Hitchhiking with Nature: exploring snake venom peptides to fight cancer and superbugs

Clara Pérez-Peinado

TESI DOCTORAL UPF / year 2019

THESIS SUPERVISOR
David Andreu
Department of Experimental and Health Sciences
Universitat Pompeu Fabra

A mis padres y mi hermana.

Acknowledgements

Dice Albert Espinosa en uno de sus libros: "*... Cada año de mi vida he buscado doce perlas. Doce personas que no conociera pero que se me aparecieran y marcaran mi mundo de tal manera que yo virara. Son joyas que el mundo te da y que, aunque pasen los años aún conservas. El tiempo no les quita nada de su brillo ni de su intensidad. Además de las perlas, a lo largo de tu vida, encontrarás personas que son más que perlas. Son energías especiales que debes encontrar, que se funden con la tuya. Con el tiempo, algunas perlas pasan a ser diamantes...*".

Lo cierto es que he sido muy afortunada en este camino. He contado con la ayuda y el apoyo incondicional de las perlas y diamantes que ya atesoraba, y he encontrado muchas otras nuevas! Es a ellos, a quien quiero agradecer y dedicar este trabajo:

En primer lugar, a mi supervisor y guía, David Andreu. Esta tesis no hubiera sido posible sin ti. Gracias por la oportunidad, la confianza, la libertad, el apoyo y las enseñanzas de todo tipo, no sólo científicas. Gracias por ser mi mentor y un referente a seguir.

Esta tesis tampoco hubiera sido posible sin la colaboración del grupo de Miguel Castanho y Gandhi Rádis-Baptista. Gracias en especial a Claudio, quien me enseñó mis primeras lecciones en el laboratorio. Y también a Salomé, que me recibió con los brazos abiertos en Portugal y guió mi trabajo. Con especial cariño, gracias a los dos diamantes que encontré en Portugal, Susana y Sara, por acogerme como una compañera y amiga, pero sobre todo, gracias por quedaros.

A mis compañeras/os en este viaje: estas páginas se quedarían cortas para agradecer el apoyo y la amistad a los tres grandes diamantes que he encontrado en este laboratorio: María, Sira y Mar. Habéis sido compañeras de todo; laboratorio, deporte, salidas, batallas espartanas, risas y algún que otro lloro... gracias por ser compañeras de vida y la familia que se escoge! Un especial agradecimiento también a Javi, nuestro capitán eterno; gracias por las enseñanzas y la ayuda, pero sobre todo, por tu eterna paciencia! También me gustaría agradecer a todos aquellos con quien he compartido bonitos momentos

de laboratorio; Yolanda, Beatriz, Carolina, Gerard, Ferran, Maribel, Zhoreh, Dani... Muchísimas gracias por compartir ciencia, experiencias, buenos momentos y algún que otro partido de vóley. No podría olvidarme tampoco de mis biólogas favoritas, las Ramonas! Gracias por todos esos momentos que nos han llevado hasta aquí.

Gracias también a mi último diamante en bruto... João. Tampoco tendría suficientes líneas para agradecerte la ayuda, el apoyo y las enseñanzas. Has sido partícipe de este proyecto de formas muy diversas, con buenos consejos, ideas, correcciones, muchas conversaciones...Gracias por todo ello. Aunque mi agradecimento va mucho más allá: gracias también por "remover" mi mundo. Ah! Y sí; #whateverworks... and it actually worked.

Hay tres personas a las que el agradecimiento por el apoyo a este trabajo debería ser extensible a toda una vida, mis tres diamantes de sangre; mis padres Eva y Jorge, y mi hermana Ana. Sois el motor que va siempre en mí, y es por vuestro apoyo incondicional que mis metas se hacen realidad. Así que un enorme GRACIAS para vosotros siempre va a quedarse corto! También a Naia, nuestro pequeñito diamante, que sólo con su sonrisa y su "Hola tita!" es capaz de llenarme de felicidad... Y a Fernando; muy a tu pesar, ya eres uno más de la familia Addams!

Finalmente, me gustaría agradecer el apoyo y la confianza depositada en mi trabajo a diversas fundaciones e instituciones que me han facilitado el camino: al Departament de Ciéncies Experimentals i de la Salut (DCEXS-UPF), a las unidades de Citometría, Microscopía y Proteómica del PRBB, a la Sociedad Española de Química Terapéutica (SEQT), a la Sociedad Española de Bioquímica y Biología Molecular (SEBMM), a la Sociedad Europea de Péptidos (EPS) y la Unión Europea.

Clara Pérez-Peinado

Barcelona, 2019

Abstract

This work describes the discovery and optimization of efficient, selective, cost-effective and proteolytic-resistant antimicrobial and antitumoral peptides. To this end, we first identified novel cathelicidin-related peptides from the venom gland of South American pit vipers, collectively named vipericidins.

In particular crotalicidin (Ctn), a 34-mer helical peptide from the *Crotalus durissus terrificus* venom, highlighted due to its promising antimicrobial and antitumoral properties. Its fragment Ctn[15-34] also stood out due to its overall preserved activity, improved selectivity, serum stability and significant size reduction.

Their mechanisms of action were stepwise characterized against *Escherichia coli* and a leukemic cell line. The unusually high lifespan of Ctn[15-34] in serum was also investigated to ascertain its structural determinants and molecular interaction with serum proteins.

Altogether, this thesis proposes two novel bioactive peptides as potential drug candidates to fight bacterial infections and cancer, particularly leukemia. Beyond specific results, this thesis provides a set of valuable methodologies to discover, optimize and evaluate bioactive molecules from natural sources.

Keywords: antimicrobial peptides, anticancer peptides, peptide-membrane interaction, peptide optimization, peptide degradation.

Resumen

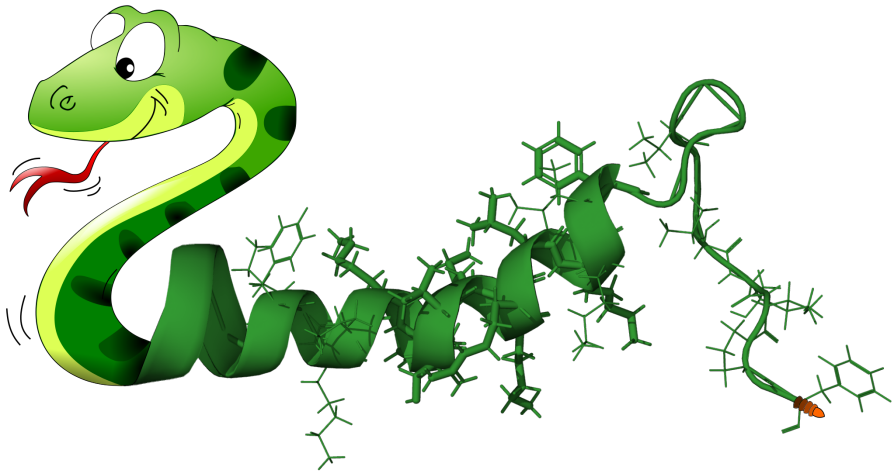
Esta tesis aborda el descubrimiento y optimización de péptidos antimicrobianos y antitumorales eficientes, selectivos, rentables y resistentes a la proteólisis. Con este fin, inicialmente identificamos nuevas catelicidinas en la glándula venenosa de diferentes víboras de América del Sur, denominadas vipericidinas.

En particular la crotalicidina (Ctn), un péptido helicoidal de 34 residuos aislado del veneno de la serpiente *Crotalus durissus terrificus* destacó por sus propiedades antimicrobianas y antitumorales, así como su fragmento Ctn[15-34], que mostró una selectividad y estabilidad en suero mejoradas y una reducción de tamaño considerable.

Los mecanismos de acción de ambos péptidos contra *Escherichia coli* y una línea celular de leucemia fueron investigados. También se investigó en profundidad la inusual estabilidad en suero de Ctn[15-34], con el fin de conocer sus determinantes estructurales y las posibles interacciones con proteínas del suero.

En conjunto, esta tesis propone dos nuevos péptidos bioactivos como posibles candidatos para combatir infecciones bacterianas y la leucemia, además de proporcionar un conjunto de metodologías útiles en el descubrimiento, optimización y caracterización de moléculas bioactivas a partir de fuentes naturales.

Palabras clave: péptidos antimicrobianos, péptidos antitumorales, interacciones péptido-membrana, optimización de péptidos, proteólisis.



