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Design and optimization of new antimicrobial peptides against gram-negative bacteria

PhD Thesis presented by Daniel Sandín García

Thesis director: Marc Torrent Burgas

December 2022

Department of Biochemistry and Molecular Biology of Universitat Autònoma de Barcelona

Prólogo

Tras estos cinco largos años que han supuesto esta etapa y aunque no soy muy dado a hacer este tipo de cosas, creo que es un buen momento para empezar a agradecer a todo el mundo que me ha ayudado o apoyado, o al menos me ha hecho sentir así durante este largo trayecto. Es cierto que ha habido tiempos mejores y peores, pero creo que haciendo un computo global de toda la tesis, los aspectos positivos siempre van a ser más y mejores. Más allá de que haya aprendido a utilizar maquinitas y a maltratar seres unicelulares, creo que este doctorado me ha aportado otras muchas cosas. Me ha enseñado a ser capaz de hacer las cosas por mi mismo, a no darme por vencido, a confiar más en otras personas para sacar adelante las cosas (y también en sentido contrario) y a hacerme valer dentro de un ambiente en ocasiones demasiado competitivo y hostil (aún estoy por entender porque esto es así). Creo que todas las experiencias vividas estos ya más de 5 años me ayudarán sin duda alguna en lo que está por venir.

Ahora viene la parte complicada, ya que mostrar mis emociones nunca ha sido uno de mis mayores valores. Igualmente haré este "esfuerzo", ya que en papel me va a costar un poco menos. No soy muy dado a reconocer lo que hayan podido hacer por mí, además de darme la existencia, educación y esas cosas, pero está claro que, si no fuera por ellos, yo no estaría aquí ahora mismo haciendo esta pantomima. Tanto mi padre, Patxi como mi madre, Beli, dentro de lo diferentes que pueden ser en formas y modo, han conseguido que haya llegado hasta aquí y no me pierda en el camino. Por eso, este trabajo siempre será tan mío como vuestro (si no os gusta lo que ha salido, os aguantáis). Evidentemente, tampoco lo habría conseguido sin su otra creación, mi hermano Juan y Dory, que aunque hace mucho que no los tengo cerca al irse a Bélgica por amor y claro está por el amparo capitalista de un sueldo digno, siempre me ha apoyado desde la distancia y cuando se ha hecho alguna visitilla. Puede que en muchas cosas no coincidamos, pero igualmente siempre has sido el que mejor me ha entendido desde épocas más vestigiales, por decir de algún modo. Esto lo estoy escribiendo mientras el

pequeño Nico está en camino, así que soló espero que os vaya genial con el peque y que pueda hacer mínimamente por el lo mismo que habéis hecho por mí. Eso sí, ya se que es difícil, pero si es más cerca de casa, mejor que mejor. Dentro de la familia, y teniendo en cuenta que me he ido de casa para llevar a cabo esto, tanto mi tío Ángel como mi primo Mikel han sido un gran apoyo. Siempre he esperado con muchas ganas las comidas de los fines de semana para poder charlas con vosotros sobre cualquier cosa. Si bien es verdad que no sé al final si voy a saber más sobre arquitectura que de todas las ciencias de la vida juntas. Voy a hacer un alto para hacer una mención especial. La verdad es que el doctorado me ha consumido mucho tiempo y al final no he podido pasar por allí lo que me habría gustado. Sin embargo, parte de la culpa es mía y espero tener aún tiempo para poder disfrutar de tiempo contigo. No sé hasta qué punto serás consciente de esto que estoy haciendo, pero también te lo dedico abuela María y al abuelo Abel, que lo estará viendo allí donde esté.

Como esto va por fascículos, como las buenas series, ahora llega el tramo de amig@s y compañer@s. Voy a empezar sin embargo antes con mi director de tesis Marc. Igual si tuviera que hacer ahora mismo una descripción en la que se explique la relación amor-odio podría añadir tu imagen, por las largas temporadas de frustración que he vivido. Sin embargo, está claro que me has enseñado mucho de lo que sé y a la hora de la verdad me has apoyado cuando he tenido un problema. Espero que siga habiendo una conexión al terminar la tesis y que os vaya muy bien a Natalia y a ti en la UAB (sin monopolizarla). También tengo que mencionar a David Andreu, que, aunque no he podido pasar tanto tiempo trabajando contigo salvo el primer año, y que por temas burocrático de simple dejadez no vayas a aparecer en un lugar tan destacado como me gustaría en esta tesis, para mi eres sin lugar a dudas el codirector de esta tesis. Volviendo a los estratos del proletariado al que pertenezco, tengo que mencionar especialmente a mis compañeros aquí, Núria, Javi y Roberto. Vosotros mejor que nadie comprendéis lo que ha sido pasar por este camino de "gloria" y siempre nos hemos apoyado en los malos momentos y reído en los buenos. Más que un compañero, sabéis que aquí tendréis un amigo para lo que sea (ah bueno, y mi más sentido pésame a las nuevas generaciones del lab Sysbio).

Igualmente, también me tengo que acordar a mis otros compañer@s del PRBB. María, Javi, Clara, Sira, Mar y un largo etcétera de gente con el trasiego que había en ese laboratorio. Solo lamento no haber podido contribuir a conseguir más trofeos de volley para el equipo. En mi último año, he tenido la suerte de realizar una estancia en Oporto en el laboratorio de Paula Gomes, donde pude aprender cosas nuevas y conocer a Cátia, Ana, Melanie y Teresa. Más allá del estrés que tenía con tesis y publicaciones, fueron tres meses maravillosos que me aportaron mucho personal y profesionalmente. Esto tampoco me habría salido tan bien sin mis compis de piso en Sabadell, con quien he tenido grandes momentos. Está claro que aquí hay que hacer especial mención a la cuadrilla de "krakos", mi familia de no consanguineidad que me habéis dado fuerza de tantas formas diferentes. Las noches de partidita, las visitas exprés a Gasteiz para alguna excursión más fiesta tonta, los viajes y mil cosas más. Todos (incluso los que no estáis tan cerca): Josu, Antxon, Javi, Iñigo, Andrés, Ander, Miguel, Mikel y David. Gracias por toda una vida de fechorías, risas y experiencias. Espero que siga así para siempre.

Por último, pero no por ello menos importante, tengo que dedicarte este último párrafo a ti, Sara. Igual no llevas tanto tiempo en mi vida como muchas de las personas aquí mencionadas, pero esto del cariño y felicidad por suerte no se mide en unidad de tiempo. Tuve la suerte de que nos encontráramos y desde entonces te has convertido en mi mejor apoyo y con quien más disfruto de los momentos fuera de la universidad. Nunca tendré palabras ni tiempo suficiente para agradecerte que me hayas aguantado en mis tiempos más bajos y solo espero poder seguir aportándote para que seas feliz. Sé que estos últimos meses no estoy siendo la mejor persona que puedo ser para ti, pero estoy convencido que dentro de muy poco las cosas cambiarán (con esto no quiero decir que estén mal ahora, ya lo sabes) y poder hacer todas las aventuras que tenemos pendientes sin tener tantas preocupaciones. ¡Japón nos espera! Entre otras muchas cosas.

Ahora es el momento de que los valientes y/o los que estén obligados se coman el pedazo de tostón que va detrás de esto. Los que continúen la lectura a partir de aquí serán los verdaderos héroes.

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ABSTRACT

English

The emergence of new microbial drug resistances has conferred increasing harm to human health every year, of which multi-drug resistance is exceptionally problematic. Antimicrobial peptides (AMPs) are a bundle of molecules originating from all eukaryotic and prokaryotic kingdoms that show extraordinary activities not only against bacteria, as many studies have demonstrated their efficacy against virus, parasites, and tumors as well. Regarding their antibacterial activity, they are one of the most promising molecules to help conventional antibiotics to overcome drug-resistant bacteria. Their capacity for bacterial membrane disruption makes them ideal treatments to take the lead against the conventional ones already available, with incipient activity exhaustion, or to be used as coadjuvants to conventional treatments.

The purpose of the different studies performed for this thesis was to improve the understanding on the mode of action of AMPs, to discover new candidates against bacterial pathogens, and to improve the features of already discovered AMPs. During the study, the development of tolerance to several well-known peptides was evaluated. The results suggest that bacteria develop tolerance after several cycles of peptide application by lag. This tolerance appears to affect the use of conventional antibiotics depending on the mechanism of action, possibly affecting single and combinatorial therapies. The mutational landscape found in the evolved strains was varied, affecting different pathways. Some mechanisms were affected, as demonstrated in tolerance studies performed with conventional antibiotics.

In addition to this conceptual study, there are the *de novo* discovery and improvement of AMPs. A battery of peptides from heparin-binding proteins were synthetized. This peptide discovery was performed from a structural rational design point of view. The disaccharide core of gram-negative lipid A resembled that of heparin. Hence, sequences from heparin-binding proteins with antimicrobial activity may bind to this bacterial compound. The results obtained matched well with the hypothesis, which states that peptides are highly active against gram-negative bacteria but not much against gram-positive strains. High affinities to heparin and LPS binding were obtained from the designed peptides. We also found structural refolding in the presence of these molecules. Finally, the hECP30 peptide with origin in human RNase 3 was rationally modified at usual protease points with non-natural and synthetic residues substitutions to improve its stability. The last modifications offered more than 40-fold increase in human serum stability. Moreover, we found a similar activity in most stable analogs and toxicity reduction.

These findings provide interesting insights into the new ways of conducting basic research on AMP behavior and antibiotics. The projects focused on drug development in

addition to introducing new promising antimicrobial agents, opening the scope to innovative approaches for the development of antimicrobial drugs.

Español

La aparición de nuevas bacterias resistentes cada año se está convirtiendo en uno de los mayores peligros para la salud pública, siendo especialmente preocupantes las variantes multirresistentes. Los AMPs son un grupo de moléculas con origen en ambos reinos eucariota y procariota con una extraordinaria actividad no solo contra bacterias, sino que diversos estudios han mostrado que son efectivos contra virus, parásitos y células tumorales también. Respecto a su actividad bacteriana, son unos de los tratamientos más prometedores que podrían ser utilizados con bacteria resistentes. Su capacidad para romper la membrana los convierte en los perfectos sustitutos y/o coadyuvantes para ayudar a los antibióticos convencionales, cuya actividad frente a este tipo de bacterias se está viendo cada vez más comprometida.

El objetivo de los diferentes estudios realizados dentro del marco de esta tesis está dirigido a mejorar la comprensión sobre el funcionamiento de los AMPs, descubrir nuevos candidatos frente a bacterias patógenas y mejorar las cualidades de moléculas ya descubiertas. Durante la tesis, se desarrolló tolerancia frente a diferentes péptidos en *E.coli*. Los resultados obtenidos sugieren que las bacterias se convierten en tolerantes tras cierto número de ciclos de tratamiento por aumento de lag. A parte de esto, se ha observado que estos mecanismos de tolerancia pueden afectar al tratamiento con antibióticos convencionales, y que el resultado dependería de su modo de acción. Esto podría resultar de especial importancia en el caso del uso de terapias combinatorias. El perfil mutacional observado en las cepas tolerantes afecta a diferentes vías del desarrollo de la bacteria. Se han observado con estudios previos que algunas de las vías afectadas son compartidas con los eventos de tolerancia en antibióticos.

Además de este estudio más conceptual, otro proyecto ha estado enfocado en el desarrollo de nuevos AMPs. Se sintetizó una batería de péptidos a partir de proteínas con afinidad a heparina (HBPs). El descubrimiento de estas moléculas está basado en un diseño racional por homología estructural. Se debe a la similitud que existe entre el disacárido de heparina y el núcleo glucídico del lípido A presente en la pared celular de las bacterias gram-negativas. Por ello, secuencias provenientes de estas HBPs con actividad antimicrobiana mostrarían afinidad también por el lípido A y, por tanto, ser específicas para este tipo de bacterias. Los resultados obtenidos confirman la hipótesis generada, ya que los péptidos sintetizados han mostrado afinidad tanto por heparina como por LPS, además de observar un reordenamiento estructural en presencia de ambas moléculas. También se ha visto una mayor actividad frente a bacterias gram-negativas que contra gram-positivas. Por último, se modificó racionalmente la estructura del péptido hECP30, obtenido previamente de la

RNasa 3 humana. Se modificaron residuos propensos al ataque por proteasas con aminoácidos no naturales o sintéticos para intentar mejorar su estabilidad, uno de los mayores puntos débiles de los AMPs. Los últimos análogos sintetizados en este estudio fueron capaces de aumentar más de 40 veces la estabilidad del péptido original. Además, se observaron actividades similares a las del hECP30 original y una reducción en cuanto a la toxicidad referida en diferentes células humanas y glóbulos rojos.

Todos los hallazgos realizados dentro del marco de esta tesis proporcionan información valiosa para la investigación básica en antibióticos y nuevos enfoques para el desarrollo de nuevos nuevas posibles moléculas que podrían llegar a ser utilizadas en la clínica.

INTRODUCTION

1. Brief introduction to antibiotics history

Antimicrobial agents have been present on the Earth long before their massive use due to the discoveries made in the early 20th century or even before the presence of human beings. In fact, ancient Egyptians have been known to already use some aliments containing antibiotic-producing microorganisms to heal wounds[1]. Studies have demonstrated the use of tetracycline, which was found in bones from more than 1,000-year-old samples[2]. However, the golden era of antibiotics started after the discoveries made by Paul Ehrlich and Alexander Fleming[3]. Fleming's contribution in 1928 added to the unravelling of the purification and massive production of penicillin that were achieved by further research and its first use in clinics[4], which was inaugurated in this period. Later, in the mid-century, the name “antibiotic” was used for the first time, authored by Selman Waksman and given to natural products expressing either bacteriostatic or bactericidal activities against microbes. Moreover, Waksman is responsible for the discovery of streptomycin and was the promoter of research for the massive development of new antimicrobial compounds[5]. A few years later, Waksman obtained the Nobel prize in physiology and medicine owing to his findings[6].

Between the early 1940s and 1980s, the detection and commercialization of new antibiotics were constant[7] (**Table 1**). The techniques for new antimicrobial discovery through soil sample screenings, optimized by Waksman et al., were mainly responsible for the enormous set of natural antibiotics found throughout this period[7]. Some examples of drugs found during this time were clavacin, fumigacin, actinomycin, and neomycin[8–10]. This set of achievements was one of the direct causes of the sharp boost in lifespan during the last century. For example, in the United states, life expectancy almost doubled. Before the 20th century, the average lifespan of Americans was 47 years, whereas now is 79 years[11]. Although many antimicrobial agents were found at that time, only a couple new agents have been discovered since the end of the 1980s. Synthetic modifications from already used natural ones have been introduced to the market since then[12,13]. The current situation opens a new era of uncertainty where the need for new treatments is becoming more urgent because of the causes that will be exposed in the next pages of this thesis. Are we living the end of the antibiotic era and, subsequently, the loss of life profits that they provide us?

Table 1. List of all the antibiotic classes discovered up to date.

Class	First discovery	First FDA approval
Aminoglycosides	1943	1946
Antituberculous drugs	1952	1952
β-Lactams	1928	1938
Carboxylic acid	1971	1987
Chloramphenicols	1946	1948
Fosfomycin	1969	1989
Glycopeptides	1953	1958
Ketolides	1997	2004
Lincosamides	1963	1964
Lipopeptides	1986	2003
Macrolides	1948	1951
Nitrofurans	1952	1953
Nitroimidazoles	1960	1960
Oxazolidinones	1987	2000
Polypeptides	1947	1959
Quinolones	1960	1967
Rifamycins	1957	1958
Steroids	1962	1983
Streptogramins	1953	1998
Sulfonamides	1961	1961
Tetracyclines	1948	1952

Table modified from Durand et Al.[7].

2. Antimicrobial resistance (AMR) and multi-drug resistance (MDR)

Even before the first use of the first antibiotic in patients, scientists have already been alerted about the first case of antimicrobial resistance. In fact, the same group responsible for the discovery of penicillin also showed the existence of penicillinases prior to their massive sale[14]. Decades later, as with antibiotics, bacterial resistance was shown to be

already present in nature thousands of years before to its discovery. D'Costa et al. showed the presence of more than 30,000-year-old resistance-codifying gene sequences[15]. Until now, more than 20,000 potential resistance genes have been described, although only a little portion from this huge amount has been confirmed[16].

Bacterial resistance could be defined as the collection of changes that these microorganisms undergo during its lifetime, both naturally owing to spontaneous mutations or under stress produced by antimicrobial agents, which leads them to survive antimicrobial medicines. This interpretation is supported by the World Health Organization (WHO) and has stablished the antimicrobial resistance problem as one of the 10 most important global health problems that humanity will have to face until 2050[17]. By this year, more than 10 million deaths are expected each year, regardless of whether no actions were taken to revert the situation, whereas approximately 700,000 deaths have been reported each year[18]. Such kind of infections also derive in a socioeconomic issue due to a sharp increase in treatment costs, which include both the treatment itself and the time spent by patients in health-care institutions[19,20]. Among the resistant bacteria, the Infectious Diseases Society of America (IDSA) established a group of species as the most dangerous ones because of their infecting, resistance development, and transmitting capacities[21], named under the acronym “ESKAPE”[22]. This assembly of bacteria is formed by *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species[23].

In the next pages, the main reasons why bacteria have become resistant to most available antibiotics and how this could jeopardize life quality and expectancy in humans will be aborded.

2.1. Causes

As previously shown, resistance to antibiotics was established much longer before the deliberate use of antibiotics in humans[24]. Antimicrobial resistance (AMR) and multi-drug resistance (MDR) may be incorporated in many ways. It could be first categorized into two groups: spontaneous or acquired mutations in stress conditions that confer resistance[25] and horizontal gene transfer (HGT of resistance determinants among prokaryotic species)[26]. This genetic material exchange has been demonstrated to occur

in phylogenetically proximate species[27,28]. However, stress conditions also help in HGT[29,30], as it happens with punctual mutations.

The current causes that contribute to the exponential increase of MDR pathogens caused by antibiotic discovery induction, are illustrated in **Figure 1**. Improper use of antibiotics by humans has directly driven an increase in antibiotic resistance development ratio[31]. Regarding this impact, three main niches have higher burdens of resistance acquisition.

2.1.1. Animal overfeeding and soil contamination

Abuse in antibiotic consumption by humans is mainly happening in parallel with the increasing demand for animal meat. Extensive production of meat or animal derivative aliments has used antibiotics as its major ally. This farm mining method was supposed to improve animal growth efficiency by reducing infection odds and getting higher food yields[32]. Approximately 80% of the antibiotics used worldwide are designated for this purpose[33]. Misuse of antibiotics in animals also affects other sources of human food. Animal wastes can be used in fertilizer production for plant growth purposes or assimilated by soil and contaminate phreatic water, which may also be available for human consumption[34,35].

2.1.2. Wrong antibiotic prescription and self-medication

It exists also a connection between the development of new resistant bacteria and misdiagnosis and later antibiotic prescription by physicians[36]. The lack of awareness about the antibiotic problem is widespread in worldwide healthcare systems. Many medical professionals prescribe antibiotics recklessly, commonly treating viral infections with inappropriate drugs, hence favoring bacterial evolution through a resistant position. Some studies have shown incorrect antibiotic prescription in many cases, even in half of the prescriptions made[37]. Self-medication is also likely to occur, mostly owing to low educational and awareness levels of the microbial resistance issue in patients[38]. Moreover, the high accessibility of drugs in some countries increases the possibility of this situation to occur.

