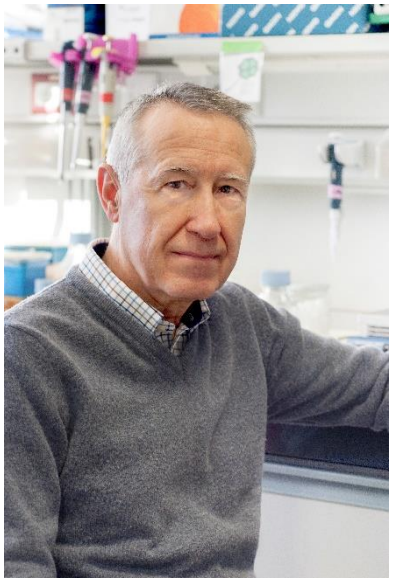

Pedro García González

Group at the Centro de Investigaciones Biológicas Margarita Salas (CSIC):

Protein engineering against antimicrobial resistance

Research line: *New enzybiotics against bacteria that cause respiratory diseases*

Description

Endolysins are phage-encoded enzymes whose function is to hydrolyze the peptidoglycan of the host bacterium at the end of the lytic cycle of the phage, allowing the dissemination of the phage progeny. In recent years, and to deal with many bacterial pathogens, the use of a wide variety of endolysins has been described which, after their recombinant expression and purification, are added exogenously to bacteria. In this therapeutic context, these specialized enzymes (enzybiotics) represent a great hope in their future clinical application due to their various advantages over antibiotics, namely: a) the majority have different levels of specificity (strain, species, genus ...) and, consequently, do not affect the usual microbiota; b) no bacteria resistant to these enzymes have been described so far, probably because their target (peptidoglycan) is a highly conserved essential structure among bacteria; c) for the same reason, they are equally effective against multi-resistant strains; d) they also display antimicrobial activity against bacteria that form biofilms which are generally refractory to the action of antibiotics; e) they are effective in all types of bacterial metabolic states; f) can be cheaper to produce than any antibiotic. The numerous articles that have been published in recent years provide an

additional argument of the interest that this strategy is awakening among the scientific and business community, and contribute to establishing these agents as a real alternative to fight against the great threat of multi-resistant pathogens to short term.

Previously, we constructed the most powerful chimeric enzymes to date against pneumococcus (Cpl-711 and PL3), which contain a catalytic domain (lysozyme or amidase) and a choline-dependent substrate-binding domain. This characteristic makes this type of enzyme strictly specific against pneumococcus. In addition, we have characterized other enzymes with a broader host range, such as Cpl-7S and Csl2. Likewise, in the case of Cpl-711 we have shown that this protein exhibits a synergistic action with certain antibiotics, as well as with another enzyme that breaks different bonds, such as PL3. More recently, a full design and development path towards obtaining phage lysin-based antimicrobials especially directed against Gram-negative pathogens have been carried out. This analysis supported a widespread appearance of antimicrobial peptide-like subdomains within a relevant subpopulation of lysins from Gram-negative bacteria infecting phages. In this way, an enzybiotic candidate, named Pae87, was selected for researching its intrinsic activity against Gram-negative pathogens, particularly *Pseudomonas aeruginosa*, and to serve as a scaffold for the developing of efficient antimicrobial molecules. In addition, a putative substrate-binding subdomain was identified within the very catalytic domain of the protein. Moreover, the ability of Pae87 to bind and disrupt the Gram-negative outer membrane was proven, and it was related to a specific C-terminal region termed peptide P87. This peptide had an antimicrobial activity of its own, comparable to that of the full protein. Furthermore, peptide P87 was enhanced by rational point mutation and thus the derived peptide P88 was obtained. This peptide P88 had a greater bactericidal efficiency against a similar range of Gram-negative bacteria but no dramatic increase in its cytotoxic effect against eukaryotic cells was found. Interestingly, the peptide displayed a potent *in vitro* synergy when used in combination with various antibiotics with intracellular targets (namely macrolides, chloramphenicol, and tetracycline). All these enzymes have been tested in different *in vitro* tests with planktonic bacterial cultures and in the form of biofilms, and the results are validated in different animal models of infection, such as mice or zebrafish.

On the other hand, we have developed a strategy for the *in vivo* enzybiotics behaviour enhancement. Since lysins *in vivo* half-life is known to be rather short (30-60 min), one of the currently explored approaches for enzybiotics administration is encapsulation and

controlled release. Based on the *Streptococcus pneumoniae* surface structure, which stands out for the presence of choline residues that serve as anchorage for physiologically relevant surface proteins, a chitosan-based polymer was designed. Such a polymer was grafted with diethylaminoethanol (DEAE) moieties, which can act as a structural and functional analogue of choline. The chitosan-DEAE nanoparticles were thus also able to bind choline-binding enzybiotics, such as the antipneumococcal Cpl-711, and release it in a controlled manner, although with some cytotoxic effect against eukaryotic cells.