Preface

Snakes are arguably among if not the most despised creatures in the entire animal kingdom. With some exceptions to the contrary (serpents protected and revered, even worshipped, in ancient and –a few– contemporary societies), for the vast majority of people the notion of a creature furtively slithering underfoot, with the added threat of a deadly, fast-acting poisonous bite, epitomizes harm, evil and treacherousness. Some examples have been amply recorded in ancient tradition (e.g., the serpent of Eden, Cleopatra's viper-assisted suicide), classic literature (e.g., Poe's *The cask of amontillado*) or colloquial language ("he's a snake in the grass").

Alongside this generalized perception of snakes incarnating terror and deceit, the ancient view of the serpent as a dual archetype of good and evil (e.g., the rod of Asclepius, Hindu mythology) has not only survived but, paradoxically, become increasingly supported by recent evidence showing that, when properly used, snake venoms can actually help saving lives rather than ending them.

Snake venoms are cocktails of toxins refined over millions of years of evolution, often encoding a variety of powerful biological effects in a single component. One is therefore entitled to consider snake venoms as rich libraries of bioactive molecules awaiting discovery to treat human disorders. A pioneer example in

this direction is the development of Captopril® from a peptide sequence in the venom of the Brazilian pit viper *Bothrops jararaca*, and the subsequent outpouring of a plethora of ACE inhibitors that have had a significant impact in hypertension control since the 80s. So imagine for a moment, the possibility of exploiting many similarly powerful bioactive components in other snake venoms, fine-tuning them and reshaping them into drugs to treat CNS diseases such as Alzheimer or Parkinson, cancer or infectious diseases. This is not a wishful or inauspicious quest, as many of these scenarios are nowadays actively explored by both public research groups and pharma companies worldwide.

An obvious question is, for starters, developing snake venom-based drugs to treat exactly what? Among the vast therapeutic challenges of the 21st century some focus is required and two main areas come to mind, if nothing else by their broad public perception, thus frequent appearance in media headlines: superbugs and cancer. Firstly, we are frightfully close to running out of effective antibiotics in critical situations, due to factors such as over prescription, non-compliance, etc. that are fueling the emergence of superbugs. Secondly, new cancer therapies are urgently needed to complement and/or replace current chemotherapeutic drugs and their often undesirable side effects.

The fundamental goal of the present work is therefore to investigate snake venoms in search for novel antimicrobial and anticancer agents. This idea is of course far from revolutionary, as the quest for therapeutic leads from Nature is a strategy widely exploited by pharmaceutical industry over years (see Introduction). A more distinctive element of the present thesis, however, is our focus on those required properties to a bioactive compound turn into a commercial drug, beyond the therapeutic activity.

From the beginning of this project, we were tremendously aware of the vast number (more than 3000) of peptides that are already known to fight bacteria or tumor cells. Unfortunately, most of them do not have any chance to turn into a drug due to selectivity, immunogenicity, bioavailability or costs issues. Thus, we sought to find a right molecule, our initial lead, and optimize it into a drug, overcoming such drawbacks.

Drug discovery and development process is a project for many years and it is beyond the scope of a PhD thesis, but we kept this idea in mind along this work by building a toolbox for bioactive drug lead discovery and refinement. We considered, from the very beginning, additional but also compulsory properties

for a drug-lead evolving into a commercial drug, such as selectivity, synthesis yield, toxicity, costs and proteolytic degradation (main bioavailability drawback of peptides).

Taken into account these ideas, the reader will easily understand the following thesis outline:

Introductory **chapter 1** is divided into three sections: i) background for the current health problems this thesis deals with (i.e., superbugs and cancer) and justification of the urgent need for new drugs in both fields; ii) description of antimicrobial and antitumoral peptides as promising alternatives in the search for novel anti-infective and cytotoxic drugs; iii) use of snake venoms as a general drug discovery arsenal, with antimicrobial and antitumoral peptides in particular.

Each of the subsequent chapters includes research papers (either published or to be submitted shortly), preceded by a specific introductory section describing key concepts necessary for better understanding the scope and the contents of each publication.

Chapter 2 (article I) constitutes the initial publication that set this project in motion, describing the discovery of a new family of antimicrobial peptides, named **viperidicins**, from different South American snake venoms. I joined the project during my Master thesis at Prof. David Andreu's lab, where I had the opportunity to work with Dr. Claudio Borges Falcão, a visiting post-doctoral researcher from Dr. Gandhi Rádis-Baptista's lab at the Federal University of Ceará, Brazil. The combined expertise of both groups in peptide synthesis and venomics, respectively, fostered the successful discovery of a novel family of pit viper-derived antimicrobial peptides, validated by both genetic studies and synthetic replicas.

Among the various vipericidins unveiled in our initial study, the most promising one, named **crotalicidin (Ctn)**, was selected for further studies and optimization. Accordingly, **Chapter 3 (article II)** describes the structural studies of Ctn, its unforeseen but exciting additional anticancer activity and, especially, its rational dissection into two fragments, one of which, named **Ctn[15-34]**, showed enhanced antibacterial selectivity and remarkably higher stability in human serum than the parental peptide. This work must be regarded as a highly successful example of structural optimization in peptide medicinal chemistry, in line with one of the goals of Prof. Andreu's research, namely designing

streamlined, minimalist synthetic versions of therapeutically promising natural peptides.

With two promising candidates, Ctn and Ctn[15-34], at hand, we set out to study their modes of action against bacteria and tumor cells. Thus, **Chapter 4 (articles III and IV)** describes mechanistic studies underlying their antimicrobial and antitumoral profiles. During this part of my thesis I had the opportunity to share my work and learn from Dr. João Miguel Freire, at the time a PhD student visiting our group from Prof. Miguel Castanho's lab at the Institute of Molecular Medicine (IMM), University of Lisbon. João mentored me into the world of flow cytometry and microscopy that allowed me to study the spatial-temporal events occurring during peptide interaction with the tumor cell membrane. In this context, I had the opportunity of a one-year secondment at the laboratory of Prof. Castanho in the frame of a Marie Sklodowska-Curie Action, with the goal of acquiring expertise in the use of biophysical techniques to study membrane-active peptides under the supervision of Dr. Ana Salomé Veiga and with the help of Susana A. Dias. About the same time Prof. Andreu spent a half-sabbatical at the laboratory of Prof. David Craik, Institute of Molecular Biosciences (IMB), University of Queensland, Australia, a worldwide reference in the study of bioactive peptides from natural sources, particularly plants and animal (spider, snake) venoms. This visit fostered an ongoing, fruitful collaboration between IMM, IMB and our UPF laboratory, joining efforts to characterize the bactericidal mechanism of both Ctn and Ctn[15-34]. Still along these lines, **Chapter 4** also includes a paper (under evaluation) in which the biophysical and biochemical studies of the antitumoral mechanism are complemented by proteomic studies where several putative intracellular targets in the antitumoral strike are identified.

In parallel with the above studies on mechanism of action, we also focused attention on the proteolytic fate of both Ctn and Ctn[15-34] peptides. This reflects another long-standing committment of Prof. Andreu's laboratory, namely optimizing bioactive peptide leads into entities with realistic drug potential, which almost always implies dealing with the proteolytic limitations of peptide therapeutics. Prof. Andreu succeeded in instilling in me the relevance of this medicinal chemistry principle, and making me aware of the lack of universal, standardized assays to evaluate peptide drug proteolysis, so together we undertook the challenge of devising a plan to evaluate the structural determinants and interactions governing the lifespan of peptides

(in particular, Ctn[15-34]) in protease-rich fluids such as human serum. Thus, **Chapter 5 (articles V and VI)** contains two articles, one aimed at defining the structural determinants that confer Ctn[15-34] its unusual residence time in serum, again in collaboration with the IMM Lisbon group, and a second paper exploring Ctn[15-34] interactions with serum components by means of proteomic analysis, completed with a validation of putative interaction with serum carriers, helped by Dr. Sira Defaus of our group.

Finally, **Chapter 6** combines the achievements with conclusions and outlook on future work. As quite often the case in scientific endeavors, my (in hindsight) evidently overambitious goal of generating one (or more!) therapeutic leads from a fascinating natural source became too tall an order for the time constraints of a PhD project. On the other hand, the thrill of that initial discovery has remained alive, and I trust that the following pages will allow the reader to share in the excitement of that quest, to understand what I learned during the journey, and to realize how fortunate I consider myself for having had the opportunity to pursue it.

Objectives

The main goal of this thesis consists on exploring **snake venom components for the discovery and development of new antimicrobial and anti-tumoral peptide-based therapeutic agents.** Aware of the challenges to successfully leading peptides into the market, we concentrated our efforts on the following specific sub-objectives:

- **Devising cost-effective, optimized synthetic replicas of the initial snake venom-derived lead.** Rational optimization/dissection steps of the primary lead will be performed in order to define the minimal structural determinants (i.e., pharmacophore) of biological activity.