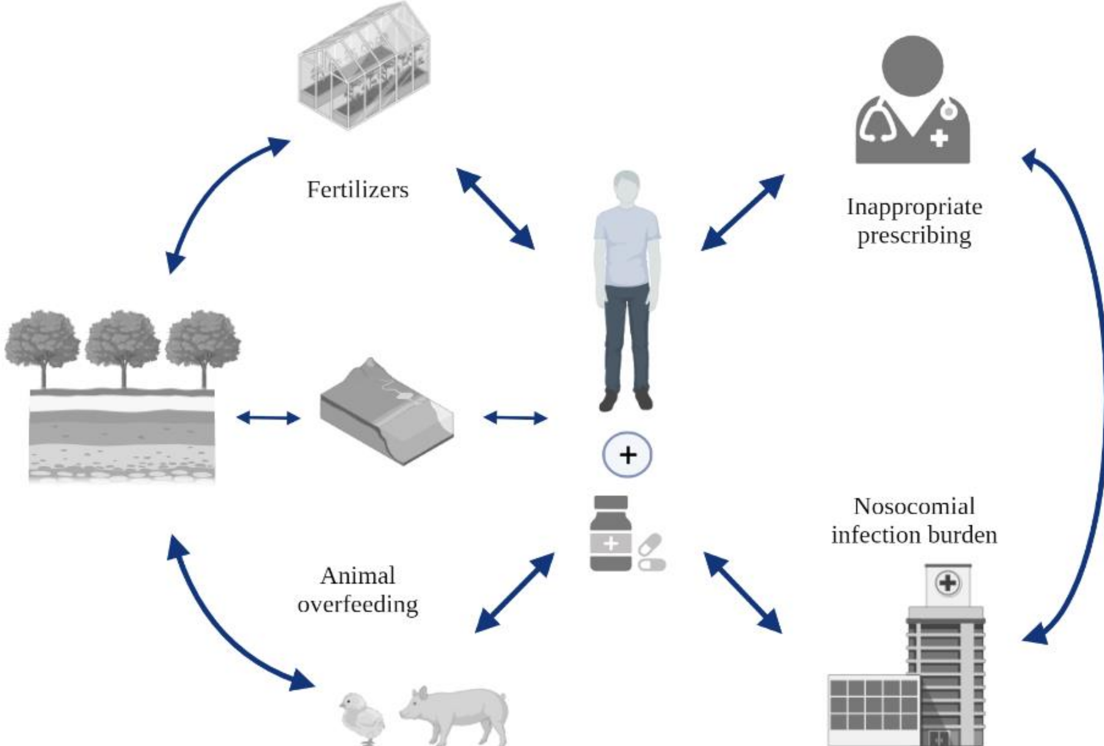


Figure 1. Graphic summary of the current main sources of MDR development. From left to right, bottom to top, antibiotic overfeeding of animals for human consumption, whose residues contaminate soils and water, is shown. The image above represents the use of contaminated fertilizers in the exploitation of plants, which finally reach human consumption. The third source are hospitals, which face the major burden of MDR infections. Finally, misuse of antibiotics through wrong prescriptions in primary health care is another cause of relevant increase in AMR ratio.

2.1.3. Nosocomial infections as niche of resistances

This last hub for human-boosted antibiotic resistance might be linked to the previous causes. However, as hospitals are the main locations where the infection burden is assimilated and diseases with poorer outcomes are treated, they must be considered as essential promoters of antimicrobial resistance. Hence, the high rate of bacterial infections makes hospitals good niches for MDR development[39–41]. Hospitals departments with higher antibiotic employment were demonstrated to present higher bacterial resistance rates[42]. Many gadgets and medical devices are susceptible to contamination, providing AMR bacteria the perfect environment to spread in the facilities[43,44]. In hospitals, there are many patients with immunosuppression due to various diseases. Many of them are continuously treated with antibiotics because they are prone to contract bacterial infections under their

immunosuppressed condition[45,46]. This situation allows bacteria to become resistant[47,48].

These three causes are encapsulated in the **One Health concept**, firstly so-called by scientists in 2003. This approach was established because of the severe acute respiratory syndrome (SARS) and avian influenza health emergencies that occurred in the same year. The scientists stated the relevance of considering health from a worldwide point of view[49]. There is a continuous interaction between human beings, wildlife, and other living beings in shared ecosystems. Such tight interconnection requires strict levels of prevention, surveillance, and control to avoid possible health harms. Several organizations settled with the purpose of awareness spreading among the population and containment of future problems[49].

2.2. Resistance acquisition and mechanisms of action

Microbes develop resistance to antibiotic molecules in many ways. As explained earlier, the mechanisms could be intrinsically present in bacteria before antibiotic treatments in many ecosystems[50] or appear after a punctual mutation or acquisition by horizontal transfer[51,52]. HGT can occur in three ways: by the achievement of environmental free DNA (this is the only mechanism that allows bacterial gene uptake from other species), by means of plasmid transfer of genetic material or by bacteriophage-mediated transduction[53]. In most cases, this gene transfer is mediated by gene sequences with high motility[54]. Nonetheless, foreign gene acquisition could eventually occur in an addressed manner mediated by other pathways[55]. Apart from these leading origins of resistance, there is a third one called adaptive resistance. These modifications are necessarily driven by previous antibiotic exposition to bacteria, which develops mechanisms to overcome the noxious effects of these compounds[56]. The changes in the bacterial genetic sequence not only provide benefits to bacteria but also generally modify their fitness and decrease their growth ratio by affecting crucial pathways[57].

The mechanisms achieved by bacteria that permit them to survive the antimicrobial effect of conventional drugs are as diverse as antibiotic targets. Antibiotics perform their activities at all structural levels of bacteria, targeting either the plasmatic membrane, cell wall synthesis, protein synthesis, or DNA replication and transcription[58–62]. Hence, resistances have been set at all prokaryotic structural levels, avoiding the effect of each

antibiotic[63]. The relevance of AMR/MDR was acknowledged by researchers long time ago. Thus, they have made many efforts to improve the understanding of the mechanisms involved in all kinds of bacterial species[64–67]. Improving the bacterial resistance background is a necessary step toward the development of new combinatorial therapies that could counteract the mechanism and revert the acquired survival capacities[68,69].

Following the scheme in **Figure 2**, the different mechanisms adopted by bacteria to achieve resistance against antibiotic action are as follows:

2.2.1. Enzymatic modification of antibiotics

Sometimes bacteria produce enzymes that can be or cannot be secreted through the membrane to their immediate environment[70]. Such molecules lead to target inactivation by different mechanisms.

The inactivation could be driven by complete degradation of the antibiotic. One of the most studied groups of hydrolytic enzymes in bacteria are β-lactamases. They break the amide bond of β-lactam compounds[71]. So far, more than 400 different β-lactamases have been reported to be present in all bacterial species[72].

However, the antibiotic does not necessarily need to be degraded to achieve the inactivation. Bacteria use other proteins that trigger antibiotic modification. They block drug activity by steric interference, using acetylation, phosphorylation, or adenylation reactions[73].

2.2.2. Drug intake avoidance

Principally, bacteria adopt two systems to prevent antibiotic absorption. Gram-negative bacteria were proven to exhibit reduced permeability that prevents the entrance of hydrophilic antibiotics, whereas this mechanism has lower prevalence in gram-positive bacteria[74]. This difference between species is related to their differences in cell wall structure. As some antimicrobial agents target the inner membrane of gram-negative bacteria, they display intrinsic mechanisms that support the release of threatening molecules from the cell. Porins with unspecific target selection are mainly responsible for this[75]. Most widely known porins involved in antibiotic resistance are OmpF, OmpC, a PhoE in *Escherichia coli*, and OprD in *Pseudomonas aeruginosa*[76,77]. Under antibiotic exposure stress conditions,

modifications in either a different kind of porin or their expression levels (both up or downregulated) may occur[78].

The second excretion mechanism involves efflux pumps that execute molecular release through active transport. They are transmembrane protein complexes that are present in both gram-negative and gram-positive bacteria[79,80]. These clusters of proteins can be both substrate specific or effective against a wide range of molecules[81,82]. Previous studies have shown that this mechanism is an example of susceptibility to common transfer by HGT. Still, they are more likely to be found in the chromosome and clustered in some operons[83]. Moreover, greater quantity and more variety of efflux pumps are usually seen when the genome of the bacteria is larger owing to the presence of the coding genes of these proteins at a certain percentage in its genome[84]. They are classified into five main groups: small multidrug resistant, resistance nodulation division, multidrug and toxic-compound extrusion, major facilitator superfamily, and ATP-binding cassette (ABC)[85].

2.2.3. Target modification by mutation

As mentioned earlier, mutation in bacteria mostly occurs in spontaneous events. In some other occasions, under antibiotic stress conditions, mutations are directed to the pathogen target, allowing it to evade the antimicrobial effect[86]. Previous studies have described evading mutations in bacteria. For example, fluoroquinolones are susceptible to undergo resistance in *E. coli* by point mutations in both A and B subunits in DNA gyrase (*gyr*) or topoisomerase IV genomes[87].

2.2.4. Target chemical alteration

Another way to make the antimicrobial target inaccessible is to produce enzymes that can add modifications to the host molecule[88]. Thereby, bacteria can protect the site of action of the antibiotic. As shown previously with the modification of antibiotics, modifications of the targets can occur in different ways. One relevant case counteracts the activity of polymyxin. Modifications in LPS charge composition avoid the recognition of the cell surface of these compounds, mediated by electrostatic forces. The addition of 4-amino-L-arabinose, phosphoetanolamine, or galactosamine

to lipid A are the most common modifications[89–91]. Methylation of rRNA in different ribosomal subunits is another example of protection against different antibiotics[92,93].

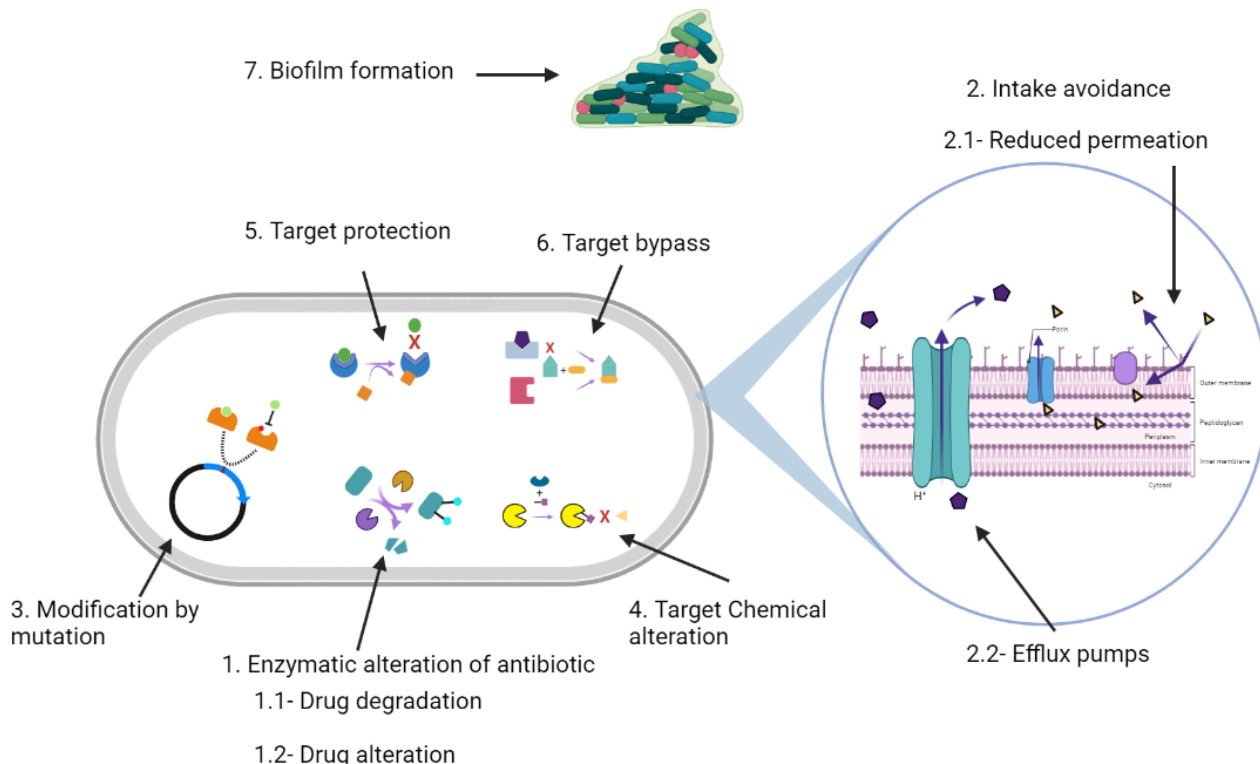


Figure 2. Scheme of the resistance strategies developed by AMR bacteria to overcome the effect of antibiotics. All mechanisms are explained in section 2.2 of the thesis. On the right side, there are exposed reduced permeation and efflux pumps related to the bacterial membrane. In the schematic bacteria, drug and target modifications, and target protection, bypass, and chemical alterations are represented. Finally, biofilm formation is displayed at the top of the figure.

2.2.5. Target Protection

In other occasions, bacteria synthetize compounds that compete with the original target of the antibiotic. Overproduction of this substitutes can eventually bind antibiotics with greater avidity, hence protecting them from their deleterious consequences[94]. For instance, Sugiyama et al. discovered a protein encoded in *Streptomyces verticillus blmA* gene, which can bind to bleomycin antibiotic, preventing its DNA-disrupting mode of action[95]. Protection mediated by allosteric binding has also been detected. It has been described in *tet*(M) and *tet*(O) genotypes

in gram-positive bacteria whose presence favors the allosteric modification of the tetracycline target and provides resistance[96].

2.2.6. Target bypass or complete switch

The following mechanism made by bacteria to avoid antibiotic activity consists of synthetizing either temporally or permanently other molecules whose activities can replace that from the original molecule affected by the antimicrobial agent[97]. One of the best-known pathways belonging to this class of alterations is mediated by alternative penicillin-binding protein (PBP2a). This peptidoglycan transpeptidase can overcome the binding of β-lactam antibiotics and continue synthetizing the cell wall[98].

2.2.7. Biofilm formation

The last mechanism observed in bacteria for resistance gain is biofilm formation. The special microenvironment created in biofilm-like colonies provides multiple mechanisms to become both tolerant and resistant to the effect of antimicrobial agents[99]. Among the vast number of tools developed in biofilms, some important ones are reduced permeability to antimicrobial agents, the appearance of persister cells with reduced growth rate or altered metabolism due to the scarcity of nutrients, alterations as a consequence of hypoxic conditions, horizontal gene transfer of resistance determinants, or quorum sensing[99].

3. New candidates against MDR bacteria

The continuous proliferation of new resistant and MDR strains has consequently provoked an immediate necessity to find new ways to stop this inexorable road to a preantibiotic era. In the last decades, only few antibiotics have reached the market, including mostly artificial modifications of other agents already in use[100]. These alterations may suppose an improvement in activity, stability, or other pharmacologically relevant parameters, but the structure remains similar to their predecessors, thus making them susceptible to resistance. Since the 1990s, daptomycin and ketolides are the only discovered compounds with new modes of action[101]. The elevated expenses needed for

the development of new antimicrobial agents and the low efficiency dampened pharmaceutical companies' economical efforts in this field to benefit more profitable ones[102].

Under this basal condition, there is an imperative necessity to find new ways to combat MDR bacteria. Primarily, a better strategy for antibiotic surveillance should be developed[103]. Controlling the administration of broad-spectrum antibiotics, both for human and veterinary purposes, may also be a powerful strategy to control the development of new resistances[104–106].

Apart from these preventive tools, it is necessary to continue developing new antimicrobial drugs that could overcome resistant populations. In spite of the few investments by pharmaceutical companies, development of novel approaches is ongoing in basic research units such as universities and research centers. In combination with companies, they are getting good revenues and advancing to clinical trials[107].

Ignoring the development of modifications or synthetic approaches from conventional antibiotics, the most interesting new models to target drug-resistant bacteria will be slightly explained (summarized in **Table 2**). Therapies could be divided into three groups: antimicrobial agents with new modes of action, combinatorial therapies, and improved delivery systems[108].

Among the new antimicrobial agents, some under development try to target bacterial biofilm interactions. The molecules in research are mostly directed to avoid or inhibit the connections between bacteria, and disrupt or hinder the synthesis of biofilm inducers[109]. Against both planktonic and biofilm bacteria, three different approaches exhibit promising results. The first one is mediated by bacteriophages. This type of virus can attack a wide range of bacteria and kill them with different grades of specificity and barely have reactivity to human cells[110,111]. Nanoparticles are also currently under research alone or in combination with other antimicrobials, with great impact[112]. The other family of compounds, on which this thesis is focused on, is antimicrobial peptides (AMPs). The following pages will be dedicated to describe in depth these molecules and the state of the art.

Combinatorial therapies consist on a combination of two or more drugs that have been shown to have synergetic activity against bacteria. This could be achieved by merging different antibiotics whose targets are involved in distinct cell development levels. Some

combinations have already demonstrated excellent outcomes in eradicating infections, mostly by combining β-lactams with other kinds of antibiotics[113]. Some combinations have also been proven to mediate the sensitization to antibiotics that bacteria were previously shown to be resistant[114]. However, molecules to combine with antibiotics do not necessarily have conventional antibiotics nor antimicrobials. There are other adjuvants that prevent the development of the proliferation of more microbes, antimicrobial resistance, or block their mechanisms of action[115,116].

Table 2. Compilation of the most recent approaches to develop new antimicrobial strategies.

Antimicrobial approach	Antimicrobial agent	Bibliography
New antimicrobial agents	Antibiofilm compounds	[117–119]
	Bacteriophages	[120,121]
	Nanoparticles	[122,123]
	Antimicrobial peptides	[124,125]
Combinatorial therapies	Antibiotic combinations	[126]
	Antibiotic + other antimicrobial agents	[127,128]
	Antibiotic + adjuvant	[129,130]
New delivery systems	Polymers	[131]
	Metal nanoparticles	[132,133]
	Liposomes	[134,135]

Regarding the improvement of the methods of drug administration, the most recent studies have been mainly focused on the use of more potent and safer strategies for antibiotic delivery. It has been widely proven that frequent systemic administration against infections fails. The main reasons are their low penetration through some tissues and their possible retention in some organs such as the liver and kidneys[108,136,137]. The carriers with the best results thus far are biopolymers, which intrinsically display antimicrobial activities but have been mostly tested to bind with other antimicrobials such as metal nanoparticles or liposomes[138–142]. In the last few years, ionic liquids, which are charged organic compounds, have been tested as well, providing better penetration

and accessibility through the skin for old antibiotics[143]. Moreover, they have been demonstrated to show antimicrobial activities[144].

4. Antimicrobial peptides

Antimicrobial peptides (AMPs) have been introduced as possible leading substitutes or adjuvants to conventional antibiotics in overcoming antimicrobial resistance. Accordingly, the introduction of this thesis will be focused on providing an exhaustive overview of all relevant aspects of these molecules, including their origins, structural determinants, different modes of action, advantages, and harms, compared with conventional treatments. Moreover, future perspectives will be discussed to provide a solid background of the work presented herein.

4.1. Discovery and milestones

It is now widely recognized that the description of the first antimicrobial protein is attributed to the same person who discovered the first antibiotic, Alexander Fleming. He discovered the antimicrobial activity of the human lysozyme in 1922 [145]. However, at that time, this achievement did not have much relevance, eclipsed by the later discovery of penicillin and the ineffectiveness of the protein as a treatment for most of the infections. However, the lysozyme has never been used as an antibiotic for clinical purposes, but it has been actually used in industries such as the beverage industry as a preservative, like other compounds later discovered[146]. Between the time this antimicrobial protein was first described and the boom during the 1980s upon the research on AMPs[147], other relevant achievements have been made. Gramicidin produced by *Brevibacillus brevis* was the molecule subsequently discovered in 1939 by Dubos et al.[148]. A few years later, in 1944, gramicidin became the first AMP approved by the US Food and Drug Administration for gram-positive bacterial injuries and is still available for topical applications[149,150]. In 1947, the colistin (polymyxin E) cyclic peptide was discovered by Stansly et al. and has been shown to have a high antimicrobial activity level against gram-negative bacteria[151].

The findings on cecropins and cathelicidins in the 1980s became crucial in provoking a boost in AMP research, as they demonstrated that they could be present in the animal

kingdom and even in mammals and confirmed their involvement in the human innate immune system[152,153]. Since the beginning, more than 3,000 AMPs have been described[154]. Some software programs have been developed in the last decades to compile all discovered sequences, of which the most complete until now are The Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.php) and the Collection of Antimicrobial Peptides (CAMP; http://www.camp.bicnirrh.res.in/)[155,156]. **Table 3** lists the software programs that made it to the market and are still used until now in the treatment of relevant infectious diseases.

In addition to the AMP-based drugs already available, others are currently in their clinical stages and may be introduced in the market in the next few years (**Table 4**). Nevertheless, considering that AMPs are potentially present in all living kingdoms[157], too many possibilities on this set of molecules are yet to be discovered.

4.2. Structure and other properties

The chemical and structural determinants of many AMPs have been thoroughly studied[158–160]. They can be classified according to different options, which will be explained next together with the characteristics that better categorize them.

