonspecific. However, the overstimulation of the immune response can increase harmful effects of proinflammatory responses and even the risk of developing sepsis(232). Due to

the complexity of the immune response on its own and of the relationship between the usage of AMPs and immune response modulation, the consequences of using novel AMPs as antibiotics should be determined in developmental studies so that AMPs can be the powerful and safe tools to fight bacterial infections that we need. The development of photoswitchable AMPs is not without limitations, for instance, first differences in antimicrobial properties between the active and inactive conformations of the photoswitchable analogues were moderate and also, regarding the specific approach taken, *in vivo* reduction of azobenzenes such as CEBA might cause free and potentially toxic anilines(340) (although other photoswitchable compounds like acylhydrazones are not without risk(311,341)). Nonetheless, we believe that it is worth investing further research efforts in having effective antibiotics while inactive upon their release into the environment. Also, our attempt in developing photoswitchable AMPs in this thesis provides novel and useful tools for future research in photocontrollable antibiotics using CEBA.

Concerning the future steps of the antimicrobial development projects and regarding the current data presented in this thesis, the different projects presented would greatly benefit from structure-activity relationship studies (e.g., PLP-3 and CAP-18 derived peptides projects would greatly benefit from microscopy assays where fluorescent labelled analogues of these peptides were used upon bacteria to illustrate bacterial localization),the production of bacterial resistant mutants to these peptides to better understand the antibiotic candidates' targets or mechanisms of action, *in vivo* toxicity studies (especially considering antimicrobial peptides are known for their toxicological liabilities), further pharmacokinetics and pharmacodynamic analyses or even other assays such as proteomic arrays or pull down assays, that would help better illustrating and understanding both the stability profile and the mechanisms behind these promising antimicrobial candidates' modes of action. As pointed out in literature, insufficient characterization of antibiotic candidates' *in vitro* activity, lack of acknowledgment of emergence of target-based resistance and lack of awareness of toxicological issues represent shortcomings in the development of novel antibacterials(72). Therefore, we consider that the results of the assays and

studies proposed would be of great aid in planning future drug development steps in each of these projects and even in the acceptance of funding applications for preclinical assays, which is crucial due to the large financial investment that takes place in this early phase of antibiotic development.

The research presented in this thesis highlights the diverse strategies that can be employed when designing and developing novel AMPs against drug-resistant pathogenic bacteria. The studies included in this work are focused on enhancing antimicrobial activity, improving stability and introduce photocontrol of antimicrobial activity. The results demonstrate powerful antimicrobial activity for these AMP candidates, especially for CAP-18 derived peptides and PLP-3, adding to much needed research against Gram-negative MDR pathogens such as *A. baumannii* and *P. aeruginosa*. The findings from these projects contribute to the global efforts to combat bacterial AMR through obtaining novel antimicrobials and provide insights into designing effective AMPs. It is crucial to continue exploring innovative approaches through multidisciplinary research to gain novel tools against ever evolving bacterial pathogens.

8 CONCLUSIONS

1. From all CAP-18 derived peptides designed and tested, CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} were the derivatives with the most potent antimicrobial activity against multidrug resistant *A. baumannii* and *P. aeruginosa* strains.

2. The peptide PLP-3 has potent antimicrobial activity against multidrug resistant strains of *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*.

3. Truncation of the N-terminus of CAP-18 to generate derivatives, especially by 5 or more amino acids, resulted in peptides showing a significant loss of antimicrobial activity when compared to longer derivatives such as CAP-18_{31} or D-CAP-18.

4. Presence of human serum albumin at physiological concentrations affects the antimicrobial activity of CAP-18 derived peptides increasing their MIC values. This effect might be a consequence of unspecific binding of derivatives to albumin, thus limiting free peptide concentration and requiring higher peptide doses to elicit an inhibitory effect on bacterial pathogens. All in all, CAP-18 derivatives MICs against *A. baumannii* in human albumin supplemented media are within attainable ranges suggesting still favourable *in vivo* concentrations could be reached against this pathogen.

5. Antimicrobial activity of PLP-3 against *A. baumannii* is maintained at physiological concentrations of human albumin, suggesting low binding of PLP-3 to human albumin.

6. Derivatives CAP-18_{31} and D-CAP-18_{31} are the least haemolytic peptides and have the widest therapeutic window against *A. baumannii* of all the

CAP-18 derivatives tested, suggesting their activity is selective against bacterial cells rather than human erythrocytes.

7. PLP-3 human erythrocyte haemolysis is low at clinically relevant concentrations that inhibit bacterial cell growth.

8. Although there are moderate differences in toxicity of each CAP-18 derived peptides against human cells, results vary between the cell types tested when comparing estereoisomers. D enantiomers of CAP-18 derived peptides have the lower toxicity against A549 cells while L enantiomers CAP-18 and CAP-18_{31} are less toxic than D-CAP-18 and D-CAP-18_{31} against HeLa cells.

9. Cytotoxicity assays of PLP-3 upon A549 and HeLa show this peptide is not cytotoxic against the cell types tested and that cytotoxicity is low at clinically relevant concentrations that inhibit bacterial growth.

10. Peptides CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} have bactericidal effect against *A. baumannii* strains within 2 to 4 hours of incubation. Bactericidal effect on *P. aeruginosa* took wider incubation times of 2 to 8 hours for CAP-18 and D-CAP-18 while CAP-18_{31} and D-CAP-18_{31} showed bactericidal effect between 2 to 8 hours only against *P. aeruginosa* R2 while they did not show bactericidal effect against the strain *P. aeruginosa* 121110.

11. Transmission Electron Microscopy studies of CAP-18 derived peptides against *A. baumannii* and *P. aeruginosa* strains show alterations of the bacterial outer membranes, empty vesicles and clear spots in the cytoplasm, aggregation and membrane rupture. These phenomena are

consequent with peptide membrane targeting and interaction, a well-known mechanism of action of antimicrobial peptides.

12. Membrane permeability assays suggest PLP-3 has a rapid and dose-dependent permeabilization of *A. baumannii* and *P. aeruginosa* membranes suggesting a mechanism of action related to membrane disruption.

13. Peptides PLP-3, CAP-18, D-CAP-18, CAP-18_{31} and D-CAP-18_{31} are potential candidates for clinical development as effective antibiotics against drug resistant bacterial pathogens.

14. Development of a novel photoswitchable tetra-ortho-chloro-azobenzene amino acid that can be operated with visible light enables gaining photocontrol of the antimicrobial activity of peptides.

15. Linear and cyclic photoswitchable tyrocidine A analogues show promising antimicrobial activity results and photocontrol, in particular linear analogue 2 against *A. baumannii* strains tested.

16. Photoswitchable tyrocidine A analogues quickly turn into an inactive state upon daylight exposure, providing a tool to better control de release of antibiotics into the environment, thus reducing the chances for emergence of resistance.

17. Research on photoswitchable tyrocidine A analogues offers a novel approach in fighting antimicrobial resistant bacteria, by using visible light as a safe stimulus for controlling antimicrobial activity.

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