- **Evaluating and minimizing the potential toxicity of the proposed peptide leads.** Selectivity of the peptide candidates against bacterial/tumor cells as a prerequisite to further development and consideration as final candidates.

- **Studying the metabolic processing of therapeutic peptide candidates.** In order to optimize the proteolytic degradation of peptide candidates, their behavior on serum will be evaluated in depth.

- **Studying the mechanism of action of the final candidates against bacteria and tumor cells.**

List of Publications

Full papers involving work of this thesis[I]

I Falcao CB, de la Torre BG, **Pérez-Peinado C**, Barron AE, Andreu D, Radis-Baptista G.
Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers.
Amino Acids. (**2014**);46(11):2561-71. DOI: 10.1007/s00726-014-1801-4.

II Falcao CB, **Pérez-Peinado C**, de la Torre BG, Mayol X, Zamora-Carreras H, Jimenez MA, Radis-Baptista G, Andreu D.
Structural dissection of crotalicidin, a rattlesnake venom cathelicidin, retrieves a fragment with antimicrobial and antitumor activity.
J Med Chem. (**2015**);58;(21):8553-63. DOI: 10.1021/acs.jmedchem.5b01142.

III **Pérez-Peinado C**, Dias SA, Domingues MM, Benfield AH, Freire JM, Radis-Baptista G, Gaspar D, Castanho MARB, Craik DJ, Henriques ST, Veiga AS, Andreu D.
Mechanisms of bacterial membrane permeabilization by crotalicidin (Ctn) and its fragment Ctn(15-34), antimicrobial peptides from rattlesnake venom.
J Bio Chem. (**2018**);293(5):1536-49. DOI: 10.1074/jbc.RA117.000125.

IV **Pérez-Peinado C**, Freire JM, Valle J, Andreu D.
Selective tumor cell attack by crotalicidin (Ctn) and its fragment Ctn[15-34]: insights into their dual membranolytic and intracellular targeting mechanism.
(**To be submitted**)

V **Pérez-Peinado C**, Dias SA, Mendonça DA, Castanho MARB, Veiga AS, Andreu D.
Structural determinants conferring unusual long life in human serum to rattlesnake-derived antimicrobial peptide Ctn[15-34].
J Pept Sci. (**2019**);25(8):e3195. DOI: 10.1002/psc.3195.

VI **Pérez-Peinado C**, Defaus S, Sans-Comerma L, Valle J, Andreu D.
Decoding the human serum interactome of snake-derived antimicrobial

[i] Supplementary movies and excel tables included in articles III, IV and VI are available on the CD attached to this thesis.

peptide Ctn[15-34]: Toward an explanation for unusually long half-life. J Proteomics. (**2019**);204:103372. DOI: 10.1016/j.jprot.2019.04.022.

Communications involving work of this thesis

I **Pérez-Peinado C**, Falcão CB, de la Torre BG, Jiménez MA, Radis-Baptista G, Andreu D. (2015, June).
Structure-activity studies of crotalicidin and analogs, antimicrobial and antitumoral peptides from rattlesnake venom.
Oral communication presented at the II Simposio de Jóvenes Investigadores de la Sociedad Española de Química Terapéutica, Madrid, Spain.

II **Pérez-Peinado C**, Falcão CB, de la Torre BG, Mayol X, Zamora-Carreras H, Jiménez MA, Radis-Baptista G, Andreu D. (2016, February).
Structure-activity studies of crotalicidin and analogs, antimicrobial and antitumoral peptides from rattlesnake venom.
Oral communication presented at the 15$^{\text{th}}$ Iberian Peptide Meeting. 2016, Porto, Portugal.

III **Pérez-Peinado C**, Falcão CB, de la Torre BG, Mayol X, Zamora-Carreras H, Jiménez MA, Radis-Baptista G, Andreu D. (2016, September).
Rational dissection of the snake-derived peptide crotalicidin retrieves a fragment with enhanced antimicrobial and antitumoral properties.
Poster presented at the 4$^{\text{th}}$ European Peptide Symposium and the 8$^{\text{th}}$ International Peptide Symposium, Leipzig, Germany.

IV **Pérez-Peinado C**, Dias SA, Domingues MM, Benfield AH, Freire JM, Radis-Baptista G, Gaspar D, Castanho MARB, Craik DJ, Henriques ST, Veiga AS, Andreu D. (2018, February).
Mechanisms of bacterial membrane permeabilization of snake venom-derived peptides crotalicidin (Ctn) and Ctn[15-34].
Oral communication and poster presented at the 16$^{\text{th}}$ Iberian Peptide Meeting and 4$^{\text{th}}$ ChemBio Group Meeting, Barcelona, Spain.

V **Pérez-Peinado C**, Dias SA, Domingues MM, Benfield AH, Freire JM, Radis-Baptista G, Gaspar D, Castanho MARB, Craik DJ, Henriques ST, Veiga AS, Andreu D. (2018, June).
Mechanisms of bacterial membrane permeabilization of snake venom-derived peptides crotalicidin (Ctn) and Ctn[15-34].

Oral communication at the 16th Naples Workshop on Bioactive Peptides, Naples, Italy.

VI **Pérez-Peinado C**, Dias SA, Domingues MM, Benfield AH, Freire JM, Radis-Baptista G, Gaspar D, Castanho MARB, Craik DJ, Henriques ST, Veiga AS, Andreu D. (2018, August).
Mechanisms of bacterial membrane permeabilization of snake venom-derived peptides crotalicidin (Ctn) and Ctn[15-34].
Oral communication at the 35th European Peptide Symposium, Dublin, Ireland.

Other related publications and communications not included in this thesis:

I Dias SA, Freire JM, **Pérez-Peinado C**, Domingues MM, Gaspar D, Vale N, Gomez P, Andreu D, Henriques ST, Castanho MARB, Veiga AS.
New Potent Membrane-Targeting Antibacterial Peptides from Viral Capsid Proteins.
Front Microbiol. (**2017**);8:775. DOI: 10.3389/fmicb.2017.00775.
(Appendix I)

II Zamora H, **Pérez-Peinado C**, de la Torre BG, Andreu D and Jiménez MA. (2014, June).
NMR insights into the structural basis for antimicrobial activity of a snake cathelicidin peptide.
Oral communication at the XIV Congress of the Spanish Biophysical Society, Alcalá de Henares, Spain.

III Belo R, Gomes J, Vaz S, **Perez-Peinado C**, Valle J, Andreu D, Sebastião A, Neves V, Castanho MARB, Diógenes MJ. (2017, February).
Elucidating the effects of kyotrophin on the brain.
Oral communication at the XLVII Reunião Anual da Sociedade Portuguesa de Farmacologia, Coimbra, Portugal.

IV Freire JM, Dias SA, **Pérez-Peinado C**, Domingues MM, Gaspar D, Andreu D, Henriques ST, Castanho M, Veiga AS. (2017, June).
Exploring viral proteins for membrane-active peptides.
Poster presented at the 25th American Peptide Symposium, Whistler, Canada.

V Dias SA, Freire JM, **Pérez-Peinado C**, Domingues MM, Gaspar D, Vale N, Gomes P, Andreu D, Henriques ST, Castanho MARB, Veiga AS. (2018, February).
Novel membrane-targeting antibacterial peptides derived from viral capsid proteins.
Oral communication presented at the 16th Iberian Peptide Meeting and 4th ChemBio Group Meeting, Barcelona, Spain.

VI Cavaco M, Neves-Coelho, S, **Pérez-Peinado C**, Gaspar D, Andreu D, Castanho MARB, Neves, V. (2018, February).
The Effect of Fluorophores on Peptide Properties.
Poster presented at the 16th Iberian Peptide Meeting and 4th ChemBio Group Meeting, Barcelona, Spain.

VII Cavaco M, **Pérez-Peinado C**, Gaspar D, Andreu D, Castanho MARB, Neves, V. (2019, July).
Targeting brain metastases with a peptide conjugate.
Oral communication presented at the 19th FEBS Young Scientists' Forum, Krakow, Poland.