The first and most coherent way is to take into account the structures of the peptides. The sequence length of AMPs mainly range from 5 to 50 amino acids (aa) but could have up to 100 aa or even more. In fact, the APD database recently increased its sequence acceptance threshold to 200 aa, expanding it to include larger antimicrobial proteins[161]. However, in the databases described earlier, the number of AMPs with a three-dimensional structure, does not even reach 20% of the total inputs[162]. Regarding their secondary structure, AMPs can have either only α-helical, β-strand sheet (habitually stabilized with disulfide bonds), and αβ mixed structures or a random coil (Figure 3)[163]. It should be highlighted that peptides in solution tend to be unstructured and become restructured in the presence of cell membranes or particles that mimic this kind of structures[164]. In addition to this secondary structure-related classification, AMPs can also be organized considering the primary structure. Many peptides have some concrete residues enriched within their sequence. Some AMPs have high tryptophan, proline/arginine, or histidine levels in their sequences[165–167].

Table 3. AMPs already available in the market.

AMP	Release year	Structure	Mode of action	Activity Spectrum*	Origin
Bacitracin	1948		Inhibits cell wall development	G+	*Bacillus subtilis*
Gramicidin	1952		Membrane pore forming	BS	*Bacillus brevis*
Polymyxin B	1952		LPS binding and membrane disruption	BS	*Bacillus polymyxa*
Vancomycin	1955		Inhibits peptidoglycan synthesis	G+	*Amycolatopsis orientalis*
Colistin	1958		LPS binding and membrane disruption	BS	*Paenibacillus polymyxa*

Teicoplanin	1988		Inhibits peptidoglycan synthesis	G+	*Actinoplanes teichomyceticus*
Daptomycin	2003		Membrane disruption	BS	*Streptomyces roseosporus*
Telavancin	2009		Inhibits peptidoglycan synthesis	G+	Semi-syntetically derived from Vancomycin
Dalbavancin	2014		Inhibits peptidoglycan synthesis	G+	Semi-syntetically derived from Teicoplanin
Oritavancin	2014		Inhibits peptidoglycan synthesis	G+	*Amycolatopsis orientalis*

Data obtained from Gan and collaborators review[168].

*Antimicrobial activity: G+, gram-positive bacterial strains; BS, broad spectrum.

Up to now, AMPs from all living domains (prokaryotes, archeon, and eukaryotes) have been discovered. In addition, they are present in all living kingdoms (bacteria, protists, plants, animals, fungus, and archeon)[161]. For this reason, this has become another extensive way of classifying peptides.

Table 4. AMPs under clinical trials.

Clinical trial phase	AMP
Phase 1	Melittin, Friulimicin, NVB-302, hLF1-11, LTX-109, Wap-8294A2, PL-5, IDR-1
Phase 2	LL-37, Murepavadin, P113, EA-230, C16G2, Novexatin, Melamin, Mel4, LFF571, Delmitide, DPK-060, GSK1322322, PXL01, AP-214, PMX-30063, XF-73, CZEN-002, Ghrelin
Phase 3	Gramicidin[a], Polymyxin B[b], Colistin[c], Daptomycin[d], Neuprex, Iseganan, Surotomycin, Pexiganan, XOMA-629, Omiganan, SGX942, Ramoplanin, p2TA, D2A21

Marketed AMPs in clinical trials for other purposes: [a,b]Gramicidin and polymyxin B as eyedrop formulations; [c]Colistin as an alternative treatment for ventilator-associated pneumonia (VAP); [d]Daptomycin, as treatment for acute hematogenous osteomyelitis. Some AMPs may already be commercialized for other purposes. The data presented were obtained from Dijksteel and collaborators[169].

The hydrophobicity is one of the most important physicochemical features of AMPs [170]. Overall, the percentage of hydrophobic residues in AMPs is approximately 50%[163]. Otherwise, in almost all cases, peptides have a positive net charge. This cationicity ranges from +2 to +9 positive charges within their sequences, corresponding to arginine and lysine residues[171]. The correlation between the two is crucial for the development of the activity. An appropriate balance is necessary during the selective interactions with cell membranes[172]. In the next chapter, how these factors are involved

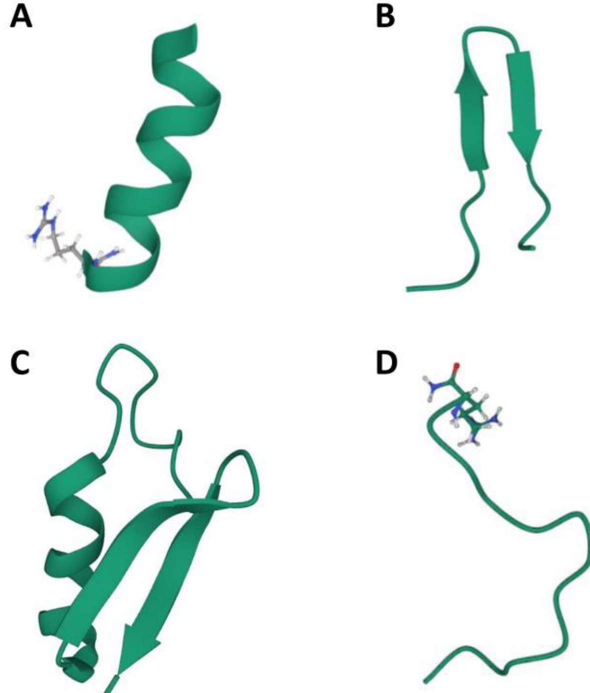


Figure 3. Scheme of the secondary structures acquired by AMPs. **A** – α-helical (LL-37), **B** – β-strand (Tachyplesin), **C** – αβ mixed structure (Sapecin) and **D** – random coil (Indolicidin).

in the development of this mode of action will be explained in depth. It is also important to know where the two types of residues are located in the sequence. Studies have tested both the effects of charged and hydrophobic residue proportions and placement[173–175].

4.3. Therapeutically relevant activities

This section is probably the most relevant and informative part of the introduction about why AMPs are so mainstream for worldwide researchers. AMPs can develop a great variety of activities in cells. Some of them are of great interest because they could be repurposed for clinical use. In this work, all studies performed were focused on antimicrobial activities. This will be discussed in detail in the second part of the chapter. First, other relevant roles will be summarized with given examples.

Apart from the extensively demonstrated activity against a wide range of bacteria, AMPs have been shown to target other pathogens or cells. Antiviral activity has already been reported for up to 200 peptides until now (checked by search in APD3). More than half of them have been concretely proved to exhibit activity against the human immunodeficiency virus (HIV). For example, the dermaseptin peptide was demonstrated to be active. However, it has been also shown to be toxic at lower concentrations to immune system host cells[176]. It has also been reported to have an activity against many other viruses than the HIV. It has also been demonstrated to inhibit the replication of SARS coronavirus 2[177].

AMPs exhibit potent activities against fungal infections. A search in peptide databases showed more than 1,000 entries of antifungal peptides. Again, antifungal activity can be found in all living kingdoms, but peptides originating from plants are the biggest source of these compounds[178]. They have broad-spectrum activity and can act at many levels in the fungal cell, with cell membrane or intracellular targets[179]. In fact, echinocandins and their analog pneumocandins have been used in *Candida* infections for many years after the discovery of the first compound of this family in 1974 [180].

Antiparasitic activity is another deeply studied function of AMPs. A review from Torrent et al. compiled the mechanisms of action attributed to peptides against parasites and identified the parasites for which they are effective[181]. Leishmaniosis is one of the

most studied illnesses caused by parasites in animals, and antimalarial compound findings have monopolized the discovery of antiparasitic compounds in humans[182].

Lastly, peptides have been meticulously tested as anticancer compounds for clinical purposes. As in other functions promoted by AMPs, the activity against cancer cells is based on the electrostatic interactions with their plasmatic membrane[183]. Cancer cells present alterations in lipid content compared with healthy cells. For instance, cancer cells switch their content to phosphatidylserine in the outer leaflet of the plasmatic membrane, which results in total lipid content levels ranging from 3% to 9%, whereas in healthy mammalian cells, this lipid is present in the inner membrane[184]. Gaining a negative charge makes tumor cells targetable by AMPs more selectively than normal cells. Many ongoing studies are trying to deliver AMPs as anticancer drugs[185–187].

Besides these exclusively therapeutic potential uses of AMPs, there are other protective functions developed during human illnesses in relation to the parental activity of the innate immune system of AMPs in mammalian cells. Many works have depicted the mediation by AMPs as immune system regulators during an injury. Cathelicidins and defensins are the best examples of these immunomodulatory ability[188]. LL-37 was demonstrated to participate in wound healing and reepithelization. The expression of this protein was found to be upregulated during these processes[189]. Furthermore, AMPs were also shown to be involved in angiogenesis, chemotaxis for immune system cells, and anti-inflammatory procedures[190–192].

Finally, as remarkable counterparts of all exposed positive aspects of AMPs, hemolytic and cytotoxic activities were observed in AMPs most of the time. These side activities are also structure dependent. The proportions of hydrophobicity and polar and charged residues can determine the propensity of this event to occur[192]. Although the therapeutic window of AMPs has been expanded by approximately 100 times the concentration where toxic effects appear, it is important to design good strategies in preclinical studies to accurately determine the cutoff limit before toxicity appears, apart from developing molecule improvement strategies to minimize toxicity[193].

4.4. Antibacterial activity and mechanisms of action

This introduction will be focused on its main goal, which is to present the antibacterial properties of AMPs. Bactericidal activity has already been proven in a wide range of bacteria, including gram-positive, gram-negative, and mycobacteria[194]. As in other activities of AMPs and those previously described in the thesis, the mode of action of the antibacterial activity is mainly via electrostatic interactions with the bacterial cell envelope and by provoking its loss of integrity[195]. However, is not the only mechanism described for them[196]. All mechanisms are summarized in **Figure 4**.

The principal cell membrane-disrupting model was hypothesized through the years to be performed in diverse mechanisms. However, the three most studied and accepted ones by experts in the subject are as follows: the barrel-stave, toroidal, and carpet models. In all three models, the mechanism starts with recognition of the positive charges of the AMPs and the negatively charged molecules in bacterial cell walls (e.g., lipopolysaccharides in gram-negative bacteria and teichoic acids in gram-positive ones)[197]. Apart from this, the lipid composition in the outer leaflet of the membrane is different in bacteria with a much higher proportion of negatively charged ones (i.e., phosphatidylserine) than in host mammalian cells (zwitterionic phospholipids and cholesterol)[198].

In the **barrel-stave model**, after the recognition of the molecules to the membrane, owing to the hydrophobic patches present in AMPs, the bilayer is narrowed. This ultimately makes AMP units trespass the lipidic bilayer, binding to the hydrophobic backbone of the lipids and interacting with the AMP units themselves, forming a pore-like structure that reminds of a protein ion channel through the membrane[199]. These channels can have different pore sizes depending on the number of peptides recruited for its formation. Pores lead to the loss of integrity and release of the inner content of the cell, thus becoming necessarily lethal for the bacteria[200]. Alamethicin is a peptide that forms this kind of pores when they are bound to the cell membrane. This α-helical peptide was demonstrated to interact through this barrel-stave transmembrane mechanism and is characterized by small hydrophilic regions[201].

The basis of the mechanism explained in the **toroidal model** is similar to the ones described earlier. It differs in how the pore is formed after peptide binding. The polar regions of the peptide interact with the polar head of the lipids. It also creates a pore,

which in this case is an invagination of the bilayer instead of a transmembrane insertion[202]. Many peptides that were previously supposed to create pores through the barrel-stave model were later proved to act in this manner[203]. Melittin is the best representative of the toroidal pore-forming mechanism regarding its relevance in studies throughout the years[206]. Other studies have shown a subclass of the toroidal model, where peptide monomers could lay in the pore in different orientations, called the disordered toroidal pore model[204].

The **carpet model** theory is different from the other theories exposed before, notwithstanding the same beginning of the process. The key here is the excessive accumulation of peptide monomers in the membrane surface. High concentrations of acidic lipids in the cell membrane significantly favor the development of this mechanism[205]. When the accumulation of peptide particles increases, a non-reversible situation emerges where the bilayer is solubilized through a detergent mechanism[206]. The disrupted membrane forms micelles with the AMPs, hence destroying the cell. Dermaseptin is a peptide demonstrating antimicrobial activity via the carpet model[207].

In addition to the three main models shown, other theories of the modes of action of AMPs have been described, as summarized in **Figure 3**. The previous models described earlier could be grouped in a membranolytic mechanism, whereas the ones described next are gathered in a non-membraolytic one. Such models are thought to be an initial step for intracellular targeting AMPs[208].

The **electroporation model** occurs when the membrane interacts with highly charged molecules. When the binding molecule has enough charge density to create an electric potential of 0.2 V, peptides can establish transient pores of 2 to 4 nm in diameter in the membrane[209]. Peptides need minimum features to develop such mechanism (net charge of +4 and length of 12 aa at least)[209]. The formation of these pores has already been described for polymyxin B and melittin AMPs[210,211].

Some peptides interplay with the membrane by the **sinking-raft model**. Occasionally, AMPs get bound preferentially to some concrete lipids, creating a mass ratio imbalance that provokes abnormal curvatures in the bacterial membrane. This again triggers the formation of transient pores whereby peptides penetrate the cell[212]. Using this membrane-binding model, Pokorny and Almeida described that δ-lysin cross the cell

membrane. They found that the peptide penetrates the membrane by forming transient trimers that trigger lipidic layer permeabilization[213].

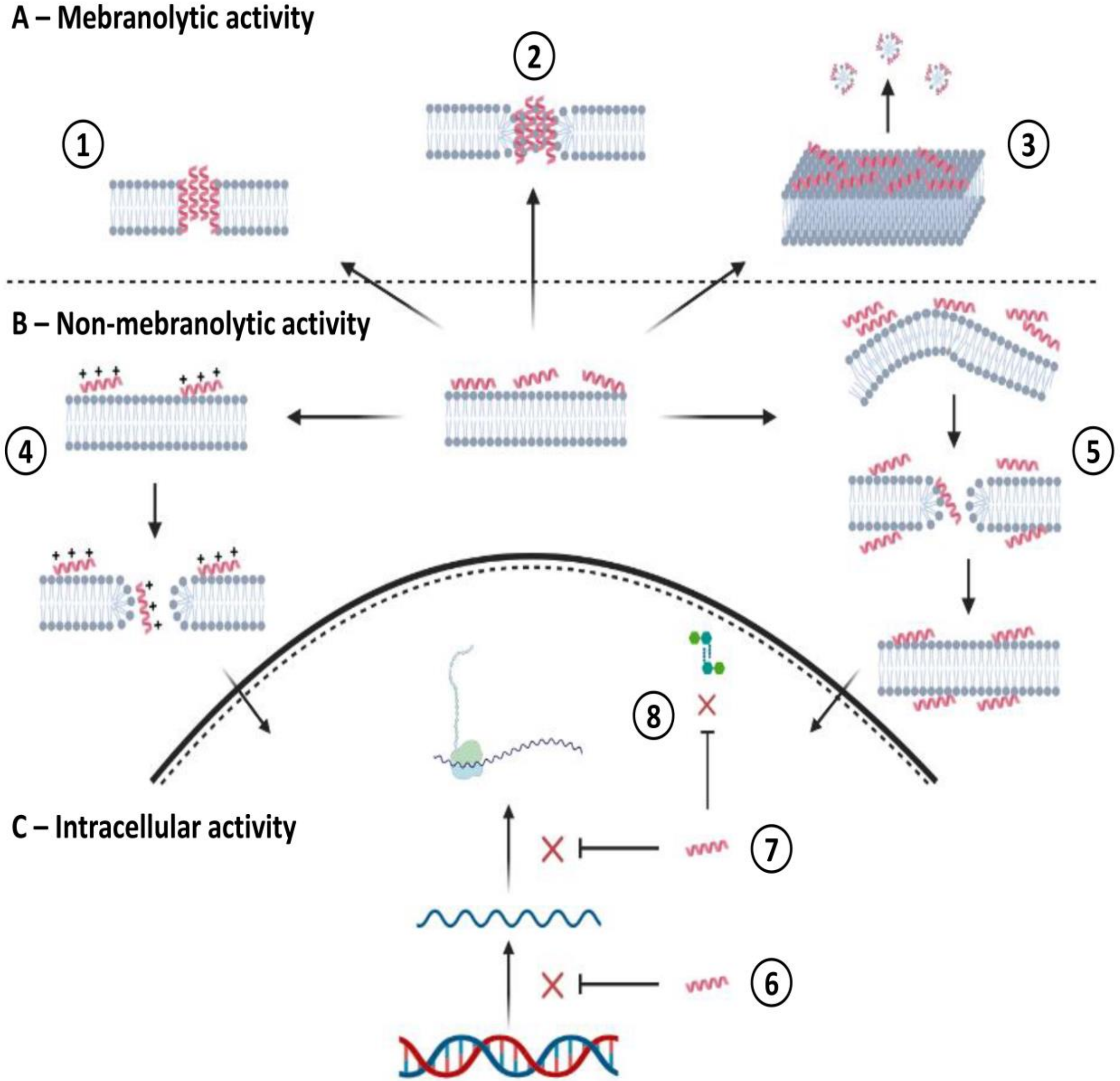


Figure 4. Scheme of the different mechanisms of action of AMPs against bacteria. First, in **A**, the ways that end in cell membrane disruption are described. **1** The barrel-stave model describes the formation of mimicking transmembrane pores. **2** The toroidal model also ends in pore formation, but peptides interact with lipid polar heads, provoking membrane invagination. **3** Acting more like a detergent, the carpet model implies the saturation and micellation of the membrane due to high accumulation of AMPs on the surface. In **B**, the models whose interactions with the membrane are not involved with its degradation are described. **4** The electroporation theory is driven by AMPs with high net charge. Regardless of whether they can create electric potentials higher than 0.2 V, they create transient pores and can be internalized. **5** Transient pore formation is the outcome of the sinking-raft model. However, the basis is the appearance of abnormal curvatures in the membrane because of mass-ratio impairment. Peptides that cross the bacterial membrane can execute intracellular activities, as simplified in **C**. AMPs exhibit interactions at **6** the DNA and RNA levels, **7** protein translation and protein folding, and **8** cell wall synthesis. All models against the inner components inhibit bacterial growth.

Otherwise, AMPs are not just limited to membrane-linked activities. Once they pass through the membrane via non-membranolytic mechanisms, they can interact with the cell at every level of its development. It was previously found that many AMPs exhibit both mechanisms of action, and each one is triggered depending on the concentration used during the treatment[214,215]. When AMPs are supplied at concentrations higher than the minimum inhibitory concentration (MIC), they practically always exert a membranolytic mode of action, whereas at their MICs or sublethal concentrations, some of them trespass the cell membrane and block cell growth and propagation[216,217].

Starting from the most basic level of cell development, AMPs have been described to target **DNA** and **RNA**. Park et al. demonstrated that even at concentrations several folds higher than the MIC of buforin II peptide, it showed great affinity to DNA and RNA, hindering metabolic procecesses[218]. Indolicidin is another peptide that has been shown to block DNA biosynthesis, inactivating DNA topoisomerase I and binding itself to dsDNA and ssDNA[219].

Other AMPs were shown to block **macromolecular synthesis**. This involves protein synthesis and maturation[220]. Proline-rich AMPs are a group of peptides shown to have protein synthesis affection even at concentrations higher than the MIC. Their mechanism of action was shown to be slower than those of strictly membranolytic peptides. Moreover, the D-enantiomers of these peptides exhibited a huge loss of activity compared with the L-naturally produced ones. This fact was barely appreciable for lytic AMPs[221]. Pleurocidin and dermaseptin were found to inhibit macromolecular synthesis at concentrations lower than the MIC[217]. DnaK bacterial chaperone activity was also proven to be disrupted when treated with drosocin and L-pyrrochoricin AMPs. They were hypothesized to close the peptide-binding pocket at the C-terminal terminal region of the chaperone, but this has not been experimentally confirmed yet[222].

Some studies have also tested the ability of some AMPs to block **cell wall biosynthesis**. The lantibiotic family of peptides was studied in depth for its activity that affects cell wall development[223]. Hasper et al. demonstrated that nisin sequestrates the cell wall component Lipid II, which is essential for cell wall biosynthesis in bacteria. This mechanism could also be attributed to other lantibiotics such as gallidermin and epidermin[224].

4.5. Benefits towards bacterial infection treatment

Over the decades, AMP treatment has presented advantages to the use of conventional antibiotics. Now that the background of infection mechanisms is clearer and the spread of bacterial resistance is becoming more dangerous, the benefits from AMPs are more relevant than ever.