Members of the group:

Roberto Vázquez Fernández (until July 2021)

Susana Ruíz García

Selected publications

Díez-Martínez, R., de Paz, H. D., Bustamante, N., García, E., Menéndez, M. y García, P. 2013. Improved lethal effect of Cpl-7, a pneumococcal phage lysozyme of broad bactericidal activity by inverting net charge of its cell wall-binding module. Antimicrob. Agents Chemother. **57:**5355-5365.

Díez-Martínez, R., de Paz, H. D., García-Fernández, E., Bustamante, N., Euler, C. W., Fischetti, V. A., Menéndez, M. y García, P. 2015. A novel chimeric phage lysin with high in vitro and in vivo bactericidal activity against *Streptococcus pneumoniae*. J. Antimicrob. Chemother. **70:**1763-1773. doi: 10.1093/jac/dkv038.

Retamosa, M. G., Díez-Martínez, R., Maestro, B., García-Fernández, E., de Waal, B., Meijer, E. W., García, P. y Sanz, J. M. 2015. Aromatic esters of bicyclic amines: a new generation of antimicrobials against *Streptococcus pneumoniae*. Angew. Chem. Int. Ed. **54:**13673-7. doi: 10.1002/anie.201505700.

Díez-Martínez, R., García-Fernández, E., Manzano, M., Martínez, A., Domenech, M., Vallet-Regí, M. y García, P. 2016. Auranofin-loaded nanoparticles as a new

therapeutic tool to fight streptococcal infections. Sci. Rep. **6:**19525. doi: 10.1038/srep19525.

Blázquez, B., Fresco-Taboada, A., Iglesias-Bexiga, M., Menéndez, M. y García, P. 2016. PL3 amidase, a tailor-made lysin constructed by domain shuffling with potent killing activity against pneumococci and related species. Front. Microbiol. **7:**1156. doi: 10.3389/fmicb.2016.01156.

Vázquez, R., Domenech, M., Iglesias-Bexiga, M., Menéndez, M. y García, P. 2017. Csl2, a novel chimeric bacteriophage lysin to fight infections caused by *Streptococcus suis*, an emerging zoonotic pathogen. Sci. Rep. **7**:16506. doi: 10.1038/s41598-017-16736-0.

Vázquez, R., García, E. y García, P. 2018. Phage lysins for fighting bacterial respiratory infections: a new generation of antimicrobials. Front. Immunol. **9:**2252. doi:10.3389/fimmu.2018.02252.

Vázquez, R. y García, P. 2019. Synergy between two chimeric lysins to kill *Streptococcus pneumoniae*. Front. Microbiol. 10:1251. doi.org/10.3389/fmicb.2019.01251.

Vázquez, R., García, E. y García, P. 2021. Sequence-function relationships in phage-encoded bacterial cell wall lytic enzymes and their implications for phage-derived products design. J. Virol. 95:e00321-21. https://doi.org/10.1128/JVI.00321-21.

Vázquez, R., Blanco-Gañán, S., Ruíz, S. y García, P. 2021. Mining of Gram-negative surface-active enzybiotic candidates by sequence-based calculation of physicochemical properties. Front. Microbiol. 12:660403. doi:10.3389/fmicb.2021.660403.

Vázquez, R., Caro-León, F. J., Nakal, A., Ruiz, S., Doñoro, C., García, L., Vázquez-Lasa, B., San Román, J., Sanz, J., García, P. y Aguilar, M. R. 2021. DEAE-chitosan nanoparticles as a pneumococcus-biomimetic material for the development of antipneumococcal therapeutics. Carbohydr. Pol. https://doi.org/10.1016/j.carbpol.2021.118605.

Funding

· CIBER de Enfermedades Respiratorias (CIBERES) (2006-)

· Enzibióticos contra bacterias patógenas respiratorias formadoras de biofilm. Entidad financiadora: Agencia Estatal de Investigación. Referencia: SAF2017-88664-R. (2018-2021). Inv. Principal: Pedro García.

· Ultraestructura y alcance biotecnológico de los módulos de unión a colina: una aproximación de biología sintética (SYNTHECHOL). Entidad financiadora: Agencia Estatal de Investigación. Referencia: PID2019-105126RB-I00. (01/06/2020 - 31/05/2023). Inv. Principal: Jesús M. Sanz.

Recent Doctoral Thesis:

- **Roberto Díez Martínez**: Estudios estructurales y funcionales de la lisozima Cpl-7 del bacteriófago Cp-7 de neumococo. Universidad Complutense de Madrid. Facultad de Ciencias Biológicas. 2014. Sobresaliente *cum laude*.

- **Roberto Vázquez Fernández**: New strategies for the design and development of protein antimicrobials based on phage products. Universidad Complutense de Madrid. Facultad de Ciencias Biológicas. 2021. Sobresaliente *cum laude*.

Link web page:

https://www.cib.csic.es/research/microbial-plant-biotechnology/protein-engineering-against-antimicrobial-resistance