Glossary

Abbreviations & Acronyms

aa - amino acid
ACE - angiotensin-converting enzyme
ACN - acetonitrile
ACP - anticancer peptide
AFM - atomic force microscopy
AFM - afamin
AGC - auto gain control
Ahx - aminohexanoic acid
AMP - antimicrobial peptide
AMR - antimicrobial resistance
ANS - 1-anilino-8-naphthalenesulfonate
AP - affinity purification
AP-MS - affinity purification followed by mass spectrometry
ApoL1 - apolipoprotein L1
ApoM - apolipoprotein M
ATCC - American Type Culture Collection
ATP - adenosine triphosphate
b- - biotin
BF - Bodi Fluor^{TM} 488 acid
BFDR - bayesian false discovery rate
BP - band pass
CAP - cationic antimicrobial peptide
CATH - cathelicidin
cathelin - cathepsin L inhibitor
CBG - corticosteroid-binding globulin
CD - circular dichroism
cDNA - complementary DNA
CF - 5(6)-carboxyfluorescein
Chol - cholesterol
CI - clinical isolate
CL - cardiolipin
CLSI - Clinical and Laboratory Standards Institute
COSY - correlation spectroscopy
CPP - cell-penetrating peptide
CPZ - chlorpromazine
CRAMP - cathelicidin-related antimicrobial peptide
Ctn - crotalicidin
DBP - vitamin D-binding protein
DCM - dichlorometane
DDA - data-dependent acquisition
DIEA - N,N-diisopropylethylamine
DIPCI - N N'-diisopropylcarbodiimide
DLS - dynamic light scattering
DMEM - Dulbecco's modified Eagle medium
DMF - N,N-dimetilformamide
DMPG - 1,2-dimyristoyl-snglycero-3-phospho-rac-(1-glycerol)
DNA - deoxyribonucleic acid
DPC - dodecylphosphocholine
DPC-d38 - [$^{2}H_{38}$]dodecylphosphocholine
DTT - dithiothreitol
EC_{50} - half-effective concentration
EDTA - ethylenediaminetetraacetic acid
EIPA - ethyl-isopropyl amiloride
EMA - European Medicines Agency
FA - formic acid
FACS - fluorescence-activated cell sorting
FBS - fetal bovine serum
FCA - flow cytometry assay
FDA - Food and Drug Administration

FDR - false discovery rate
Fibroblast 1BR3G - human skin fibroblasts
FLIM - fluorescence-lifetime imaging microscopy
Fmoc - fluorenylmethyloxycarbonyl
FSC - forward scatter
GI - gene identification
GO - gene ontology
HBTU - 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HC_{50} - half-hemolytic concentration
HDL - high-density lipoprotein
HDP - host defense peptides
HeLa S3 - human cervix carcinoma cells
HEPES - 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid
HL-60 - human promyelocytic leukemia cells
HOAc - acetic acid
HPLC - high-performance liquid chromatography
HSA - human serum albumin
HSQC - heteronuclear single quantum coherence spectra
HTS - high-throughput screening
IC_{50} - half-inhibitory concentration
Jurkat E6.1 - human leukemic T-cell lymphoblast
KEGG - Kyoto Encyclopedia of Genes and Genomes
L-AAO - L-amino acid oxidase
LC-MS - liquid chromatography–mass spectrometry
LfcinB - lactoferricin
LPS - lipopolysaccharide
LTA - lipoteichoic acid
LUV - large unilamellar vesicle
MAPK - mitogen-activated protein kinase
MBC - minimal bactericidal concentration
MBCD - methyl-β-cyclodextrin
MeCN - acetonitrile
MHB - Mueller-Hinton broth
MHB II - Mueller-Hinton broth cation-adjusted
MIC - minimal inhibitory concentration
MM6 - human monocytic leukemia cells
mRNA - messenger RNA
MRSA - methicillin-resistant *Staphylococcus aureus*
MS - mass spectrometry
NF- - nuclear factor kappa-light-chain-enhancer of activated B cells
NMR - nuclear magnetic resonance
NOE - nuclear Overhauser effect
NOESY - nuclear Overhauser effect spectroscopy
NrTPs - nucleolar-targeting peptides
OD - optical density
Omw - omwaprin
OPEP - optimized potential for efficient structure prediction
PBMC - peripheral blood mononuclear cell
PBS - phosphate buffer saline
PC - phosphatidylcholine
PCR - polymerase chain reaction
PDB - Protein DataBbank
PE - phosphatidylethanolamine
PG - phosphatidylglycerol
PI - phosphatidylinositol
PLA2 - phospholipase A2
POPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline
POPE - 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-ethanolamine
POPG - 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-1-glycerol)

POPS - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine
PPI - protein-protein interaction
PpTh - thionin from *Pyrularia pubera*
PS - phosphatidylserine
PTM - post-translational modification
qPCR - quantitative PCR
RBC - red blood cell
RC - random coil
RhB - rhodamine B
rmsd - root-mean-square deviation
RNA - ribonucleic acid
ROI - region of interest
ROS - reactive oxigen species
RP-HPLC - reverse-phase high-performance liquid chromatography
RT - room temperature
SAINT - significance analysis of interactome
SD - standard deviation
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM - standard error mean
SGF - simulated gastric fluid
SIF - simulated intestinal fluid
SM - sphingomyelin
SPPS - solid phase peptide synthesis
SPR - surface plasmon resonance
SSC - side scatter
STAT-1 - signal transducer and activator of transcription 1
STRING - search tool for retrieval of interacting genes/proteins
SUV - small unilamellar vesicle
SV - snake venom
TC_{50} - half-toxic concentration
TD-PCR - touchdown-PCR
TFA - trifluoroacetic acid
TFE - 2,2,2-trifluoroethanol
THP-1 - pro-monocytic, human monocytic leukemia cells
TIS - triisopropylsilane
tM2 - thresholded Manders' coefficient
TOCSY - total correlation spectroscopy
TR-FCA - time-resolved flow cytometry assay
TSA - trypcase soy agar
U937 - pro-monocytic, human myeloid leukemia cells
UTR - untranslated region
VEGF - vascular endothelial growth factor
VISA - vancomycin-intermediate *Staphylococcus aureus*
VRE - vancomycin-resistant *Enterococcus faecium*
VRSA - vancomycin-resistant *Staphylococcus aureus*
WHO - World Health Organization

Symbols

$[\theta]_{MRW}$ - mean residue ellipticity
f - cooperativity binding factor
If - fluorescence intensity
k - rate constant
k_D - dissociation constant
\# - FCA event
AG^+ - apoptotic cells gate
C_t - threshold cycle
DG^+ - damaged cells gate
E - efficiency
H - hydrophobicity
IG^+ - peptide-bound/internalized cells gate
MRW - mean residue weight

MW - molecular weight
P - permeabilized bacteria (FCA)
P:L - peptide-to-lipid ratio
PG^+ - permeabilized cells gate
R - FSC/SSC ratio
R_{eq} - response at equilibrium
R_H - hydrodynamic radius
R^2 - Pearson correlation coefficient
RhB^+/RhB^- - positive/negative RhB gates
RU - resonance units
S - slope
SG^+/SG^- - positive/negative SYTOX Green gates
$t_{1/2}$ - half-life
T_m - melting temperature
U - peptide uptake (FCA)
$\Delta\delta - chemical shift$
$\theta - ellipticity$
$\lambda - wavelength$
$\lambda_{em} - emission wavelength$
$\lambda_{exc} - excitation wavelength$
$\mu_H - hydrophobic moment$

Notes & Definitions

AKT pathway - Intracellular signal transduction pathway that promotes metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals.

Billions - Long numeric scale; 1,000,000,000,000.

Cathelicidin - Family of peptides with sequence homology to the cathepsin domain, found in the lysosomes of neutrophils and macrophages. Cathelicidins act as effectors of innate immunity in vertebrates.

Hippo pathway - Conserved animal signal cascade that controls cell proliferation, growth, differentiation and death.

Human plasma - Biological fluid that acts as the liquid base for whole blood and is formed by coagulants, proteins, electrolytes and immunoglobulins (i.e., whole blood minus RBCs, PBMCs and platelets)

Human serum - Liquid part of the blood after coagulation. Sometimes mistakenly considered synonymous with plasma, serum consists of plasma but without clotting factors (fibrinogen).

MAPK pathway - The mitogen-activated protein kinase (MAPK) cascade is a highly conserved signal transduction route involved in cell function regulation including cell proliferation, differentiation and migration.

Superbugs - In the particular context of microbial resistance, superbug refers to microorganisms (mainly bacteria) that have developed a resistant phenotype to antibiotics.

$t_{1/2}$ - Time required for a substance to be half removed by a biological process.

Contents

List of Figures

List of Tables

1

INTRODUCTION

Contents

1.1 Major challenges for 21st century therapeutics

1.1.1 The rise of antibiotic-resistant bugs

Antimicrobial resistance (AMR) has become a worldwide human health and economic emergency, bringing to light the urgent need for new anti-infective drugs with a long-lasting commercial life. According to the World Health Organization (WHO), we are running out of efficient antibiotics (1). Recent forecasts regarding human costs and economic burden associated to AMR are quite pessimistic: by 2050, the number of deaths due to AMR will increase from currently 700,000 a year to 10 million, becoming the main cause of death by that year (2, 3) (see Figure 1.1). From an economic standpoint, the worst-case scenario predicts that by that same year the worldwide gross domestic product will globally decay by a 3.8 % average due to AMR –much worse (5.6 %) for low-income countries– with worldwide losses rising up to $6 trillion (4). Indeed, the magnitude of the economic impact of AMR is comparable to that of the 2008 – 2009 financial crisis.