As slightly explained earlier, one of the most important advantages of AMPs is the ability to be active against an extensive range of microbes. Many peptides have been demonstrated to be bactericidal to a broad spectrum of bacteria. Chen et al. showed that a frog-based brevinin peptide was active against both gram-negative and gram-positive bacteria. It was even active against *Candida albicans* at micromolar levels. In all cases, brevinin exhibited microbicidal activity at the same inhibitory active concentrations[225]. Nonetheless, this feature is present in many AMPs[226,227].

In the same work, Chen et al. found the next interesting determinant that was found improved by AMPs compared with conventional antibiotics. They tested the kinetics of the antimicrobial activity of brevinin. The killing speed was shown to be concentration dependent. However, after disregarding the effect of the amount of peptide, brevinine killed a significant number of bacteria after only 30 minutes of treatment application[225]. As in the case of broad-spectrum activity, this is not an exception. AMPs usually express almost the totality of their activity within the first 30 to 60 minutes after addition[228]. In the case of conventional antibiotics, to obtain similar killing ratios, the range is usually moved to a few hours after the start of treatment[229].

The previous mechanism, together with the membrane-linked mechanism of action, whose target is less specific than those of conventional antibiotics, could be the trigger of another advantage of AMPs. As the activity of AMPs is not involved with specific proteins and binding sites (mainly with membranolytic AMPs), the propensity toward bacterial resistance development is lower[230]. Although the resistance mechanism of AMPs have already been addressed[231], no signs of affection to their innate immunomodulatory capacity, which is also decisive to overcome infection, was found[228]. Apart from this, the possibility of cross-resistance events between AMPs is low[232]. Other studies have shown that strains already resistant to conventional antibiotics were unlikely to develop cross-resistance or collateral sensitivity to AMP treatment[233].

Apart from the mild hemolytic and toxic activities described earlier, considering that the concentration normally needed is several orders of magnitude higher than the antimicrobial activity levels, AMPs are unlikely to develop relevant toxic processes[234,235]. However, toxic processes have been described in antibiotic treatments. For example, when antibiotics act upon bacteria, they display pathogen-associated molecular patterns in the environment. These particles can be deleterious to the host, inducing sepsis and, in worst cases, death[236].

4.6. Limitations and future perspectives

Despite the numerous advantages offered by AMP treatment, critical flaws remain to be addressed to make peptides a reliable therapeutic option for clinical infections. The necessity of obtaining new antimicrobial agents have led to the excessive development of AMPs. They have been introduced in clinical phases with an unappropriated optimization of molecules. In most cases, these conditions ended in premature failure[237].

First, as AMPs are synthetically produced molecules rather than spontaneously occurring in nature, they are prone to be detected by proteolytic enzymes and easily degraded. Their usual composition makes them highly reactive as well. This means that they bind to other molecules and proteins apart from their intended target. Such effects have a direct consequence to their bioavailability, reducing it considerably[238]. Protease degradation greatly affects the stability of AMPs, jeopardizing their activity against microbes. To improve the half-life of peptides, several approaches have been used, with successful outcomes. Modifications include the reduction of the sequence to remove the most susceptible regions, substitutions with synthetic or non-natural amino acids, sequence cyclization, or conjugation with other molecules[237].

As peptides are usually small portions from larger proteins synthetized in prokaryotic and eukaryotic cells, they cannot be directly obtained from nature. The most extended method for their production is chemical synthesis. Solid-phase peptide synthesis (SPPS) is the most widely applied method. Other methods of peptide production have also been used, involving the recombinant expression in bacterial, fungal, mammalian, or plant cells. The common issue present in all methods is the high production cost. Moreover, the yields obtained with these processes are low[239,240]. The production method must be improved to make the future commercialization of AMPs competitive against that of

conventional antibiotics. In fact, improvements have been made that increased production yields. For example, the use of microwaves in SPPS samples achieved a sharp improvement in peptide production[240].

Strains resistant to the few AMPs that have reached the market and those under development have already been found. The mechanisms of resistance are mainly focused on cell envelop and membrane changes. However, other mechanisms involving AMP degradation and sequestration or release by efflux pumps have been described[241].

Some of the most relevant resistance mechanisms that have been found will be broadly exposed. **Membrane modifications** are one of the most studied mechanisms in both gram-negative and gram-positive bacteria. The structural modifications of LPS are acquired under many restrictive conditions such as nutrient scarcity, extreme pH decrease, and low trace element availability[242]. The PhoPQ, PmrAB, and Rcs regulatory complexes are responsible for these changes. Addition of 4-aminoarabinose to lipid A mediated by the *pmr* family of genes has been demonstrated to confer resistance to *S. typhimurium*[242]. Mostly, *pmr* genes are regulated at the same time by the PhoPQ system. However, this family of genes is also associated to other modifications on the bacterial cell surface caused by direct regulation. Some examples are the monophosphorylation of lipid A by the *ugtL* gene or palmitoylation by another protein encoded by the *pagP* gene[243,244]. The *Pgm* gene that encodes the phosphoglucomutase protein involved in LPS biosynthesis is also a direct mediator of the development of resistance to various AMPs[245]. Modification of the *almEFG* operon was found to trigger the glycosylation of LPS, causing bacterial resistance to polymyxins[246]. In gram-positive bacteria, the resistance mechanism is directed to the teichoic acids present in the outer membrane of these bacterial family. The changes in teichoic acids are aimed at reducing the amount of negative charges necessary for the interaction with AMPs[242]. Usually, the changes observed are the addition of lysylphosphatidylglycerol to the membrane or *dlt* operon activation inducing the incorporation of D-alanine into teichoic acids[247,248]. The MprF protein expression was also found to be altered in gram-positive bacteria. This protein mediates the incorporation of lysine to phospholipids in the cell membrane. This diminishes the negative charge on the cell membrane, hence conferring resistance to AMPs[249].

Apart from the predominating membrane modifications, other important mechanisms have been reported in the literature. **Efflux pumps** adapted for AMP exportation have been observed in several bacteria. This mechanism is combined with the resistance mechanisms observed in response to conventional antibiotics. For example, some transporter complexes such as *sap* and ABC putative transporters mediated by *yejABEF* were found in *S. typhimurium*[242,250]. Finally, the last resistance mechanism to be mentioned against AMPs is the use of **proteases** by bacteria toward peptide degradation. The most studied ones are PgtE from *Salmonella* and OpmT from *E. coli*[251,252].

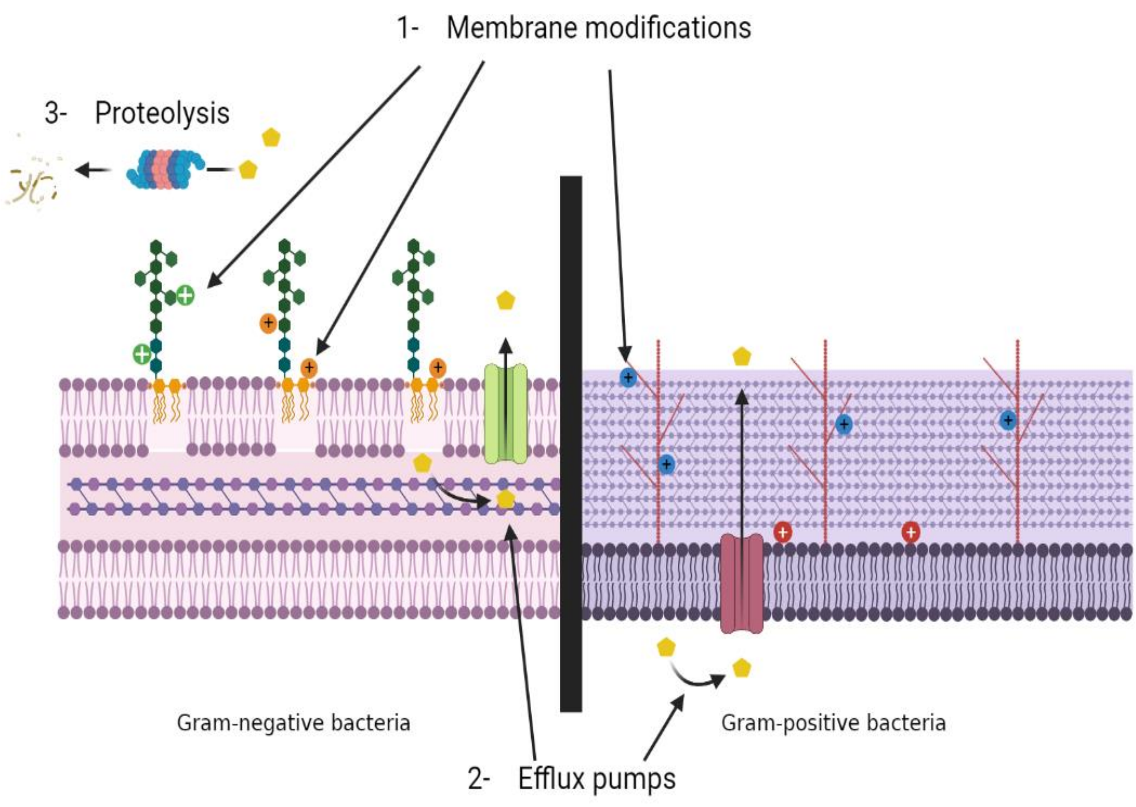


Figure 5. Summary of the main resistance methods developed by bacteria against AMPs.

Lastly, toxicity in the host is a main problem to tackle in AMP treatment. Even if many peptides show no toxicity or levels higher than the therapeutic window, these results come from *in vitro* studies and thus could not be extrapolated to clinical stages. Otherwise, AMPs usually exhibit a broad spectrum of activities, which can be harmful for commensal microbiota in the human body[253].

All these reasons, in addition to the urgency to treat resistant bacteria, focusing on multi-drug resistance organisms, warrant the rapid discovery of new molecules and mechanisms of action against them. Although AMPs are good candidates for overcoming this problem, new candidates must be identified, and their structures and properties must be improved to restrict their adverse effects, and their antimicrobial activities must be enhanced. Only more investment in AMP research can lead to the use of AMPs as a solution to overcome bacterial resistance.

OBJECTIVES

This study focused on the development of new AMP-based agents because, as explained earlier, the need to discover new antimicrobial agents against drug-resistant pathogens is becoming greater, and no effective solution can be expected in the short term. Apart from this, the improvement of basic knowledge of AMPs is of high importance for the safer release of new molecules in the market in the future.

The work presented herein was aimed at finding answers to the questions that have been raised over the decades regarding AMP development by conducting three main projects. The strategies were directed to different levels of drug development, ranging from amplifying the knowledge of the molecular basis of their mechanisms of action to the improvement of the antimicrobial activity-related properties of already discovered AMPs.

Next, a recap of projects performed is presented as follows:

- Study of the tolerance mechanisms involved in several AMPs with different modes of action described in the literature, and understand the phenotypic consequences derived and the possible effects on conventional antibiotic treatments.
- Identify new peptides from heparin-binding proteins by a structural analogy to the bacterial Lipid II present in gram-negative cell walls.
- Improve the stability of a peptide derived from human RNase 3 protein by rational modification, with the aim of maintaining the antimicrobial activity and reducing the collateral toxicity in host cells.

CHAPTER 1

ANTIMICROBIAL PEPTIDES CAN GENERATE TOLERANCE BY LAG AND INTERFERE WITH ANTIMICROBIAL THERAPY

CHAPTER 2

CRYPTIC ANTIMICROBIAL PEPTIDES IN HEPARIN-BINDING PROTEINS EXHIBIT SELECTIVE ANTIMICROBIAL ACTIVITY AGAINST GRAM-NEGATIVE BACTERIA THROUGH SPECIFIC LIPOPOLYSACCHARIDE BINDING

ABSTRACT

Glycosaminoglycan (GAG)-binding proteins regulating essential processes such as cell growth and migration are essential for cell homeostasis. These proteins bind to GAGs using specific structural signatures, like the CPC' clip motif, which encompasses two cationic residues and a polar residue in a defined structural arrangement. As both GAGs and the lipid A disaccharide core of gram-negative bacteria both contain negatively charged disaccharide units, we hypothesized that GAG-binding proteins could also recognize LPS and enclose cryptic antibiotic motifs. In this context, we herein report novel antimicrobial peptides (AMPs) derived from heparin-binding proteins (HBPs), with specific activity against gram-negative bacteria and high LPS binding. We used computational tools to locate antimicrobial regions in 82% of HBPs, most of those colocalizing with putative heparin binding sites containing a CPC' clip motif. To validate these results, we synthesized five candidates [**HBP1-5**] that showed remarkable activity against gram-negative bacteria, as well as a strong correlation between heparin and LPS binding. Structural characterization of these AMPs shows that heparin or LPS recognition promotes a conformational arrangement that favors binding. Among all analogs, HBP-4 and HBP-5 displayed the highest affinities for both heparin and LPS, with antimicrobial activities against gram-negative bacteria at the submicromolar range. These results suggest that GAG-binding proteins are involved in LPS recognition, which allows them to act also as antimicrobial proteins. Some of the peptides reported here, particularly HBP-5, constitute a new class of AMPs with specific activity against gram-negative bacteria.

INTRODUCTION

Glycosaminoglycan (GAG)-binding proteins are a heterogeneous group of proteins mostly associated with the cell surface and the extracellular matrix[254]. They mediate a plethora of functions including signaling, cell proliferation, and coagulation[255,256]. Up to date, most studies of the GAG interactome have focused on protein interactions with heparin, a highly sulfated form of heparan sulfate, due to the commercial availability of heparin and heparin-Sepharose[257]. This has allowed defining the heparin interactome, a highly interconnected network of proteins functionally linked to physiological and pathological processes[258]. Although the structural nature of these proteins is diverse,

they share common features, such as the presence of certain domains and motifs. In particular, the CPC' clip motif is the major contributor to the attachment of heparin (and other sulfated GAGs) to GAG-binding proteins[259]. The motif involves two cationic (Arg or Lys) and one polar (Asn, Gln, Thr, Tyr or Ser, more rarely Arg or Lys) residues with conserved distances between the α carbons and the side-chain center of gravity, defining a clip-like structure where heparin is lodged[260]. The CPC' clip motif is conserved among all HBPs deposited in the PDB and can be found in many proteins with reported heparin-binding capacity[260].

Recently, we showed that negatively charged polysaccharide-containing polymers, such as heparin and lipopolysaccharides (LPS), can compete for similar binding sites in peptides, and that the CPC' clip motif is essential to bind both ligands[261]. Our results provide a structural framework to explain why these polymers can cross-interact with the same proteins and peptides and thus contribute to the regulation of apparently unrelated processes in the body. A paradigmatic example is FhuA, an *E. coli* transmembrane protein involved in the transport of antibiotics such as albomycin and rifamycin[262]. FhuA can bind glucosamine phosphate groups in LPS[263], and we confirmed that a short peptide (YI12WF) retaining most of the LPS-binding affinity of the original protein can also bind heparin with high affinity. When the CPC' residues in these peptides are mutated, heparin- and LPS-binding activities are largely lost, proving the motif as essential for both ligands. Heinzelmann & Bosshart also showed that human lipopolysaccharide-binding protein (hLBP) can bind heparin and enhance the pro-inflammatory responses to LPS of blood monocytes[264]. Again, the crystal structure of hLBP bound to N-acetyl-D-glucosamine shows a CPC' clip motif that could potentially bind heparin. Such observations may prove generalizable to other LPS-binding proteins and may reveal a biological interplay between LPS and heparin. Whether the reverse is true –i.e., HBPs playing a role in LPS binding and potentially in antimicrobial activity– is currently unknown.

Here we show that HBPs contain cryptic AMPs that overlap with heparin-binding regions containing a CPC' motif. These AMPs show strong selective antimicrobial activity for gram-negative bacteria. They also bind heparin and LPS with high affinity, and disrupt the bacterial cell wall. Our results suggest that LPS and heparin bind similar regions in proteins, provided they contain a CPC' clip as minimal required structure. HBPs therefore represent a source for new antimicrobials effective against antibiotic-resistant pathogens.

RESULTS

Linking heparin affinity and antimicrobial activity

Despite the differences between GAGs and LPS, both contain negatively charged disaccharides in their structure. GAGs are polymers based on variably sulfated repeating disaccharide units. For example, the most common form of heparin is a sulfated disaccharide composed of iduronic acid and glucosamine linked through a β (1→4) bond [IdoA(2S)-GlcNS(6S); Figure 6A]. For its part, LPS is composed of a polysaccharide antigen linked to a lipid A molecule, which is, in turn, a phosphorylated glucosamine (GlcN) disaccharide decorated with multiple fatty acids. The two GlcN units are linked by a β (1→6) bond, and normally contain one phosphate group each (Figure 6B). Based on these structural similarities, we hypothesized that HBPs could also potentially bind the phosphorylated GlcN units of LPS. As heparin-binding sites are commonly associated with short sequential motifs, we reasoned that specific short regions in HBPs could behave as AMPs, binding first to LPS and later destabilizing the outer cell wall and the bacterial membranes.

To probe our hypothesis, we inspected all reported HBPs (Supplementary File 1) using the AntiMicrobial Peptide Analyzer (AMPA), a prediction algorithm that can detect the presence of cryptic antimicrobial segments in proteins[265]. Using the default parameters, AMPA detected potential antimicrobial regions in 82% of the HBP set (Supplementary File 2), suggesting that most HBPs contain cryptic AMPs that can be mined by AMPA (Supplementary File 2). According to our hypothesis, these regions should colocalize with heparin-binding sites in HBPs. To ascertain whether the AMPA-retrieved cryptic AMPs could indeed bind GAGs, we first resorted to molecular docking. In AutoDock Vina, a docking region (grid) centered on the antimicrobial segment detected by AMPA was defined and docking with a heparin disaccharide (I-S, α-ΔUA-2S- [1→4]-GlcNS-6S; Supplementary File 2) was performed. Results show 76% of the cryptic antimicrobial regions as potential binders of I-S, with affinity comparable to well-defined heparin-binding motifs (Figure 6C). We also examined the presence of CPC' clips in HBPs with a docking score higher than the average energy calculated for experimentally validated HBPs (-6.8 kcal/mol, 30 proteins) and found that 74% of such regions contain a CPC' motif with geometric distances compatible with GAG anchoring

(Figure 6D). Sequential heparin-binding motifs were also found in 48% of the antimicrobial regions with detected CPC' motifs (XBBXBX and XBBBXXBX, where B is Arg or Lys, and X is undefined; Supplementary File 2). We therefore concluded that heparin-binding regions significantly overlap with cryptic antimicrobial regions in HBPs, hence structural co-localization of antimicrobial activity and GAG recognition can be posited.

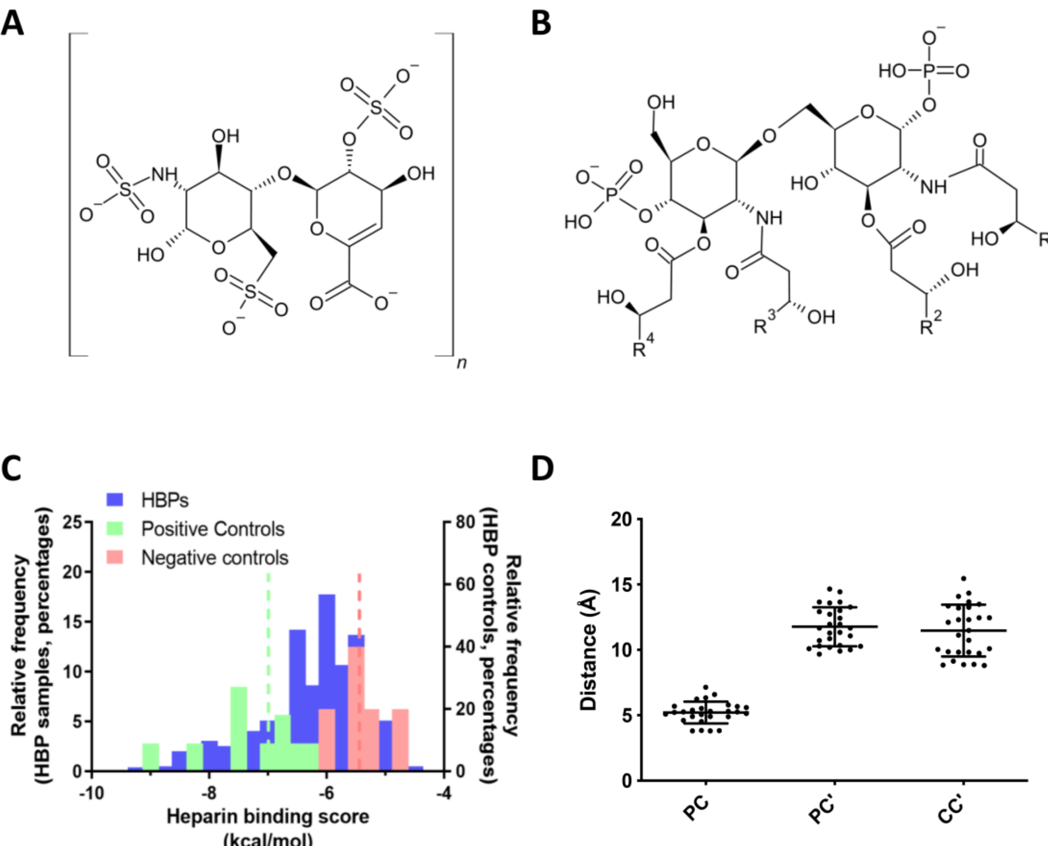


Figure 6. Antimicrobial and heparin binding affinity of HBPs. Structure of (A) heparin disaccharide and (B) Lipid A disaccharide central axis. (C) Affinity score distribution of AMPs (blue), positive controls (green, dotted line in the green refers to their mean, -7.0±1.1 kcal/mol) and negative controls (red, dotted line in red refers to their mean, -5.4±0.5 kcal/mol). (D) Distances between cationic and polar residues in the best candidates with CPC' motifs detected. Reference values for PC, PC' and CC' residues in CPC' motifs are 6.0±1.9 Å for PC, 11.6±1.6 Å for PC' and 11.4±2.4 Å for CC'[7].

Synthesis and validation of cryptic AMPs from HBPs

To confirm our hypothesis, we synthesized five peptides reproducing the regions with highest AMPA score that also contained a CPC' clip motif (Table 5, Supplementary Table S1). We used first affinity chromatography to check whether the peptides were able to bind heparin, hence proving that the binding region had been successfully delimited. Indeed, we found the retention times for all peptides in a heparin column to be higher

than control, antimicrobial peptide LL-37 (Table 5). In two cases, **HBP-4** and **HBP-5**, affinity was so high that up to 98% buffer B had to be used to dislodge them from the column. So, we could safely conclude that all peptides showed medium-to-strong heparin binding evoking that of parental HBPs.

Table 5. Description table of synthetized peptides and measured heparin affinity.

Peptide	Sequence	HPLC retention time (min)[a]	Molecular mass (Da)		Heparin affinity (% Elution buffer)[b]
			Theory	Found	
HBP-1	RWHLTHRPKTGYIRVLVH	1.5	2269.7	2268.7	60
HBP-2	RFYLSKKKWVMVP	1.7	1682.1	1681.1	59
HBP-3	FRFKRKLPKYLLF	1.9	1756.2	1755.2	68
HBP-4	GWKDKKSYRWFLQHRPQVGYIRV RFY	1.9	3414.9	3414.0	82
HBP-5	HNLFRKLTHRLFRRNFGYTLRSV	1.9	2932.4	2931.4	98
LL-37	LLGDFFRKSKEKIGKEFKRIVQ RIKDFLRNLVPRTES	2.1	4493.3	4492.3	50

[a] 10 to 60% of solvent B (ACN with 0.036% TFA) into solvent A (H_2O with 0.045% TFA) in a 3-minute run.

[b] Elution buffer was 2 M NaCl in Na_2HPO_4 10 mM.

We next inspected antimicrobial activity. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined on a panel of gram-negative and gram-positive bacteria. The synthetic peptides displayed strong activity against gram-negative (*Escherichia coli*, *Acinetobacter baumannii,* and *Pseudomonas aeruginosa*) while being much less active against gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecium,* and *Lysteria monocytogenes;* Table 6). This observation is consistent with our hypothesis that, lacking LPS, gram-positives are much less susceptible than gram-negatives to AMPs. Also, in tune with the hypothesis, peptides with the strongest heparin binding (**HBP-4** and **HBP-5)** had the best antimicrobial activity, correlating both observations. In contrast **HBP-2**, the peptide with the lowest affinity for heparin, did not show any significant difference in activity between gram-positive and -negative bacteria, except for *S. aureus*. Antimicrobial activity was also retained against clinical isolates of gram-negative strains (Supplementary Table S2).

Specifically, **HBP-4** and **HBP-5** were remarkably active, including multidrug-resistant *P. aeruginosa* strains. Given these encouraging results, we inspected the hemolytic capacity of the peptides as a benchmark of their therapeutic potential as antimicrobials (Supplementary Table S3). Erythrocyte lysis was low for all peptides; only 15% was observed up to 125 µM peptide, in contrast to >30% lysis for LL-37 at the same concentration. On mammalian (MRC-5 and HepGS) cells, again similarly favourable results were found. For **HBP-4**, the (relatively) more cytotoxic peptide, LC_{50} was comparable to LL-37, but **HBP-5** was significantly better. Overall, HBP-5 emerges as the most attractive analog, with a selectivity ratio (LC_{50}/MIC) between 50 and 800 (depending on bacterial strain) that must be regarded as outstanding for AMPs and that confirms the hypothesis that HBPs contain cryptic AMPs.

Table 2. MIC and MBC values (µM) for all peptides in reference bacteria strains.

Peptide	*E. coli*	*A. baumannii*	*Pseudomonas sp*	*S. aureus*	*E. faecium*	*L. monocytogenes*
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
HBP-1	1.6 / 1.6	1.6 / 1.6	3.1 / 3.1	50 / >100	≥100 / >100	6.3 / 12.5
HBP-2	12.5 / 25	50 / 50	25 / 50	>100 / >100	>100 / >100	37.5 / 150
HBP-3	3.1 / 3.1	0.8 / 0.8	6.3 / 6.3	25 / 50	>100 / >100	6.3 / 12.5
HBP-4	0.2 / 0.2	0.8 / 0.8	0.8 / 0.8	3.1 / 12.5	12.5 / 25	1.6 / 3.1
HBP-5	0.4 / 0.4	0.2 / 0.2	0.8 / 0.8	6.3 / 6.3	25 / 25	0.8 / 3.1
LL-37	1.6 / 3.1	6.3 / 0.2	0.8 / 0.8	25 / 25	0.8 / 3.1	0.8 / 3.1

Mechanism of action

Given the interesting antimicrobial profiles of **HBP-4** and **HBP-5,** we investigated their mechanism of action to determine if activity could be related to the interaction with LPS, hence on the cell wall. First, we analyzed LPS binding affinity with the BODIPY-cadaverine assay. The peptides with best antimicrobial activity, **HBP-4** and **HBP-5**, also exhibited strongest LPS binding, comparable to LL-37, while the remaining analogs showed moderate binding, **HBP-2** being the poorest one, again in tune with low

antimicrobial activity (Figure 7A, Supplementary Table S4). This correlation between heparin and LPS affinities strongly suggests that both activities are related (Supplementary Figure S1). The results are also consistent with the lethality curves measured in *E. coli*, in which **HBP-4** and **HBP-5** are fast acting, even more than LL-37, while **HBP-1** and **HBP-2** are the slowest ones (Figure 7B). All peptides showed membrane depolarization abilities comparable to LL-37, according to the DiSC$_3$(5) assay (Figure 7C, Supplementary Table S4), with **HBP-5** again scoring highest and **HBP-2** lowest among all analogs. Finally, to directly observe cell wall damage, the morphology of peptide-incubated *E. coli* cells was observed by scanning electron microscopy. In all cases we could detect a clear disruption of the bacterial envelope (Figure 7D), confirming that the peptides act at the outer membrane level, disrupting cell structure and promoting depolarization, eventually resulting in cell death.

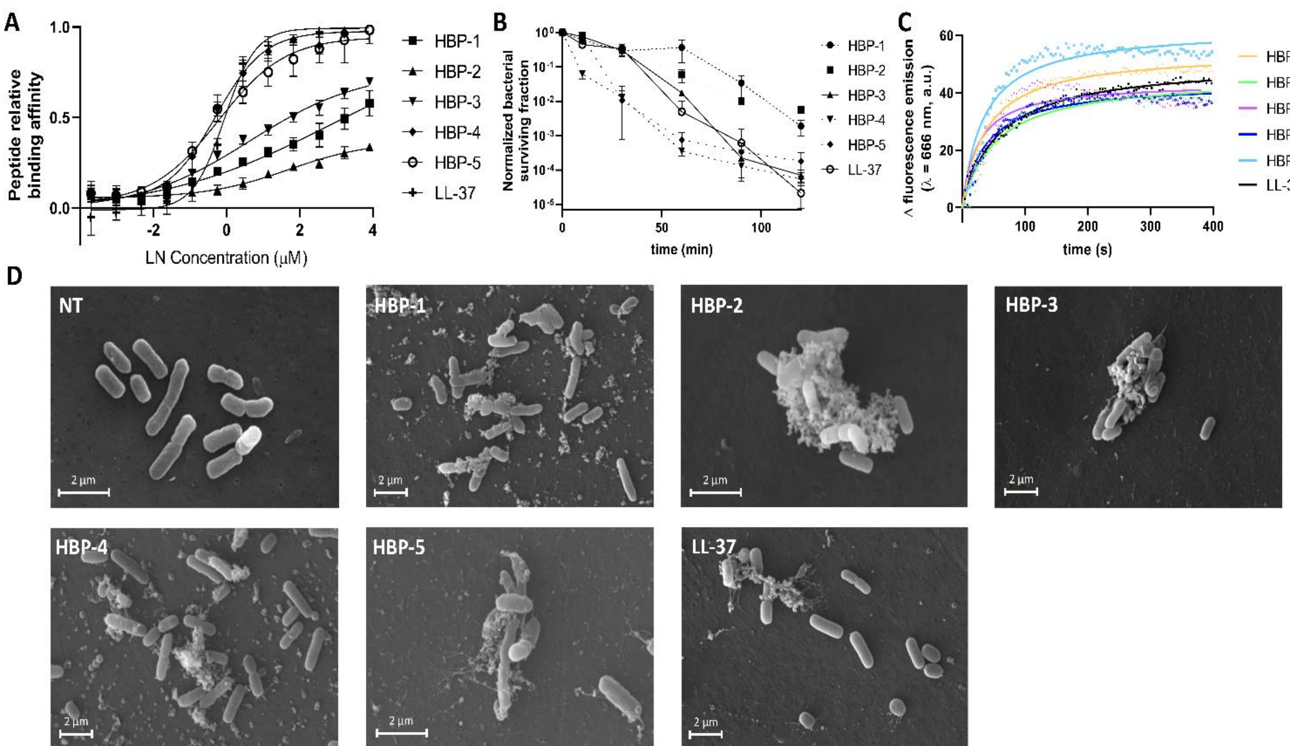


Figure 7. Mechanism of action of HBP-derived antimicrobial peptides (A) LPS affinity measured as an increase of the fluorescence emission (λ_{em}=620 nm) of BODIPY-cadaverine at different peptide concentrations. (B) Bactericidal activity kinetics obtained by treating E. coli planktonic cultures with HBPs. Peptides concentrations used for this assay were 50 µM HBP-1, 150 µM HBP-2, 50 µM HBP-3, 25 µM HBP-4, 6.3 µM HBP-5 and 50 µM LL-37. (C) Cell depolarization measured as DiSC3(5) fluorescence emission increase after incubating E. coli cells with HBPs. LL-37 was used as positive control of membrane depolarization. All peptides were tested at 10 µM, but HBP-2, tested at 20 µM. (D) SEM pictures of E. coli cells treated with HBPs at same concentrations used in C, after 2h of incubation at 37ºC. NT= non peptide control. All measurements were performed in triplicate.

Structural characterization

To investigate any structural changes occurring upon interaction of the peptides with heparin or cell membranes, we obtained circular dichroism (CD) spectra in buffer, SDS micelles, LPS micelles, and heparin (Figure 8A, Supplementary Tables S5-8). In almost all cases, the structures in water were random, with minima at ~200 nm. In the presence of SDS micelles, peptides **HBP-3** and **HBP-5** displayed minima near 208 and 222 nm, with a positive band at ~190 nm, evidencing a shift towards helical conformation. For peptides **HBP1**, **HBP-2**, and **HBP-4**, a shift towards a minimum at 218 nm was observed, suggesting a beta strand structure. This behavior is typical of AMPs; the random-to-structure transition favors partial insertion into the membrane, promoting depolarization. With LPS micelles again a transition from random to either helix or beta strand was observed for **HBP-4** and **HBP-5**, less pronounced for the other analogs. This behavior was repeated for all peptides in the presence of heparin, except for **HBP-2,** which remained in disordered conformation. These results are consistent with the above antimicrobial and LPS binding assays in suggesting that LPS and heparin binding triggers a structural arrangement into a more defined, antimicrobially effective structure which, in all cases, is similar to that adopted by the peptide segment in the corresponding original protein (Figure 8A).

As **HBP-5** was the most interesting analog in terms of antimicrobial activity and heparin-binding, we decided to inspect its solution structure by NMR in (i) water, (ii) DPC micelles, and (iii) in the presence of heparin analogs. First, we performed a qualitative analysis of the $\Delta\delta_{Ha}$ and $\Delta\delta_{Ca}$ conformational shifts ($\Delta\delta = \delta^{observed} - \delta^{random\ coil}$, ppm; see Methods and Supplementary Figure S2). The fact that $\Delta\delta_{Ha}$ and $\Delta\delta_{Ca}$ values are within the random coil range indicates that **HBP-5** is mainly disordered in aqueous solution, as observed previously by CD. In DPC micelles, the stretches of negative $\Delta\delta_{Ha}$ and positive $\Delta\delta_{Ca}$ values indicates the presence of helix structures spanning residues 3-11. Structure calculation, which includes medium and long-range distance restraints derived from the observed NOE cross-peaks (see Methods and Supplementary Table S9 and S10), showed a well-defined N-terminal helix spanning residues 3-15, three-residues longer than deduced from qualitative analysis of $\Delta\delta_{H\alpha}$ and $\Delta\delta_{C\alpha}$ values, and a less ordered non-regular turn-like motif involving residues 18-22 (Figure 8B). The relative disposition of the a-helix and the turn-like motif is very poorly defined Unfortunately, attempts to retrieve an NMR 3D structure in the presence of heparin analogs were unsuccessful. Spectra of **HBP-**

5 with either the fondaparinux (Arixtra®) pentasaccharide or the simpler H-IIIS disaccharide acting as heparin analogs did not provide any NOE cross-peaks evidencing intermolecular peptide-sugar contacts, in great part due to the substantial sample precipitation observed particularly in the case of the fondaparinux pentasaccharide. When incubated with the H3S disaccharide, many cross-peaks are shifted relative to free **HBP-5** (see Supplementary Figure S4). To identify which residues are most affected upon disaccharide interaction, weighted chemical shift differences ($\Delta\delta_w$, ppm; see methods) were plotted as a function of peptide sequence (Figure 8C). It is clear in this plot that significant differences are mainly located at central peptide section, residues 8-14 (Figure 8C). In view of this, and to obtain additional insights into heparin binding of **HBP-5**, we performed a molecular dynamics simulation with the fondaparinux pentasaccharide. The results show that the pentasaccharide remains in contact with the peptide all along the simulation time, suggesting strong binding. Specifically, we observed a persistent salt bridge between the Arg13 side chain and the S6 sulfate group of pentasaccharide (Figure 8D). Another salt bridge between Arg10 and the S6 sulfate, plus a loosely defined hydrogen bond between His9 and the S3 were also identified. These three residues (His9, Arg10, and Arg14) form a CPC' motif with their relative distances maintained throughout the simulation (Figure 8E, Supplementary Figure S4), altogether suggesting a CPC' clip as a key binding element.

DISCUSSION

Inflammation and coagulation are closely related, with inflammatory proteins often interacting with GAGs and influencing their anticoagulant activity[266]. Some proteins play important roles in both processes; a case in point is histidine-rich glycoprotein, an adaptor protein released by platelets, that regulates angiogenesis, immunity, and coagulation[267].

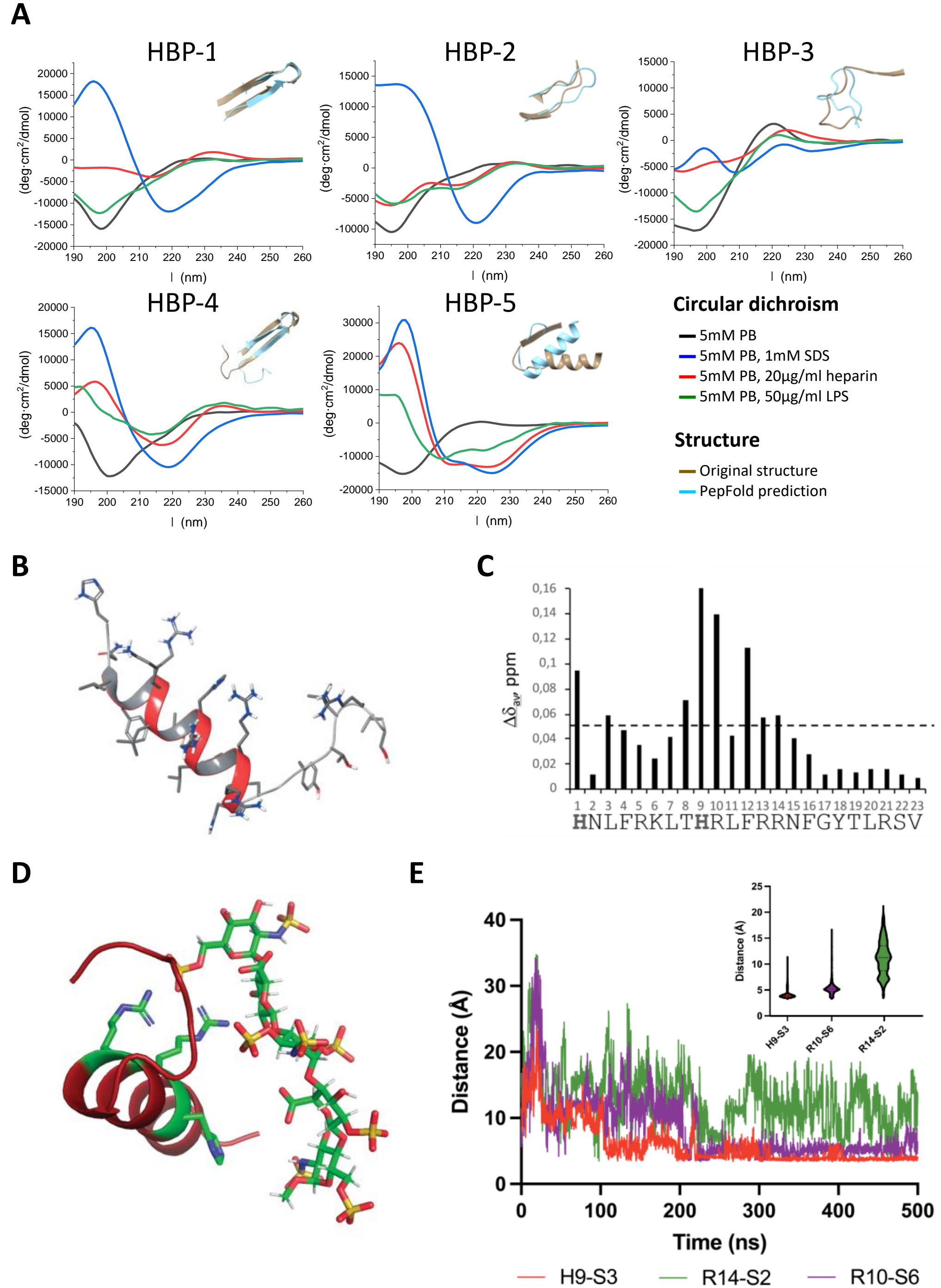


Figure 8. Structural characterization of HBP-5 in different conditions. **(A)** Circular dichroism spectra of HBPs in PBS 5 mM (black lines); PBS 5 mM and SDS 1 mM (blue lines); PBS 5 mM and heparin 20 µg/mL (red lines), and PBS 5 mM and LPS 50 µg/ml (green lines). Overlapping original peptide structures in native protein (in light blue) and PepFold predicted structures (in brown) are added for each peptide in the upper-right corner of each plot. **(B)** Structure of the peptide in DPC micelles as solved by NMR in. **(C)** Weighted chemical shift differences ($\Delta\delta_w = [(\delta_{HN}^{bound} - \delta_{HN}^{free})^2 + (\delta_{H\alpha}^{bound} - \delta_{H\alpha}^{free)2}]^{1/2}$, ppm; see methods) induced by the presence of the heparin disaccharide H1S plotted as a function of peptide sequence. Peptide/disaccharide ratio 1:1. The horizontal line indicates the $\Delta\delta_w$ = 0.05 ppm; Residues with values below this line are considered as unaffected by interaction; and **(D)** structure of the peptide bound to the heparin analog Arixtra as defined by MD simulation. **(E)** Distances between the CPC' residues involved in heparin binding as observed in the MD simulation and summary of calculated distances.