Among the causes contributing to AMR emergence, overuse and misuse of antibiotics stand out as the major ones (5, 6). Foresightedly, it was the discoverer of penicillin himself, Sir Alexander Fleming, who ended his Nobel acceptance speech with a warning for the future use of antibiotics, that we have ignored at our peril: "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to on-lethal quantities of the drug make them resistant... Moral: if you use penicillin, use enough."

Moreover, inappropriate antibiotic prescribing (as prophylactics, for treating viral infections, wrong dosage, lacking diagnostic test, etc.) adds to misuse by patients; it has been estimated that antibiotic treatment is inappropriate, suboptimal or unnecessary in 30 – 60 % of cases (7, 8) (Figure 1.1). Furthermore, the agricultural industry usually supplies livestock animals with antibiotics not only for infection control but also for growth stimulation, both practices

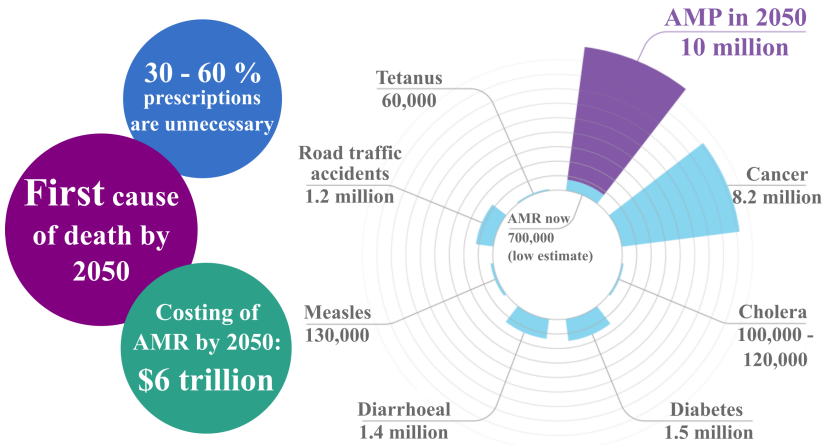


Figure 1.1: Human lives and economic costs of AMR by 2050. Estimation of annual deaths due to AMR by 2050 and comparison to current major causes of death. Data and graphs adapted from the *Review on Antimicrobial Resistance* (https://amr-review.org) (11). Predicted economical impact data by 2050 extracted from the World Bank Group 2017 report (4).

promoting the emergence of bacterial resistance, as both antibiotics and/or resistant bacteria pass to humans through food consumption (9, 10).

The AMR threat has also been aggravated by pharmaceutical industry practices and regulatory barriers. Antibiotic development has lost economic appeal for pharma due to low price/revenue, brief usage periods, availability of cheap generic versions and often limited commercial lifespan (new market arrivals initially reserved for the worst multirresistant cases are gradually moved into general usage, upon which resistances emerge and efficacy is lost) (6). These factors make anti-infectives less profitable that other drugs, and some companies have either held off or even entirely discontinued their R&D on this field (12, 13).

At the molecular level, AMR development may occur via spontaneous gene mutations resulting in acquired beneficial advantage for bacterial survival, which then get passed on to successive generations. Most commonly, AMR appears due to horizontal gene transfer, which consists on taking up genetic material (e.g., plasmids or transposons) encoding for AMR from strains of the same or even different species (14). In one way or another, phenotypically these chromosomic or extra-chromosomic changes may result in, inter alia, reduced antibiotic uptake, increased efflux pumping, target site modification

with consequently reduced or suppressed affinity, target overexpression, or drug modification/inactivation by enzymatic cleavage, (for an extensive review of AMR, refer to (15, 16)).

1.1.2 The AMR crisis opens new opportunities

In 2017, the WHO disclosed the priority pathogen list for which new antibiotics are urgently needed (17). Critical carbapenem-resistant strains of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, as well as diverse strains from the *Enterobacteriaceae* family resistant to both carbapenem and 3rd generation cephalosporin (including *Klebsiella pneumonia*, *Escherichia coli* and *Enterobacter* spp., among others) were highlighted. The vancomycin-resistant *Enterococcus faecium* (VRE), methicillin- and vancomycin-intermediate and resistant *Staphylococcus aureus* (named as MRSA, VISA or VRSA respectively), clarithromycin-resistant *Helicobacter pylori*, fluoroquinolone-resistant *Campylobacter* and *Salmonella* spp., and *Neisseria gonorrhoeae* resistant to both 3rd generation cephalosporin and fluoroquinolone are also referred with high priority as pathogens without proper and efficient treatment.

In such a disturbing scenario of AMR crisis, not only should current antibiotics be more prudently used than ever but novel anti-infective drugs are urgently imperative (18). WHO also published in 2017 a report analyzing the scarce current pipeline of antibacterial agents under clinical development, and claiming for more investment in basic science, drug discovery and clinical development to fight the AMR crisis (1). Moreover, WHO reported that most agents under clinical trials are modifications of existing drugs, thus providing only short-term relief, and emphasized the urgent need for innovative products to treat multi- and pan-resistant pathogens.

In response to this challenge, different approaches have been proposed to identify and design new antibacterial drugs. They include chemical modification (prodrugs or analogs), fusion of existing antibiotic structures with one another or with molecules overcoming resistance, or *de novo* design based on rational computer-aided or HTS approaches (19, 20). Potential alternatives include phage therapy (bacterium-specific lytic phages), lysins (bacteriophage enzymes able to cleave the bacterial cell wall), genome editing by CRISPR/Cas9, antibacterial antibodies or host defense peptides (HDPs) such as bacteriocins

(21-24). Drugs with targets and/or modes of action different from classical ones, and with reduced adaptative pressure to develop resistance are of particular interest. In this context, antimicrobial peptides (AMPs) stand out as a promising alternative to fill the niche of future, novel anti-infective drugs (extensively described in section 2.1).

1.1.3 Cancer

Despite undeniable progress over the last decades, cancer remains the paramount challenge to the medical and research community in the 21st century. Costs related to cancer are awesome, not only in human lives but also in terms of socio-economical wellness. The statistics below (summarized on Figure 1.2), should not just be seen as mere numerical records; underneath them lies the suffering of families, friends and colleagues reflecting the broad impact of cancer disease in our lives.

According to WHO, cancer is nowadays the second leading cause of death worldwide; one out of six deaths is cancer related (25). Annual statistics from the Global Cancer Observatory (26) for 2018 highlight 18 million new cases and 9.6 million deaths (27, 28), roughly the total population of European countries such as Portugal or Sweden. Unfortunately, future outlooks remain unfavorable, with the annual incidence expected to rise up to 29.5 million global cases and 16.4 deaths by 2040 (26) (Figure 1.2).

From an economic perspective, the financial costs associated to cancer are staggering for both patients and society, and the trend is increasing. In 2014, direct health costs associated with cancer in the EU amounted to 86 billion (Figure 1.2), with a similar figure ($87.8 billions) for the US in the same period (29, 30). The socioeconomic burden is expected to rise as prevalence grows, population aging/life-span increases and new, usually expensive therapies are introduced in the healthcare system.

Despite differences across countries regarding sex, age groups, economic class, etc., the five predominant cancers diagnosed are lung, breast, colorectum, prostate and stomach, with death rankings being lung, colorectum, stomach, liver and breast cancer (26). Treatment success rates are highly correlated with cancer type and disease stage, but on average the 5 year survival rate was ca. 67 % between 2009 – 2015 in the U.S. (31).

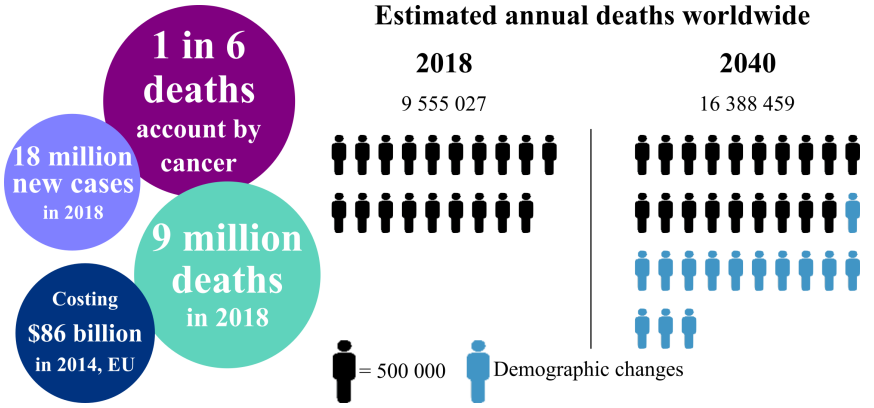


Figure 1.2: Cancer burden. Current global statistics and future forecasts of cancer in terms of human lives and economic costs. Data extracted from the Global Cancer Observatory (26) and from Wilking et al. (29, 30).