Many proteins, particularly those involved in host defense, can act as reservoirs of AMPs, silently embedded in these protein sequences but produced on demand by host proteases during events such as inflammation, clotting e[268,269]. After a wound, processes to prevent bleeding, remove damaged tissue and keep the lesion free from pathogen entry and subsequent infection are called for. In such scenarios, proteases hydrolyzing surrounding proteins and releasing (formerly) cryptic AMPs to achieve preventive antimicrobial action can play a crucial role. A relevant example is thrombin. While the whole protein does not display antimicrobial activity *per se*, after cleavage, its C-terminal displays strong and broad activity[270]. It is therefore not surprising that proteins involved in GAG binding can become an important source of AMPs, hence contribute to preventing infection. This dual action, GAG binding and antimicrobial activity, can be interpreted in structural terms by the similarities between GAG and lipid A structures, both containing negatively charged disaccharide units. It is therefore reasonable to suggest that the ability to bind GAGs could also foster LPS recognition, hence allow interaction with the outer membrane of gram-negative bacteria.

Here, we have shown that GAG-binding proteins can be a source of new AMPs, some with remarkable activity. The fact that these peptides can bind to both heparin and LPS is consistent with the above structural similarity hypothesis, and with the fact that these peptides have much higher activity on gram-negative bacteria. With further optimization, HBP-derived cryptic AMPs should prove useful for treating infections by gram-negative bacteria that are resistant to classic antibiotics and pose huge risks for hospitalized patients.

MATERIALS AND METHODS

Materials. *Escherichia coli* BW25113 was obtained from the Coli Genetic Stock Center. *Acinetobacter baumannii* (ATCC 15308), *Pseudomonas sp.* (ATCC 15915), *Staphylococcus aureus* (ATCC 12600), *Enterococcus faecium* (ATCC 19434), *Listeria monocytogenes* (ATCC 19112), and *Streptococcus pyogenes* (ATCC 8668) were obtained from CECT (Valencia, Spain). Clinical strains were obtained from the Vall d'Hebron Hospital Microbiology Service (Barcelona, Spain). MRC-5 and HepG2 cell lines were purchased from ATCC. Horse defibrinated red blood, DiSC3(5) (3,3'-

dipropylthiadicarbocyanine Iodide), BODIPY® TR cadaverine, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were from Thermo Fisher (Hampshire, England). *E. coli* lipopolysaccharide and heparin were acquired from Merck (Darmstadt, Germany). Heparin disaccharide I-S trisodium salt was from TLC Pharmaceutical Standards (Newmarket, ONT) and fondaparinux (Arixtra®) was a gift from the Hospital del Mar (Barcelona, Spain) pharmacy.

Antimicrobial activity prediction and docking. The library of heparin-binding proteins was obtained from Ori et al. All sequences were processed with the AMPA antimicrobial peptide predictor (http://tcoffee.crg.cat/apps/ampa) to define the antimicrobial regions. Best candidates according to AMPA score were used for docking studies using AutoDock Vina using the heparin disaccharide H1S (Heparin disaccharide I-S, α-ΔUA-2S- [1→4]-GlcNS-6S). Grid boxes were adjusted to the regions as delineated by AMPA. To control for significant binding energy values, we docked H1S to the binding regions of proteins with a solved crystal structure containing a heparin analog. The proteins used as positive controls were, angiogenin (4QFJ), heparin lyase I (3IN9), palmitoleoyl-protein carboxylesterase (4UYW), stromal cell-derived factor 1 (2NWG), peptidoglycan recognition protein 1 (3OGX), C-C motif chemokine 5 (1UL4), heparin cofactor 2 (1JMJ), antithrombin III (1SR5), annexin A2 (2HYV), plasma serine protease inhibitor (3DY0), and heparin lyase (2FUT). Non heparin-binding proteins with a solved crystal structure with a non-sulfated disaccharide were used as negative controls, i.e., aconitase (7ACN), R-methyltransferase (R30Q), phytase (3ZHC), bifunctional epoxide hydrolase 2 (1EK2) and calpain-3 (6BGP). The proteins included in the HBPs list with the highest affinity score (higher than the average of positive controls) were checked for the presence of CPC' motifs within their sequence using UCSF Chimera.

Peptide Synthesis. Peptides in this work were synthesized as described previously[271]. They were produced on H-Rink Amide-ChemMatrix resin in a Prelude instrument (Gyros Protein Technologies, Tucson, AZ) running Fmoc solid-phase peptide synthesis (SPPS) protocols. After sequence assembly, the resin-bound peptides were deprotected in TFA/H_2O/triisopropylsilane (95:2.5:2.5 v/v), isolated by cold diethyl ether precipitation and centrifugation at 4800 rpm for 5 min, and lyophilized. Purification was performed on

a Luna C18 column (21.2 mm x 250 mm, 10 µm; Phenomenex) in a LC-8 preparative RP-HPLC instrument (Shimadzu, Kyoto, Japan) using a linear gradient of solvent B (0.1% TFA in ACN) into A (0.1% TFA in H_2O) for 30 min at 25 mL/min flowrate. Peptides prior and after purification were inspected by analytical RP-HPLC and LC-MS. RP-HPLC was performed on a Luna C18 column (4.6 mm x 50 mm, 3 µm; Phenomenex) in an LC-20AD instrument (Shimadzu) using a linear gradient of solvent B (0.036% TFA in ACN) into A (0.045 TFA in H_2O) over 15 min at 1 mL/min flowrate. LC-MS was done in an LC-MS 2010EV instrument (Shimadzu) connected to an Aeris Widepore XB-C18 column (4.6 mm x 150 mm, 3.6 µm; Phenomenex) with a liner gradient of solvent B (0.08% formic acid (FA) in ACN) into solvent A (0.1% FA in H_2O) over 15 min at 1 mL/min flowrate. Peptides with the expected mass and >95% HPLC homogeneity were lyophilized and stored at -20 ºC.

Heparin-binding affinity assay. Heparin binding was evaluated by affinity chromatography on a Heparin HP column (Cytiva, Marlborough, MA) linked to an ÄKTA go FPLC instrument (Cytiva, Marlborough, MA). 10 mL of peptide stocks at 10 µM were loaded in the column, previously equilibrated with binding buffer (10 mM sodium phosphate). Peptides were eluted in linear gradients of elution buffer (10 mM sodium phosphate, 2 M NaCl) in binding buffer. Heparin affinity for each peptide was measured as the percentage of elution buffer at the maximum elution peak intensity.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Antimicrobial activities were determined, following the classical microtiter broth dilution recommended by the National Committee of Laboratory Safety and Standards (NCLSS), adapted for AMPs[272]. Briefly, overnight bacterial cell cultures were brought to an exponential growth state (OD_{600}=0.4) in MH broth and diluted to a final concentration of $5 \cdot 10^5$ CFU/mL. 1:2 peptide serial dilutions were prepared in 96-well polypropylene plates (Greiner, Frickenhausen, Germany), in MH medium containing 0.2% (w/v) of bovine seroalbumin (BSA) and 0.02% glacial acetic acid. Samples were incubated overnight at 37ºC, and 230 rpm, and the MIC was determined as the last peptide concentration without appreciable visual growth. MBC was determined by transferring

the content of the wells to Petri plates with Luria Bertani (LB) agar and incubated overnight at 37ºC. The lowest concentration with no colonies was considered the MBC.

Killing curve assay. Peptide antimicrobial activity was also tested by lethality curve in *E. coli* cultures[273]. First, 50 µL of the peptide stock solution were added to 450 µL of an *E. coli* culture at $5{\cdot}10^5$ cfu/mL in a 1.5 mL polypropylene tube to obtain a final concentration of 1x MIC. Samples were then incubated at 37ºC and 600 rpm in an Accutherm microtube shaking incubator (Labnet, Edison, NJ) for 2 h. Samples of 50 µl were taken at several intervals and plated in LB agar. Plates were incubated overnight at 37 ºC and colonies were counted and compared with the initial inoculum to define the percentage of surviving bacteria.

Hemolytic activity. Peptide toxicity against erythrocytes was tested in horse erythrocytes[274]. Horse defibrinated blood was washed three times in phosphate buffer saline (PBS), pH 7.2 to remove the excess of hemoglobin in the supernatant and then diluted 10x in PBS. Then, 50 µL of erythrocytes were added to a 1.5 mL polypropylene tube and incubated with 50 µL of 1:2 serial dilution of peptides. An erythrocyte disruption (ED) control was prepared by adding 50 µL of 0.1% TritonX-100 in PBS instead of the peptides and an intact erythrocyte (IC) control was prepared by adding 50 µL of PBS alone. All samples and controls were incubated for 4 h at 37 ºC. Afterward, the samples were centrifuged at 3000 rpm for 3 min and the supernatants were transferred to a polystyrene 96-well plate and inspected for erythrocyte disruption by reading the absorbance at 540 nm in a TECAN Spark instrument (Tecan, Männedorf Switzerland). The hemolysis percentage was calculated as:

$$Hemolysis(\%) = \frac{Sample - IC}{ED - IC}$$

Lipopolysaccharide Binding Affinity. Displacement of fluorescent cadaverine bound to LPS was used for testing peptide affinity to LPS[275]. Briefly, 50 µL of 1:2 peptide serial dilutions in HEPES 10 mM were prepared in polystyrene 96-well plates. Then, a previously incubated mixture of 25 µL of 40 µg/mL LPS and 25 µL of 40 µM cadaverine was added to each well. A control without peptides (NP) was prepared by the addition of

50 μL of HEPES buffer, and a control without LPS (NL) was prepared by adding 25 μL of HEPES buffer instead of LPS. Plates were read in a TECAN Spark instrument (Tecan, Männedorf Switzerland) for fluorescence emission, with the following settings: 580 excitation and 620 emission wavelengths, with excitation and emission slits of 5 and 10 nm respectively. The fraction of peptide bound to LPS was calculated as:

$$Binding = \frac{Sample - NP}{NL - NP}$$

Bacterial membrane depolarization. DiSC3(5) lipophilic dye fixation was tested in *E. coli* fresh cultures to measure depolarization, as previously described[276]. In short, 5 mL bacterial suspensions in exponential phase (~0.4 OD) were washed first with 5mL of buffer A (5 mM HEPES, 20 mM glucose, pH 7.2) and later with 5 mL of buffer B (5 mM HEPES, 20 mM Glucose, 100 mM KCl, pH 7.2). Then, the bacteria were resuspended in buffer B to an OD of 0.05. Samples of 1 mL were prepared and then 0.4 μM of DiSC3(5) was added. Fluorescence emission was continuously measured in a Varian Cary Eclipse fluorescence spectrometer (Agilent, Santa Clara, California), with the following settings: excitation wavelength of 625 nm, 5 nm slit and excitation wavelength of 666 nm, 10 nm slit. 10 min after dye addition (estimated time required for DiSC3(5) quenching), peptides were added to the samples to a final concentration of 10 μM (except HBP-2, that was tested at 20 μM). Dye release was measured for, at least, 5 min.

Scanning Electron Microscopy (SEM). 1 mL of *E. coli* bacterial suspensions in exponential growth (~0.4 OD) were treated with 10 μM peptides for 2 h. After treatment, treated cells were filtered through a 0.1 μm Nucleopore filter to attach bacteria and later fixed for 2 h at 4ºC in a buffer containing 2.5% glutaraldehyde in 100 mm Na-cacodylate, pH 7.4. Afterward, cells were coated by immersion in 1% osmium tetroxide in Na-cacodylate buffer for 30 min. Samples were rinsed in the same buffer and dehydrated in ethanol with increasing concentrations (once at 30 and 70% and twice at 90 and 100% (v/v)) for 15 min each. The filters were mounted on aluminum stubs and coated with gold-palladium in a sputter coater (K550; Emitech, East Grinsted, UK). Each sample was later

inspected at 15 kV accelerating voltage in an EVO MA 10 scanning electron microscope (Zeiss, Oberkochen, Germany).

Cytotoxicity in Mammalian Cells. Toxicity in MRC-5 and HepG2 cells was tested by the MTT assay, as previously described[274]. Cell lines were maintained in Eagle's minimum essential medium (MEMα) supplemented with 10% fetal bovine serum (FBS). Cells were cultured in 75 cm^2 flasks and then transferred to polystyrene 96-well plates, at $3 \cdot 10^4$ cells per well, and incubated overnight for attachment to the well surface. Then culture media was removed and 1:2 peptide serial dilutions in MEMα were added to each well and later incubated for 4 h. After incubation, peptides were removed and 100 µL of 0.5 mg/mL MTT staining solution in MEMα supplemented with 10% FBS was added to cells and incubated for 1.5 h at 37ºC. Formazan crystals in living cells were detected after disruption with 200 µL dimethyl sulfoxide and then the absorbance was measured at 570 nm in a TECAN Spark instrument.

Circular Dichroism. CD spectra of peptides were obtained in four different conditions: 5 mM PBS, 5 mM PBS with 1 mM SDS micelles, 5 mM PBS with 20 µg/mL heparin, and PBS 5 mM PBS with 50 µg/mL LPS micelles. Peptides were dissolved in each condition to a final concentration of 10 µM. Samples were transferred to a 0.2 mm quartz cuvette (Hellma, Jena, Germany) and then analyzed in a Jasco J-815 CD spectropolarimeter (Jasco, Easton, Maryland) in the 260 to 190 nm range. For each sample, 15 spectra were acquired and averaged. Data were processed with the OriginPro analysis software and subsequently analyzed to predict secondary structure with the CDSSTR method[277] available in the Dichroweb online server[278] (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

NMR Spectroscopy. NMR samples were prepared by dissolving the lyophilized peptides at about 1 mM concentration in aqueous solution (H_2O/D_2O 9:1 v/v), in DPC micelles (30 mM [D38]-DPC in H_2O/D_2O 9:1, v/v) or in aqueous solution with the heparin analog Arixtra or the heparin disaccharide H1S (molar ratios 1:1, 1:0.5). The pH was measured using a glass micro-electrode and adjusted to 4.4 by addition of NaOD or DCl. Sodium

2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at a 0.1-0.2 mM concentration was added as internal reference for the 1H chemical shifts. A Bruker AVNEO-600 spectrometer (Bruker Biospin, Karlsruhe, Germany) equipped with a cryoprobe was used to record NMR spectra: 1D ^{1}H, 2D ^{1}H,^{1}H-DFQ-COSY (double-filtered-quantum phase-sensitive two-dimensional correlated spectroscopy), ^{1}H,^{1}H-TOCSY (total correlated spectroscopy), ^{1}H,^{1}H-NOESY (nuclear Overhauser enhancement spectroscopy), and ^{1}H-^{13}C-HSQC (heteronuclear single quantum coherence) at ^{13}C natural abundance. TOCSY and NOESY mixing times were 60 ms and 150 ms, respectively. Data were processed using the TOPSPIN software (Bruker Biospin, Karlsruhe, Germany). The NMRFAM-SPARKY software[279] was used to analyze the NMR spectra. The ^{1}H chemical shifts were assigned by analysis of the 2D homonuclear spectra using the well-established sequential assignment methodology[280], and the ^{1}H-^{13}C-HSQC spectra were analyzed to assign the ^{13}C chemical shifts. The assigned chemical shifts will be deposited at the BioMagResBank (http://www.bmrb.wisc.edu).

The conformational shifts ($\Delta\delta_{H\alpha}$ and $\Delta\delta_{C\alpha}$) were obtained as the differences between the observed chemical shifts and those in random coil (RC) peptides: $\Delta\delta_{H\alpha} = \delta_{H\alpha}^{observed} - \delta_{H\alpha}^{RC}$, ppm and $\Delta\delta_{C\alpha} = \delta_{C\alpha}^{observed} - \delta_{C\alpha}^{RC}$, ppm $\delta_{H\alpha}^{RC}$ and $\delta_{H\alpha}^{RC}$ were taken from Wishart et al.[281]. Helix populations (Supplementary Table S10) were estimated from the ^{1}Hα and ^{13}Cα chemical shifts as previously described[282]. A weighted value for the chemical shift changes ($\Delta\delta_{w}$, ppm) was defined as:

$$\Delta\delta_{w} = [(\delta_{HN}^{bound} - \delta_{HN}^{free})^{2} + (\delta_{Ha}^{bound} - \delta_{Ha}^{free)2}]^{1/2}$$

Considering all **HBP-5** residues (23 in total), the mean Dd_{w} is 0.05 ppm. Residues with $Dd_{w} > 0.05$ ppm can be considered as those mostly affected by interaction.The structure of **HBP-5** were calculated using the iterative procedure for automatic NOE assignment integrated in the CYANA 3.98 program[283]. This algorithm consists of seven cycles of combined automated NOE assignment and structure calculation, in which 100 conformers were computed per cycle. The experimental input data comprises the lists of assigned chemical shifts, and integrated NOE cross-peaks present in the 150 ms NOESY spectra, plus the ϕ and ψ dihedral angle restraints. The NOE cross-peaks were integrated using the automatic integration subroutine of the NMRFAM-SPARKY software[279]. The TALOSn webserver[284] was used to obtain the dihedral angle restraints from the ^{1}H and ^{13}C chemical shifts. The final structure is the ensemble of the 20 lowest target function

conformers calculated in the last cycle. These ensembles were visualized and examined by using the MOLMOL program[285] and will be deposited to the pdb databank.

Molecular Dynamics Simulation. MD simulations with or without Arixtra were conducted using the GROMACS v2022.3. The Glycan Reader & Modeler from CHARMM-GUI was used to prepare the system, obtaining the topology and parameter files. The force field CHARMM36 was employed as the parameters for protein and Arixtra. Initial structures were solvated in a rectangular box of TIP3P water with a minimum distance of 1.0 nm between protein and the faces of the box. K+ and Cl- ions were added to neutralize the system at an ionic strength of 0.15 M. Electrostatic interactions were calculated using the particle mesh Ewald method under periodic boundary conditions. Structures were energy-minimized and equilibrated by molecular dynamics for 130 ps. Production simulations were run on a GPU (NVIDIA GeForce RTX 3080 Ti) and 16 CPUs (Intel® Xeon® Gold 6226R CPU @ 2.90GHz) for 500 ns with a time step of 2 fs. NPT conditions were stabilized at 306 K by a Nosé-Hoover thermostat[286], and at 1 atm by a Parinello–Rahman barostat[287]. Bonds were constrained using the LINCS algorithm. Representative structures for different analyses were extracted from trajectories with the GROMACS command “gmx cluster”, using the gromos algorithm with a RMSD cutoff of 0.18 nm.

CHAPTER 3

RATIONALLY MODIFIED ANTIMICROBIAL PEPTIDES FROM THE N-TERMINAL DOMAIN OF HUMAN RNASE 3 SHOW EXCEPTIONAL SERUM STABILITY

DISCUSSION

Chapter 1: Antimicrobial peptides can generate tolerance by lag and interfere with antimicrobial therapy.

As soon as they became aware of the issue, researchers have put many efforts on the determination of the resistance mechanisms of bacteria. However, previous works have focused on elucidating how the resistant determinants are triggered in bacteria discovered at the initial phenotypic changes that allow bacteria to withstand the stress produced by antibiotics. Although the achieved mutations do not induce resistance, they permit bacteria to survive over longer periods, also called tolerance or persistence, and help in the development of new mutations that, in this case, are driven to overcome the harmful effects of antibiotic treatment[288].

Such effect was thoroughly investigated in conventional antibiotics because of the issue of antimicrobial resistance. Notwithstanding the development of new promising drugs such as AMPs, it is of high relevance to detect how tolerance is produced during the use of AMPs. As mechanisms unspecific to tolerance are altered to improve the survival time, it could be hypothesized that cross-tolerance or collateral sensitivity can occur, as previously demonstrated with resistance determinants in both conventional antibiotics and AMPs[233]. However, the acquisition of tolerant phenotypes under AMP treatment was poorly tackled, with only preliminary studies conducted so far[25].

The work presented in the first chapter yielded interesting data on bacterial adaptation to the treatments used. Tolerance was demonstrated to emerge under almost all the AMPs tested. Despite this fact, the behaviour of *E. coli* observed under the different treatments did not follow only a single pattern. Pleu treatment affected bacterial lag time and morphology much more than the other three treatments did. As observed in Figure 2, the sizes of colony-forming units appeared to be significantly affected since the first treatment. Moreover, the time to colony emergence doubled the number of untreated *E. coli* after just two treatment cycles. In the case of PolB and Derm, some modifications appear, although the effect level was lower than that observed with Pleu. By contrast, under LL-37 treatment, even after more than 10 treatment cycles, the previously described effect was not observed. Further experiments were then performed to determine how plausible the appearance of cross-tolerance events was between the same AMPs used to develop tolerance. In general, evolved strains are unlikely to be affected by cross-

tolerance after treatment with the other three tested compounds. The effect was observed only in an isolated case of PolB-evolved strain after pleu treatment. However, the evolved strain was already resistant to PolB, which indicates that the mutations acquired were directed to overcome the AMP treatment. The low tendency for cross-tolerance could be explained by the unspecific mode of action of AMPs. Their activity mainly starts with electrostatic interactions with cell membranes at the surface and are dependent on their composition and the secondary structures they adopt when in contact with the cell surface, making it unusual to find changes on the bacterial lipidic bilayer that can counteract several AMP activities without compromising their growth fitness.

Only a few already marketed antibiotics target cell membrane, mostly having a peptide-based origin[289]. Thus, cross-tolerance was also studied with conventional antibiotics. As expected from previous cross-tolerance assays, no effects were detected in the LL-37-evolved strain after treatment with four different antibiotics (ampicillin, kanamycin, ciprofloxacin, and nalidixic acid) of different mechanisms of action. In spite this result, interesting outcomes were found when a pleu-evolved strain was evaluated. Some collateral sensitivity was observed after kanamycin treatment, whereas in the case of ciprofloxacin and nalidixic acid treatments, we found cross-tolerance. The variety of responses to pleu-evolved strains could be explained by their intracellular targets in bacteria described previously in the literature[217]. Concretely, this study could open a new study window for the precise intracellular targets in the case of Pleu. Patrzycat et al. explained this intracellular action as "affected macromolecular synthesis." The results obtained in this last experiment could place the activity in DNA replication, repressing it. The cross-tolerance observed after the ciprofloxacin and nalidixic acid treatments supports this idea. However, the hypothesis must be tested further.

Later, genome sequencing obtained from evolved strains shed some light on the mutational profiles involved in tolerance development. In both genomes from the evolved strains with LL-37 and pleu, some of the most relevant mutations were already detected in pathogenic strains with described resistance to conventional antibiotics. The mutations found affect cell membrane development and macromolecular synthesis at several levels (Table S3). This means that these mutations could be mediators to finally obtain resistance-conferring mutations by increasing the survival time under certain treatments. On the other hand, we could not find any indication in the genome that could explain the development of collateral sensitivity to kanamycin that was observed in the evolved strain

obtained after pleu treatment. Comparing with previous studies that used conventional antibiotics for tolerance development, we found similarities. Fridman et al. found that the gene *sspA* was mutated in tolerant strains[290]. Although we could not find more direct coincidences on mutated genes, this most recent article[288] provides information about many other mutations that share the same affected pathways as those observed in this work. Amino acid and protein biosynthesis appeared to be affected by both conventional antibiotic and AMP continuous treatment.

The results obtained during the development of this project provide useful conclusions that can be applied in the future use of AMPs as a unique treatment or in combination with other antibiotics. Previous studies have shown the benefits of using combined AMP therapies[291,292]. Concretely, Yu et al. demonstrated that combining two or more AMP peptides improves antimicrobial kinetics and reduces the amount of peptides needed to find the same effect from the same AMP separately[292]. Our work supports this idea, as we barely found cross-tolerance effects with different AMPs. The basis of this phenomenon could be that almost all AMPs target the cell membrane, but the recognition events may be so different, as they are dependent on their structural determinants. The significant modifications of the bacterial membrane that affect the activity of several AMPs at the same time may have excessive fitness costs on their development, making them non-viable.

However, it is important to understand that when used with other antibiotics, AMPs display mechanisms of action triggered by the compounds used to avoid possible situations that favor cross-tolerance. Previous studies that used combinations of conventional antibiotics and AMPs have always highlighted the benefits obtained, unconsciously masking possible disadvantages[293,294]. Many AMPs exhibit dual mechanisms of action depending on the concentrations in which they are added while targeting bacterial intracellular pathways[217,295]. The results obtained in cross-treatments for pleu-evolved strains could be related to this. As in combinatorial therapies, AMPs are normally tested as the secondary or adjuvant treatment. Their main mechanism in the case of dual peptides is probably intracellular. Drugs with a similar intracellular target could also produce a similar tolerance pattern for bacteria, favoring the development of resistance.

Testing the efficacy of combinatorial therapies by measuring the capacity of cross-tolerance development at preclinical levels should provide more consistency to this kind of antimicrobial treatments and improve their success ratio at clinical stages.

Chapter 2: Cryptic antimicrobial peptides in heparin-binding proteins exhibit selective antimicrobial activity against gram-negative bacteria through specific lipopolysaccharide binding.

GAG-binding proteins are tightly bound to immune system mechanisms such as inflammation, coagulation, or chemokines[296]. For example, many interleukins and Toll-like receptors have been shown to possess GAG-binding regions[297]. On the other hand, human proteins with functions related to immunity have been widely known to be great sources of AMPs[298]. Many previous studies have used different techniques to obtain AMPs from GAG-binding proteins or other different protein sources linked to the immune system. The use of predictive tools and the structural homology to other peptides is the main mechanism of AMP discovery[299–302].

In this study, we present a novel combinative approach for *de novo* peptide design. We started from a structural homology point of view between the heparin disaccharide molecule and lipid A, a key component from the cell envelopes of gram-negative bacteria. Previous studies have shown the presence of putative AMPs in HBPs and found a binding affinity of CPC′ motifs to LPS[303,304]. However, in this study, we decided to combine both theories to create a brand-new discovery tool. The combination of AMP peptide-predictive software run in a huge heparin-binding protein (HBP) library and determination of heparin affinity and the presence of the CPC′ motif in best candidates allowed us to synthetize AMPs with extraordinary antimicrobial activities. Moreover, they exhibited high specificity against gram-negative bacteria. The five tested candidates showed unbalanced activity directed to gram-negative bacteria both in reference and clinical strains (Tables 2 and S3) and higher heparin affinity compared with LL-37 (Table S1), a deeply studied AMP over the last decades[305,306]. Further tests revealed a rearrangement in the presence of LPS and heparin in some cases, similar to that expected from the native protein (Figures 3 and S1).

If the purposed hypothesis were true, these peptides may have more resemblance to conventional antibiotics in terms of performing its main antibacterial activity by binding to a concrete target. If its target is lipid A, the bacteria could more easily develop resistance against the AMPs we identified. Further studies are needed to confirm concrete

binding to lipid A and the propensity to develop resistance. The stability of HBPs in the presence of host cells must be better understood. Stability was identified as a main limitation of AMPs in previous *in vivo* studies[307].

Through this work, we discovered AMPs with exceptional properties. With further research and structural optimization to improve their stability and side effects, they may reach clinical stages of development. HBP-5 assembles the best conditions for improvement. However, this is not the main message of the present research. The combination of the AMPA predictive software with structural homology approaches opens a new challenging way to develop more specific AMPs. It could also be used for the development of new drugs against gram-positive bacteria such as *Staphylococcus aureus* or *Salmonella sp.* Teichoic acids may be a great target for this tool, as they are only present in this kind of bacteria. Moreover, glycopolymers have been shown in previous studies to be involved in AMP resistance or conventional antibiotic susceptibility reduction[308].

<u>Chapter 3</u>: Rationally modified antimicrobial peptides from the N-terminal domain of human RNase 3 show exceptional serum stability.

The low stability and bioavailability of *de novo* designed antimicrobial compounds are main issues that hinder their development through preclinical and clinical stages and prevent their feasibility of being marketed[309]. In the case of peptides, switching residues commonly prone to degradation into non-natural or D-amino acids has been demonstrated to be effective[310]. This work is focused on hECP30 AMP, an improved sequence of an already discovered AMP by Torrent et al., obtained from RNase 3 human protein[311]. The peptide sequence shows a high number of arginines. This residue is a key element for the development of antimicrobial activity based on electrostatic interactions with the anionic cell surface of many pathogens. However, arginine is also a usual target for serine proteases[312]. This makes the peptide prone to fast degradation and loss of function.

The change of Arg to Orn, Dab, or Har non-proteogenic residues has led to the significant improvement of peptide stability, but not enough for therapeutic purposes (Table 2). The data obtained from high-pressure liquid chromatography and mass spectrometry showed that this low increment was due to a unique digestion product after human serum exposure. The interesting point from this cleavage product was its extraordinary stability compared with the original peptide. In peptides 2, 3, and 4, this unknown sequence appeared to maintain a great amount of peptides after 2 hours of human serum treatment, but the original peptide was almost completely digested during this incubation time (Figure 2). The cleavage product corresponded to the entire peptide, with the cleavage of the first 2 aa at the N-terminal region (Table 3). This finding was later assessed using the Expasy protease peptide cutter predictor. When the activity was tested, peptides 2, 3, and 4 exhibited some loss of activity compared with peptide 1, with peptide 4 as the most active. This could be explained by the structures of the amino acids. Har is quite similar to Arg, having the cationic group of the side chain more available to perform the activity owing to the length of the side chain being greater than that in Orn and Dab. This could also be the key factor that explains the loss of activity with Dab, with the shorter side chain. However, as the cationic groups are more exposed in peptides 1 and 4, their toxicity values (hemolysis percentages of 44% and 43%, respectively, and

mammalian cell toxicity lower than 25 µM; Table 4) are far greater than those obtained for peptides 2 and 3 (hemolysis percentages of 10% and 14%, respectively, and MRC-5 cell cytotoxicity levels higher than 100 µM; Table 4). To perform the next modifications, the Orn-based peptide was chosen, as it showed better combined results in stability, activity, and toxicity. Two additional peptides carrying the extra modifications were selected. Peptide 6 was modified only with proline in D, whereas peptide 7 was modified with both proline and ornithine in D.

As predicted on the basis of previous results and simulations, the modifications in peptides 6 and 7 provided an impressive gain of human serum stability. Both peptides exhibited a half-life longer than 8 hours, but only the change in proline conformation could achieve such improvement. Apart from this, both peptides could keep their antimicrobial activities at the same levels as that of the original peptide, especially in the case of peptide 7 (Tables 4 and 5). When toxicity assays were used to test for these modifications, the results were even better than those obtained from previous modifications and the original peptide. This suggests that modifications with non-natural or synthetic residues could improve the therapeutic window of AMPs. In both cases, the improvement in toxicity value was approximately 10-fold that of peptide 1 but was only two to four times higher when the activity changed. This means that the therapeutic window in many cases for peptides 6 and 7 was approximately 100-fold, being much better than the range obtained for peptide 1.

The main flaw of changing the peptide sequence is the associated modification of the secondary structure. Loss of the native structure could jeopardize its viability for the next preclinical steps. Hence, some structural studies were performed to test whether the native organization of the peptide within the protein was maintained. Initially, circular dichroism was performed to obtain information from a general point of view. All peptides tested (peptides 1, 2, 6, and 7) displayed an unfolded structure in aqueous solution. However, when diluted in sodium dodecyl sulfate 1 mM, the four peptides reorganized their structures into α-helix-like structures (Supplementary Figure 6). Then, nuclear magnetic resonance studies were performed to obtain detailed information about the structure acquired by the original peptide and the most relevant modifications performed. In aqueous solution, the original double α-helix structure could only barely be detected (approximately 40% between residues 5 and 13, and 10% between residues 16 and 27). Similar to the findings observed in circular dichroism, the results found when incubating

peptides in DPC micelles exhibited a rearrangement of the peptide into a double α-helix. Both helixes recover their structures to at least 70% of their residues (Figures 4 and 5). However, a remarkable change in the structure was found after the modification. The angle of the loop that links both α-helices was wider in the modified peptides than in peptide 1 (Figure 5).

This project shows how the rational modification of residues prone to protease cleavage into non-proteogenic or synthetic residues is an extraordinary tool to improve peptide stability and bioavailability. Switching just one residue of the sequence to its D-isomer achieved more than 40-fold improvement in the half-life of the peptide in human serum. Moreover, the method used for testing the stability in serum may be better than those used in similar works that used serum from animal species or single protease tests[313,314]. Better understanding of the interactions of the peptides with the serum (Table S2) and how they contribute to the loss of activity of peptides is important. Further experiments may be needed to elucidate this.

Furthermore, the modifications maintained the antimicrobial activity and even reduced its associated toxicity. The improved features of hECP30 make it a better candidate for further studies. In fact, later studies performed *in vivo* with peptide 6 (and other candidates) showed interesting data[315]. Although the survival results obtained from the candidate identified in this work are not the best (survival ratio of approximately 20%), they are still better than the vehicle results. Moreover, it is important to notice the differences between the peptides used. The same dosage was used for all candidates, taking into consideration the most toxic (the all-D analog). The toxicity of this candidate was far higher than that of peptide 6. These results may lead us to think that the therapeutic window of peptide 6 is greater than that of all-D analog. Hence, activity assays could be performed with higher dosages, probably obtaining much better survival outcomes. Such assay should be performed to avoid underestimation of the capacity of the analogs synthetized in this study.

Overall, this work provides more evidence on the benefits of rational modification and supports the relevance of using this approach during drug discovery and the progress of the development of AMPs toward clinical stages.

CONCLUDING REMARKS

Chapter 1: Antimicrobial peptides can generate tolerance by lag and interfere with antimicrobial therapy.

- Bacteria reach a tolerant state against AMPs after several cycles of treatment.
- The number of cycles needed differ according to the type of peptide.
- The tolerance obtained is driven by an increase in lag time. In some cases, the growth rate and colony morphology can also be affected. These phenotypes are alike those observed in conventional antibiotic treatments.
- Morphology changes are associated with peptides that exhibit intracellular bacterial targets.
- Cross-tolerance between AMPs is unlikely to occur.
- AMP-evolved strains alter the effect of conventional antibiotics compared with untreated strains.
- The modifications are associated with AMPs with intracellular targets.
- Cross-tolerance or collateral sensitivity development is dependent on the mechanism of action of the drugs used.
- The mutations found in the evolved strains affect cell growth at replication, translation, and cell membrane levels.
- Conventional antibiotic- and AMP-tolerant strains share affected pathways.
- This kind of experiment should be addressed for the development of new therapies to prevent the emergence of undesired new resistant strains.

Chapter 2: Cryptic antimicrobial peptides in heparin-binding proteins exhibit selective antimicrobial activity against gram-negative bacteria through specific lipopolysaccharide binding.

- Of the heparin-binding peptides obtained from the library, 82% have cryptic AMPs in their sequence.
- Of the peptides discovered, 76% show heparin affinity in some grade.
- Of candidates with higher affinity, 78% had at least one possible CPC′ motif.
- Synthetized AMPs exhibited solid antibacterial activity, even at submicromolar levels.
- The activity was clearly displaced to gram-negative bacteria, being up to 10 levels more active against them.
- HBPs present great affinity to heparin, higher than other well-stablished AMPs do.
- HBPs exhibited low toxicity levels, displaying therapeutic windows (LC_{50}/MIC) of up to 100.
- In most cases, HBPs could rearrange the same secondary structure as that in native proteins.
- HBP-4 and HBP-5 exhibited similar rearrangements in the presence of heparin and LPS.
- HBP-5 had high activity levels and toxicity outcomes, making it a good candidate for further preclinical assays.
- Combining conventional AMP-predicting tools with structural homology approaches has been shown to be a great source of new antimicrobial agents, with high specificity.
- This technique could be extrapolated for the treatment of other kinds of bacteria or illnesses.

Chapter 3: Rationally modified antimicrobial peptides from the N-terminal domain of human RNase 3 show exceptional serum stability.

- Original residue substitution by non-natural or synthetic amino acids increases peptide half-life in human serum.
- Non-natural and synthetic residue incorporation can reduce by-side hemolysis and cytotoxicity in host cells.
- Ornithine residue displays a better result profile than homoarginine and α-aminobutyric acid.
- Modifications do not remarkably affect the antibacterial activity for both the reference and clinical bacterial strains.
- Toxicity is linked to charge availability in residue side chains.
- The rational peptide modification from the MS cleavage profile increased the half-life by up to 40-fold from that observed in hECP30.
- Single-residue modification into its D-isomer engages sharp stability improvement.
- Modified peptides maintain similar secondary structures as those obtained from native peptides.
- Peptides 6 and 7 are good candidates for further testing in preclinical stages.

APPENDIXES

Appendix 1: Articles published from thesis work (until now)

- Sandín D, Valle J, Chaves-Arquero B, Prats-Ejarque G, Larrosa MN, González-López JJ, Jiménez MÁ, Boix E, Andreu D, Torrent M. Rationally Modified Antimicrobial Peptides from the N-Terminal Domain of Human RNase 3 Show Exceptional Serum Stability. J Med Chem. 2021 Aug 12;64(15):11472-11482. doi: 10.1021/acs.jmedchem.1c00795.

- Sandín D, Valle J, Morata J, Andreu D, Torrent M. Antimicrobial Peptides Can Generate Tolerance by Lag and Interfere with Antimicrobial Therapy. *Pharmaceutics*. 2022; 14(10):2169. doi: 10.3390/pharmaceutics14102169.

ANNEXES

Annex 1: Supplementary information from Chapter 1

Annex 2: Supplementary information from Chapter 2

Supplementary tables:

Supplementary Table 1. Additional data for peptides HBP-1 to HBP-5.

Peptide	Sequence	Source Protein[a]	Protein GO Biological processes
HBP-1	RWHLTHRPKTGYIRVLVH	Thrombospondin-2 (P35442)	• Cell adhesion • Negative regulation of angiogenesis • Positive regulation of synapse assembly
HBP-2	RFYLSKKKWVMVP	Alpha-1-antichymotrypsin (ACT, P01011)	• Acute-phase response • Inflammatory response • Maintenance of gastrointestinal epithelium • Negative regulation of endopeptidase activity • Regulation of lipid metabolic process
HBP-3	FRFKRKLPKYLLF	Amiloride-sensitive amine oxidase (P19801)	• Amine metabolic process • Cellular response to azide • Cellular response to copper ion • Cellular response to heparin • Cellular response to histamine • Putrescine metabolic process • Response to antibiotic
HBP-4	GWKDKKSYRWFLQHRPQVGYIRVRFY	Cartilage oligomeric matrix protein (P49747)	• Animal organ morphogenesis • Animal organ senescence • Apoptotic process • Artery morphogenesis • BMP signaling pathway • Bone mineralization • Cartilage homeostasis • Chondrocyte development • Chondrocyte proliferation • Collagen fibril organization • Growth plate cartilage development • Limb development • Multicellular organism aging • Multicellular organism growth • Musculoskeletal movement • Negative regulation of apoptotic process • Negative regulation of hemostasis • Platelet aggregation • Positive regulation of chondrocyte proliferation • Protein homooligomerization • Protein processing • Protein secretion • Regulation of bone mineralization • Regulation of gene expression • Response to unfolded protein • Skeletal system development • Skin development • Tendon development

HBP-5	HNLFRKLTHRLFRRNFGYTLRSV	Heparin cofactor 2 (P05546)	• Vascular associated smooth muscle cell development • Vascular associated smooth muscle contraction • Blood coagulation • Chemotaxis • Negative regulation of endopeptidase activity

[a] UniProt database codes added in brackets.

Supplementary Table 2. MIC and MBC values (µM) of all peptides against gram-negative clinical isolates.