The term cancer includes a wide collection of disorders, characterized by abnormal proliferation of cells (tumor), finally invading surrounding normal tissues and/or spreading into distant body sites (32). Ultimately, uncontrolled tumor progression and dissemination may lead to physical obstruction or invasion of vital organs and often competes with healthy tissues for nutrients and oxygen, compromising proper vital functions.

The transformation of healthy cells into tumor cells, known as carcinogenesis, is a complex and multistep process involving different stages (initiation, promotion and progression). It may arise from single or combined biological and genetic alterations and/or external factors (33). Briefly, as proposed by Hanahan et al., cancer molecular basis may be summarized into ten distinctive hallmarks acquired during carcinogenesis and ultimately leading to cancer cell survival, proliferation and dissemination: (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) avoiding immune destruction, (4) enabling replicative immortality, (5) tumor-promoting inflammation, (6) activating invasion and metastasis, (7) inducing angiogenesis, (8) genome instability and mutation, (9) resisting cell death and (10) deregulating cellular energetics (34).

1.1.4 Pitfalls and challenges of chemotherapy

In general, cancer treatment encompasses single or combined regimens of surgery, radiotherapy, chemotherapy and/or biological and hormonal therapy. Specifically, cancer chemotherapeutic strategies aim to inhibit or kill accelerated-dividing cells with a minimal impact on non-neoplastic host cells. Cytotoxic drugs exploit the aforementioned hallmarks of cancer, acting on specific molecules that enable the uncontrolled, fast growth of cells as well as their propagation (34, 35). Most drugs are alkylating agents, antimetabolites, anti-tumor antibiotics or mitotic inhibitors, targeting DNA synthesis and the cell-cycle (36).

However, chemotherapeutic drugs with such specific targets are often inactive due to inherent genetic resistance of some tumor cells (37) or progressively anti-tumor potency loss as tumors develop mechanisms to surpass drug cytotoxicity (35, 38-40), acquiring a resistant phenotype (40-42). Dormant cancer cells may also escape from drugs targeting fast-dividing cells, rendering them ineffective and promoting potential long-term relapses (43, 44). Other cancers are located on privileged compartments (e.g., inside the blood brain barrier or forming large solid tumors with poor blood supply) where drug delivery becomes a challenge.

Furthermore, conventional chemotherapeutic agents not only act on fast-growing cancer cells, but their cytotoxic effect also affects fast-dividing healthy cells (bone marrow, gastrointestinal tract or hair follicles among others), inducing side effects such as fatigue, neutropenia, vomits or hair loss commonly associated to current cancer treatment strategies. Low or even lack of specificity also limits the realistic dosage to suboptimal treatments in order to avoid acute toxicity. Efforts on devising new chemotherapies increasing the outcomes of cancer patients focus on higher selectivity (to avoid off-target side effects), improved drug delivery and low acquired resistances (preventing tumor relapses).

In this regard, cationic peptides are emerging as promising candidates for cancer treatment, both as active therapeutics or as drug delivery systems (addressed in the next section).

1.2 Antimicrobial and anticancer peptides

Despite a direct connection between AMR and cancer aside from their relevance as global health threats, a somewhat similar therapeutic approach in both areas is gradually emerging as a promising alternative: antimicrobial and anticancer peptides, heretofore referred as AMPs and ACPs, respectively. In this introductory section, an overview of the main properties and characteristics of AMPs and ACPs is described, in particular their origin, location, expression, sequences, structures, physico-chemical properties, functions, mechanisms and applications in clinics.

1.2.1 Why AMPs and ACPs?

In line with the urgent need of new anti-infectives linked to the AMR rise, AMPs have attracted attention mainly by their lower propensity to induce bacterial resistance (45). Considered as "dirty drugs", AMPs may display different, multiple effects, targeting the bacterial membrane while also disturbing key intracellular biological processes (46, 47). Additionally, AMPs are able to modulate the immune system, contributing to microbial clearance and control of the immune response (48). Their rapid onset of killing, non-specificity for easily-mutable targets, and multi-targeting mechanisms that include concomitant broad anti-inflammatory action, contribute to their reduced ability to induce drug-resistance in microorganisms (49). In addition, some AMPs display a broad action spectrum, showing activity against viruses, fungi and parasites (50).

In parallel, the main advantage of ACPs over conventional cancer chemotherapy lies on their ability to target slow-growing and multidrug resistant cancer cells (51) as well as their putative ability to confer protection against tumor rechallenge (52). Unlike most current chemotherapy strategies, ACPs would not be too prone to develop resistance due to their multiple and broad-spectrum mechanism of action(53). Additionally, ACPs display (or can be engineered to display) selectivity toward cancer cells, minimizing off-target side effects (54). Altogether, these advantages support ACPs as promising alternatives

to conventional chemotherapy, or be considered as adjutants with synergistic effects to current cytotoxic drug portfolio, as already demonstrated (55, 56).

1.2.2 Cationic peptides: an overview

Different nomenclatures are found in the literature to refer to AMPs and ACPs, such as HDPs or cationic antimicrobial peptides (CAPs). Herein the AMP and ACP terms will be preferentially used, with HDPs used to denote peptides from natural organisms that display anti-infective, anticancer and/or immunomodulatory properties.

Sources of cationic peptides: HDPs are ancient weapons of the host defense machinery virtually present throughout every kingdom and phylum, including plants (57), animals (58), fungi (59) and bacteria (60). Despite the broad presence in such different organisms, they share a common function: assist on the first line of defense towards microbes (61, 62).

Location and expression: To act as an initial barrier role, HDPs are typically found in surfaces directly exposed to microbes or are expressed by immune cells (58). In fact, while some HDPs are constitutively expressed, the majority are induced upon infection, inflammation or tissue damage (50, 63). In humans, HDPs have been naturally found in a wide range of mucosal secretions and epithelial cells, for instance in the skin (such as the human cathelicidin LL-37 (64), β-defensins (65, 66) or RNAses (67)), saliva (including histatins (68) and lysozyme (69)), airway tract (also containing LL-37 (70), β-defensins (71, 72) and RNAses (73)) and the intestinal tract (lysozyme (69), α-defensins (74-76), RNAses (77)) among others (78). α- and β-defensins (79, 80), RNAses (81) and LL-37 (82, 83) are also secreted by immune cells such as monocytes-macrophages, eosinophils or neutrophils in response to microbial and proinflammatorial stimuli (84-86). In other animals, expression also occurs in areas exposed to pathogen entry/contact. A great number of bioactive peptides can be found in the secretory glands on frog skin (87), or those isolated from snake venom glands (88), suggesting an overall protecting role against first microbial contact.

Sequence diversity: In terms of amino acid sequence, mature HDPs are greatly diverse even within evolutionary close species. This variability arises from the microbiome diversity that the host is forced to deal with, including

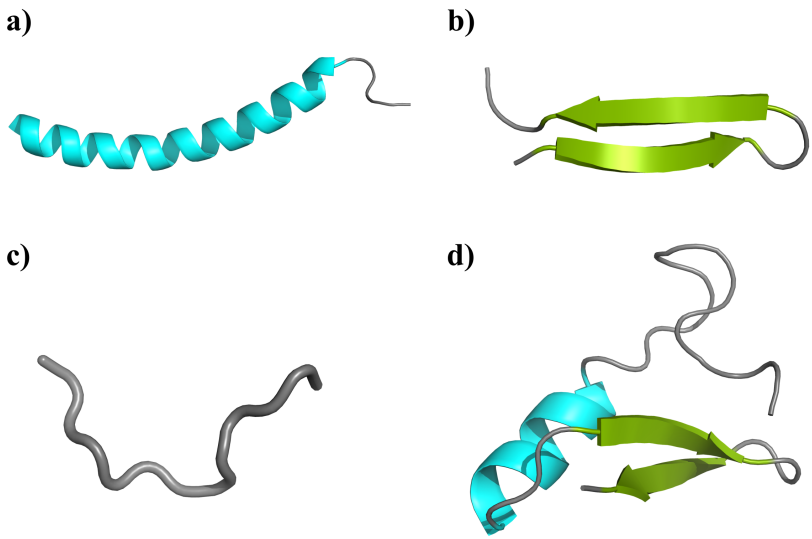


Figure 1.3: Different structures acquired by cationic peptides. Representative examples of a) α-helical peptides, such as human LL-37 (PDB ID: 2K6O); b) β-sheet containing peptides, such as cyclic gomesin (PDB ID: 1KFP); c) extended peptides rich in specific amino acids, such as Trp-rich indolicidin (PDB ID: 1G89); d) α-helix and β-sheet containing peptides, such as phormicin (PDB: 1ICA). Representations are colored by the secondary structural element; α-helix (blue), β-sheet (green), random coil (gray). Figure information extracted from (92).