Peptide	***E. coli*** **CFT073**	***E. coli*** **1166795**	***P. aeruginosa*** **827651**	***P. aeruginosa*** **827632**	***A. baumannii*** **3878**	***A. baumannii*** **3880**
	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
HBP-1	3.1 / 3.1	1.6 / 1.6	25 / 50	25 / 25	1.6 / 1.6	12.5 / 12.5
HBP-2	50 / 100	12.5 / 12.5	>100 / >100	>100 / >100	50 / 50	>50 / >50
HBP-3	12.5 / 25	1.6 / 1.6	12.5 / 12.5	25 / 25	6.3 / 6.3	6.3 / 6.3
HBP-4	0.8 / 1.6	<0.1 / <0.1	3.1 / 6.3	6.3 / 6.3	0.8 / 0.8	1.6 / 1.6
HBP-5	0.4 / 0.8	<0.1 / <0.1	0.8 / 1.6	1.6 / 1.6	0.2 / 0.2	0.4 / 0.4

Supplementary Table 3. Hemolytic and cytotoxic activities of peptides.

Peptide	Hemolysis (%)[a]	LC_{50} (MRC-5 cells, µM)	LC_{50} (HepGS cells, µM)
HBP-1	4.6 ± 0.6	>200	>200
HBP-2	2.0 ± 1.5	>200	>200
HBP-3	4.0 ± 1.3	>200	>200
HBP-4	15.5 ± 0.1	35.4 ± 1.0	38.4 ± 13.4
HBP-5	10.3 ± 0.2	69.3 ± 12.3	79.7 ± 7.0
LL-37	32.71 ± 0.7	26.1 ± 3.1	53.2 ± 1.8

[a] Hemolysis data was assayed at a peptide concentration of 125 µM.

Supplementary Table 4. EC_{50} and $t_{1/2}$ values for HBP peptides and LL-37 (Figures 3B and 3C respectively).

Peptide	LPS Affinity EC_{50} (μM)	$DiSC_3(5)$ $t_{1/2}$ (s)
HBP-1	42.0 ± 16.0	38.4 ± 8.1
HBP-2	1492 ± 653	53.0 ± 11.6
HBP-3	6.8 ± 5.5	25.0 ± 4.7
HBP-4	0.7 ± 0.6	37.2 ± 3.1
HBP-5	0.9 ± 0.7	34.4 ± 2.2
LL-37	0.9 ± 0.8	57.6 ± 3.1

Supplementary Table 5. Calculated percentage of secondary structure of peptides by circular dichroism in 5 mM PB using CDSSTR in dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

Peptide	α-Helix	β-Strand	Turns	Unordered	Total
HBP-1	0.07	0.33	0.24	0.35	0.99
HBP-2	0.04	0.32	0.24	0.37	0.97
HBP-3	0.09	0.31	0.26	0.34	1
HBP-4	0.06	0.36	0.23	0.34	0.99
HBP-5	0.12	0.27	0.26	0.34	0.99

Supplementary Table 6. Calculated percentage of secondary structure of peptides by circular dichroism in 5 mM PB, 1 mM SDS using CDSSTR in dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

Peptide	α-Helix	β-Strand	Turns	Unordered	Total
HBP-1	0.07	0.39	0.23	0.32	1.01
HBP-2	0.10	0.38	0.23	0.30	1.01
HBP-3	0.11	0.35	0.25	0.30	1.01
HBP-4	0.05	0.38	0.23	0.33	0.99
HBP-5	0.50	0.24	0.15	0.19	1

Supplementary Table 7. Calculated percentage of secondary structure of peptides by circular dichroism in 5 mM PB, LMW heparin 20 μg/mL using CDSSTR in dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

Peptide	α-Helix	β-Strand	Turns	Unordered	Total
HBP-1	0.04	0.45	0.22	0.29	1
HBP-2	0.02	0.43	0.22	0.32	0.99
HBP-3	0.06	0.46	0.19	0.28	0.99
HBP-4	0.06	0.42	0.21	0.31	1
HBP-5	0.37	0.23	0.17	0.23	1

Supplementary Table 8. Calculated percentage of secondary structure of peptides by circular dichroism in 5 mM PB, LPS 50 μg/mL using CDSSTR in dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml).

Peptide	α-Helix	β-Strand	Turns	Unordered	Total
HBP-1	0.02	0.39	0.23	0.35	0.99
HBP-2	0.01	0.39	0.21	0.36	0.97
HBP-3	0.06	0.38	0.21	0.33	0.98
HBP-4	0.06	0.44	0.2	0.3	1
HBP-5	0.2	0.27	0.25	0.29	1.01

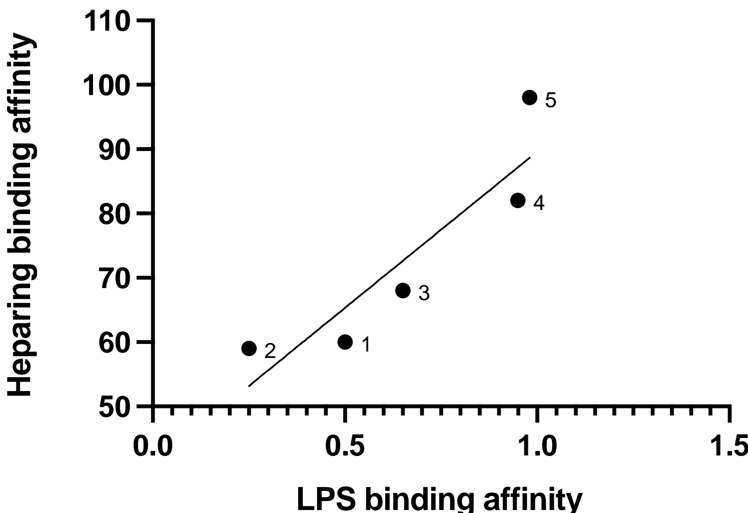
Supplementary Table S9. Averaged $\Delta\delta_{H\alpha}$ and $\Delta\delta_{C\alpha}$ values for **HBP-5** in aqueous solution at pH 5.0 and in DPC micelles (30 mM DPC) at pH 5.0 at 25ºC. Percentage of helical structure was estimated from these values. [a] Errors are reported as the standard deviation.

HBP-5						
Conditions	**Helical residues**	**$\Delta\delta_{H\alpha}$, ppm**	**% helix from $\Delta\delta_{H\alpha}$**	**$\Delta\delta_{C\alpha}$, ppm**	**% helix from $\Delta\delta_{C\alpha}$,**	**Averaged % helix[a]**
Aqueous solution	3-11	-0.039	10	+0.02	5	5±5
DPC micelles	3-11	-0.307	79	+2.95	96	85±9

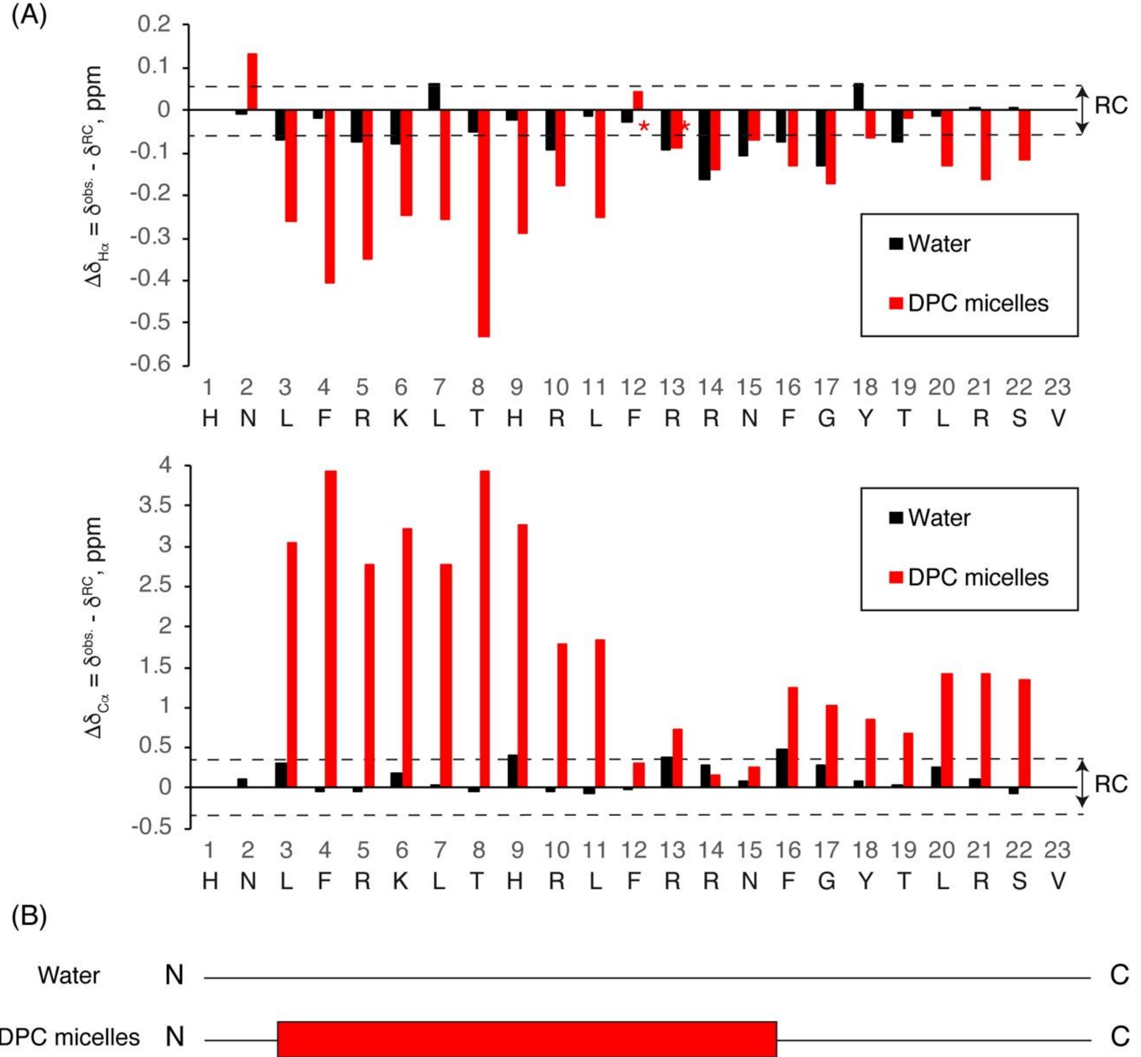
Supplementary Table S10. Summary of structural statistic parameters for the ensemble of the 20 lowest target function conformers calculated for **HBP-5** in DPC micelles.

HBP-5 in DPC micelles	
Number of distance restraints	
Intraresidue & sequential $(i - j \leq 1)$	198
Medium range $(1 < \lvert i - j \rvert < 5)$	37
Long range $(\lvert i - j \rvert \geq 5)$	3
Total number	238
Averaged total number per residue	10.3
Number of dihedral angle constraints	
Number of restricted ϕ angles	21
Number of restricted ψ angles	20
Total number	41
Pairwise RMSD (Å)	
All residues	**2-22**
Backbone atoms	1.6±0.7
All heavy atoms	2.6±0.7
N-terminal helix	**3-15**
Backbone atoms	0.3±0.2
All heavy atoms	1.6±0.3
Ramachandran plot (%)	
Most favoured regions	93.8
Additionally allowed regions	6.2
Generously allowed regions	0.0
Disallowed regions	0.0

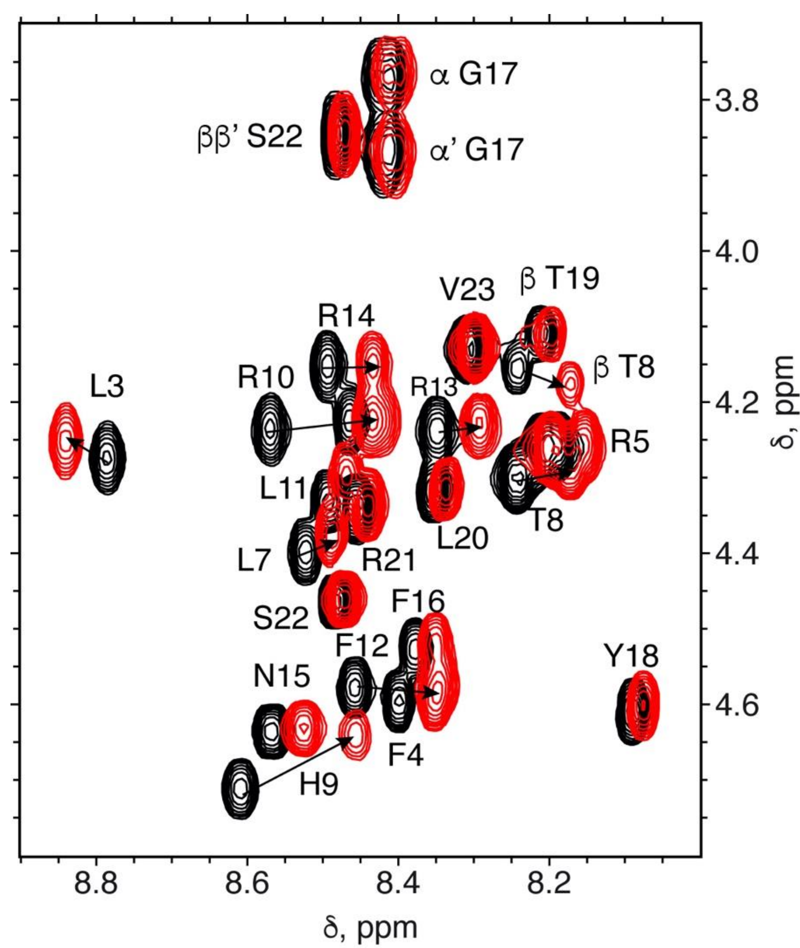
Supplementary figures:



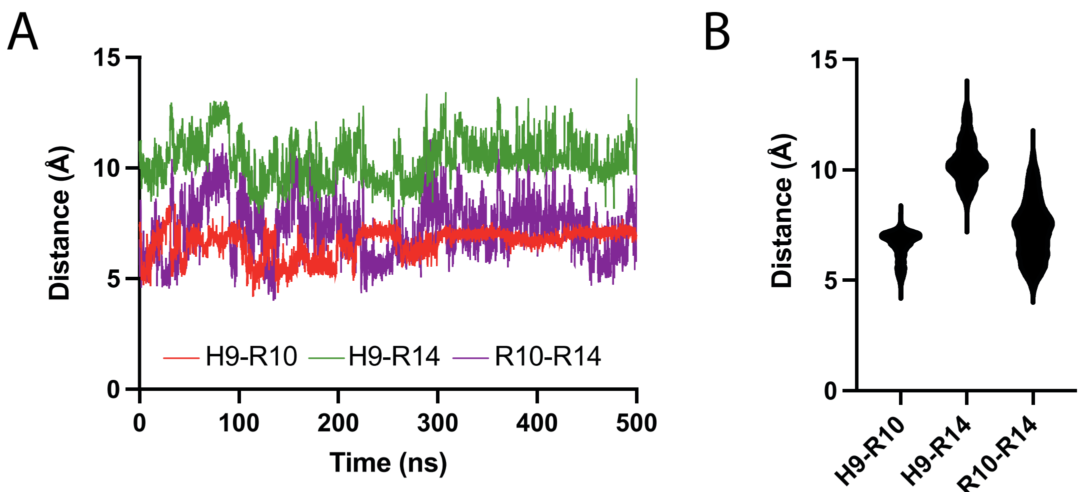
Supplementary Figure S1. Correlation of heparin-binding activity and LPS-binding activity. Heparin affinity is measured as the percentage of elution of the peptides in a heparin affinity column and LPS affinity is measured as EC_{50} values as calculated from the BODIPY-cadaverine assay.



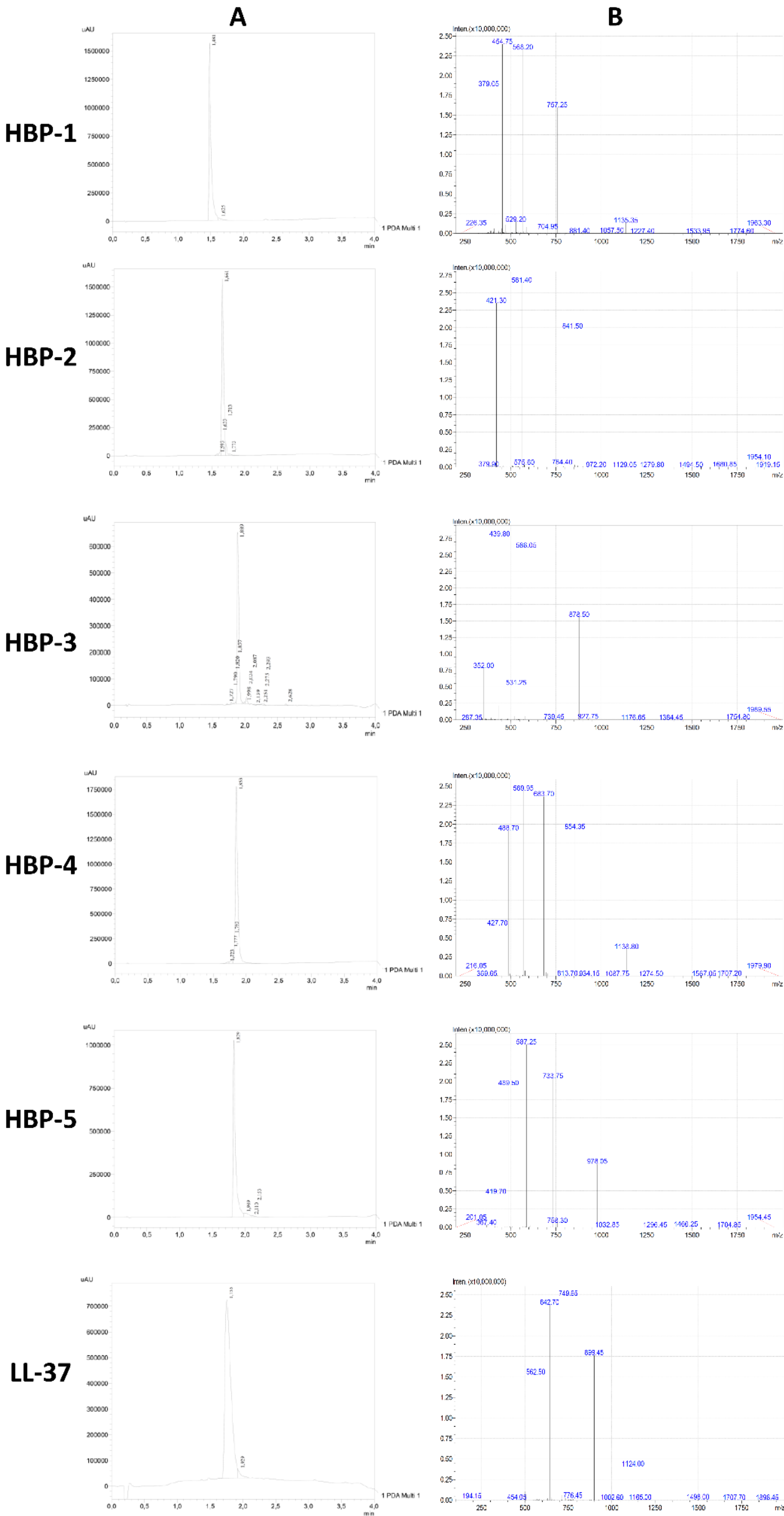
Supplementary Figure S2. **(A)** Dd_{Ha} and Dd_{Ca} conformational shifts for HBP_5 in aqueous solution (black bars) and in DPC micelles (red bars) at pH 5.5 and 25ºC plotted as a function of peptide sequence. The two dashed lines indicate the random coil range (RC). **(B)** Schematic representation of the structural features in aqueous solution and in DPC micelles. Helices are shown as red rectangles.



Supplementary Figure S3. Overlay of selected regions of 2D ^{1}H,^{1}H TOCSY spectra for free HBP-5 (black contours) and for HBP-5 in the presence of heparin disaccharide HIS at 1:1 ratio (red contours). In both cases, aqueous solution at pH 5.5 and 5ºC.



Supplementary Figure S4. Molecular dynamics simulation of HBP-5 in presence of Arixtra heparin analogue. (A) Distance between residues H9 and R10 (red), H9 and R14 (green) and R10 and R14 (purple) during the simulation. **(B)** Average distances for each residue pair over all the simulation.



Supplementary Figure 5. HPLC spectra (A) and MS spectra (B) of purified synthetic peptides.

- **Supplementary files** cited in chapter 2 may be available under request.

Annex 3: Supplementary information from Chapter 3

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