bacteria, fungi, viruses and parasites. Due to environmental and specialized habitats, these external invaders are highly divergent between species (89). However, certain types of amino acid residues are more often found in AMP and ACP sequences than others. The APD database, which contains ca. 3000 peptides (including experimentally validated bioactive peptides (90)) shows positively charged residues such as Lys and Arg as above-random at 9.6 % and 5.8 %, respectively, as well as the disulfide bond-forming Cys (6.7 %), while negatively charged amino acids such as Glu or Asp are detected less frequent ($<$ 3 %). The most enriched amino acid is Gly (11.6 %), characterized by the lack of steric hindrance. These different contributions in composition can be related to the global physico-chemical properties displayed by HDPs and ultimately with their function and mode of action.

Physicochemical properties: Despite their sequence diversity, almost all HDPs share a relatively reduced size ($<$ 50 aa), positive global net charge and high content of hydrophobic residues (91). The APD database (90) shows an average length of 33 residues, an average net charge of + 3.31 at neutral pH

and hydrophobic amino acids included with higher frequency rates than those expected by chance (> 5 %) such as Leu (8.3 %), Ala (7.6 %), Ile (5.9 %) and Val (5.7 %). These physicochemical properties reflect non-random compositions allowing the peptide to fold into three-dimensional amphipathic structures that play a crucial role in the mechanisms of action (61).

Structure: HDPs are highly diverse in 2/3D structure. A recent classification according to their complex topology (92) includes: linear α-helical peptides (e.g., melittin and human cathelicidin LL-37) (93, 94); β-sheet-containing peptides usually stabilized by disulfide bridges (e.g., porcine protegrin-1 or human α-defensins) (95, 96); peptides with α- and β-structural elements (e.g., human β-defensin 1) (97); linear extended peptides rich in particular amino acids and usually lacking any particular 3D structure (e.g., Pro/Arg-rich PR-39 or Trp-rich indolicidin) (98, 99); and finally cyclic peptides or other complex topologies (including cyclic bacteriocin AS-48, Kalata B1 or the lanthibiotic nisin A)(100-102) (see Figure 1.3, illustrating the vast diversity of structures).

Functions: The initial function proposed for cationic HDPs was to act as AMPs against an exceptionally broad spectrum of pathogens comprising Gram-positive and Gram-negative bacteria, fungi and parasites (103, 104). However, increasing studies have demonstrated an overall capability of targeting additional pathogens including viruses and biofilm-forming bacteria and fungi. Additional properties such us anticancer or modulation of the immune response have been also described (105-107). Due to their ability to translocate lipid membranes, cationic peptides have been also exploited to act as delivery vectors, in this case referred as cell-penetrating peptides (CPP) (108). Figure 1.4 summarizes the main activities of cationic peptides.

The mechanisms underlying the antimicrobial and anticancer activities are described in detail below.

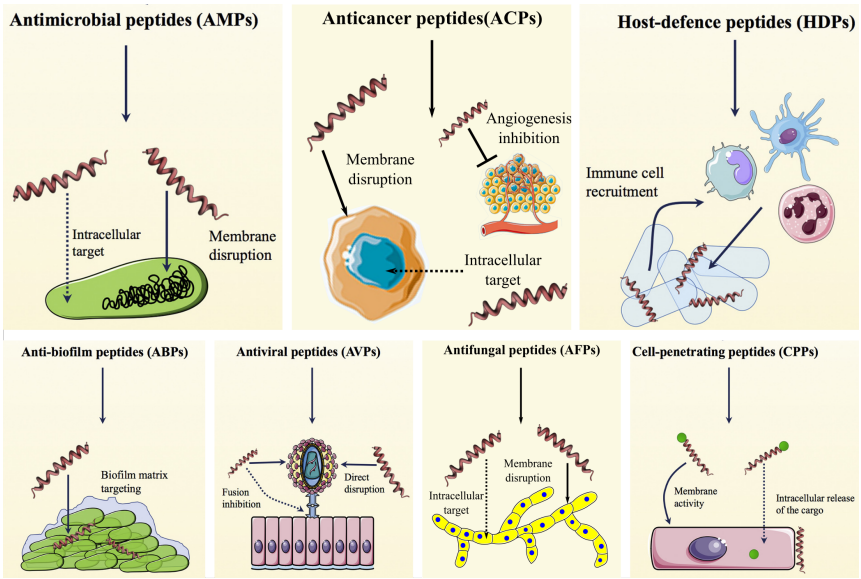


Figure 1.4: Summary of the main functions of cationic peptides. Modified from (106, 109).

1.2.3 Mechanisms of AMPs and ACPs

AMPs in particular may contribute to the microbe clearance by three complementary ways: i) direct pathogen killing by cellular membrane disruption (110); ii) interfering with key intracellular processes such as nucleic acid and protein synthesis or cell wall synthesis; iii) activation of immune response against invading microorganisms through a broad array of functions, having as ultimate goal increasing the innate immune response to assist in the removal of the pathogen, or reduce the immune response to prevent acute inflammation and sepsis processes related to infections (111, 112).

In parallel, ACPs may exert their effect also throughout single or a combination of several mechanisms, quite similar to those of AMPs; direct cytotoxicity against tumor cells, including i) membranolytic and/or ii) non-membranolytic modes of action (113, 114); iii) anti-angiogenesis (115, 116); iv) metastasis prevention (117); and v) recruitment of immune system cells to assist on the tumor clearance (118).

Important details and steps on the mechanisms are summarized in the next

pages. In addition, a description focused on the heterogeneity between bacteria, tumor and healthy cell membranes is also available, due to their pivotal role on AMP and ACP modes-of-action and selectivity.

1.2.3.1 Plasma membrane: the "gold target"

Although all biomembranes are organized as a fluid mosaic of proteins and phospholipids, fundamental differences exist between healthy and tumor eukaryotic cell membranes, as well as prokaryotic membranes. Lipid composition, architecture, overall physicochemical properties and energetics (91, 119) are, among others, some of the discriminative parameters that we can pinpoint. The main differences on composition are depicted on Figure 1.5.

Bacterial cell membrane: microbial membranes tend to be highly anionic because of their content in phosphatidylglycerol (PG), phosphatidylserine (PS) and/or cardiolipin (CL) (120, 121). Additionally, the external presence of lipopolysaccharides (LPS) in the outer leaflet of Gram-negative bacteria or teichoic and teichuronic acids adhered to the surface of Gram-positive ones, also contribute to the global negative net charge of the bacterial surface (122, 123).

Healthy eukaryotic cell membrane: in terms of lipid composition, eukaryotic cell membranes are overall zwitterionic bilayers predominantly composed of phosphatidylcholine (PC), sphingomyelin and glycolipids in the outer leaflet. Nevertheless, due to their complexity and anchoring of diverse molecules (charged glycolipids such as glycosaminoglycans, charged lipo/glycol-proteins) they may acquire a slight negative global charge (124). Refering solely to the lipid composition, the inner leaflet, contrary to the outer leaflet, contains negatively charged PS and phosphatidylinositol (PI), generating a specific asymmetry (125). Unlike bacteria, cholesterol is a major constituent of eukaryotic plasma membranes and is evenly distributed in both leaflets, regulating lipid membrane fluidity depending on temperature conditions (32).

Tumor cell membrane: like pathogens, tumor cell membranes typically have a negative net charge as a result of the inclusion or overexpression of PS in the outer membrane due to asymmetry loss, O-glycosylated mucins, sialylated gangliosides and heparin sulfates (126-128), which promote the accumulation of cationic peptides in the surface of malignant cells. Moreover, transformed cells

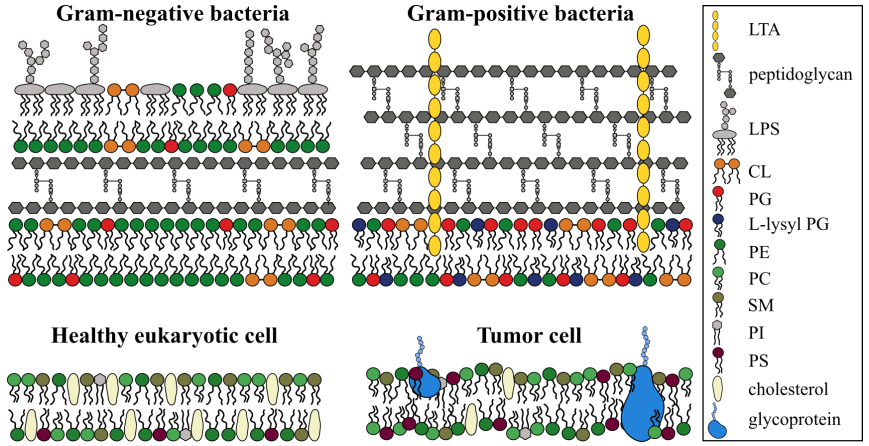


Figure 1.5: Schematic view of plasma membrane organizations in bacterial, mammalian, and tumor cells. LTA, lipoteichoic acid; LPS, lipopolysaccharide; CL, cardiolipin; PG, phosphatidylglicerol; L-Lysyl PG, L-Lysyl-phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine.

may incorporate lower levels of cholesterol in their bilayers, increasing their fluidity (129), which may facilitate insertion and/or membrane destabilization by peptides. Complementarily, transformed cells tend to present higher numbers of microvilli (130) which increase the peptide-cell interaction surface.

1.2.3.2 Membranolytic mechanisms of AMPs and ACPs

For both AMPs and ACPs, the mechanisms of membrane-targeting are not fully deciphered. Different models have been proposed to describe membrane interaction and destabilization, with the overall process divided into three steps (91): i) initial peptide interaction with membrane targets; ii) peptide adsorption/conformational change; iii) membrane destabilization and final cell death (see Figure 1.6 a for the schematic representation of the membranolytic mechanism of AMPs and ACPs).

i) Initial peptide interaction with membrane targets: it is widely accepted that the initial peptide recruitment occurs via electrostatic interactions onto cellular surfaces. The overall cationic nature of AMPs and ACPs ensures their accumulation at the bacterial/tumor cell membranes by directly binding to those negatively charged components selectively expressed or overexpressed in bacteria and tumor cell membranes (131, 132). Particularly, Hancock et al.

have proposed a so-called "self-promoted uptake mechanism" by which cationic peptides are attracted onto Gram-negative bacterial membranes displacing the divalent cations associated to LPS and destabilizing their supramolecular assembly, gaining access to the outer and inner membrane (133, 134).

ii) Peptide adsorption/conformational change: according to the two-state model proposed by Huang, upon binding peptides accumulate on the bilayer up to an effective threshold concentration, at which stage they shift from being absorbed on the membrane to start entering and/or crossing the lipid bilayer (135). Thus, a threshold can be proposed, i.e., the minimal peptide concentration [experimentally, the minimal peptide-to-lipid ratio (P:L)] necessary to induce biological effect (134, 136). Initial membrane-binding may also involve additional events promoting peptide partition into membranes such as oligomerization (i.e., human LL-37) (137), self-association (138, 139) or conformational changes such as helical structuration (i.e., magainin 2) (140). All these events contribute to the rearrangement into tertiary and quaternary active conformations that precede the membrane disruption.

iii) Membrane destabilization and final death: once inserted into membranes, peptides change the structure and organization of the bilayer. This destabilization leads to bacterial or tumor cell death by permeabilization and leakage of ions and small molecules (141). Different models for membrane damage induction have been proposed, which involve the formation of pores (barrel-stave and the toroidal pore model), peptide aggregation on the bacterial surface and subsequent membrane micellization (such as the detergent-like carpet-like model) or membrane thinning (134, 142, 143). The mechanism of membrane permeabilization/destabilization is peptide-specific, dependent on the peptide-lipid ratio and also on the specific nature of the target membrane (mechanisms extensively reviewed on refs. (144, 145) for AMPs and refs. (114, 146) for ACPs).

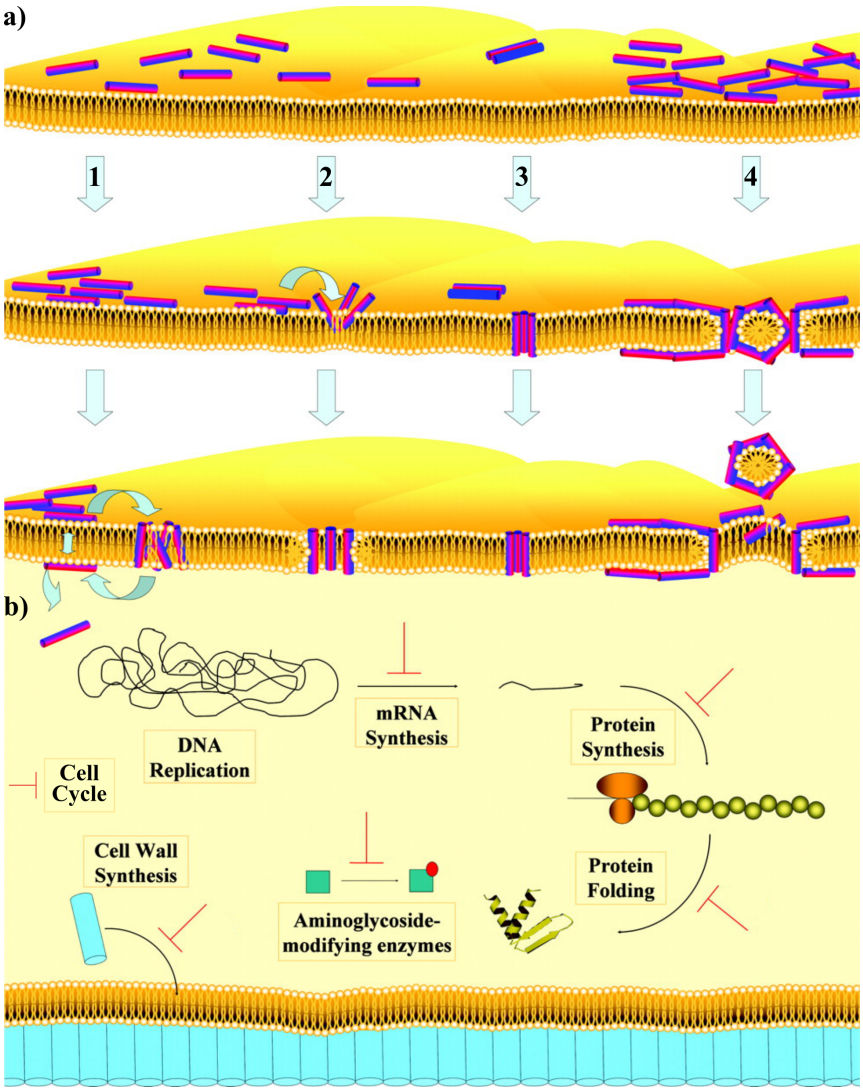


Figure 1.6: Summary of the principal modes of action of cationic peptides. a) Membranolytic mechanisms. Main steps (peptide absorption in the membrane, accumulation and conformational changes and final cell death by permeabilization, from top to bottom) depicted for the principal modes of membrane disruption proposed: 1. Aggregation model; 2. Toroidal pore model; 3. Barrel-stave model; 4. Carpet model. **b) Intracellular targeting.** Examples of key intracellular processes inhibited by AMPs and/or ACPs. Peptides are represented as cylinders, with the hydrophilic and hydrophobic regions colored in red and blue, respectively. Modified from (167).

1.2.3.3 Intracellular targeting mechanisms

Complementarily, AMPs and ACPs may enter the cell with or without directly disrupting the membrane integrity, and interact with intracellular targets. In the end they will promote as well cellular death (see Figure 1.6b). Dual mechanisms have been described for AMPs (e.g., magainin III (140, 147) and daptomycin (148, 149)) and ACPs (e.g.,peptide A(9)K (150) or temporin-1CEa (151)). Such molecules perturb the membrane and immediately gain access to the cytoplasm, while others do not disrupt the membrane and their cytotoxic effect is restricted to interfering with core metabolic pathways (i.e., apidaecidin) (152). For the latter group of peptides, however, the mechanisms to access the cytoplasm differ between AMPs and ACPs. Eukaryotic and prokaryotic cells present a fundamental biological difference: the lack of endocytic mechanisms by bacteria (153).

ACPs may translocate tumor cell membranes and access intracellular compartments using several mechanisms, including endocytic mechanisms or direct diffusion through the bilayer (154, 155). Once inside the cytoplasm, ACPs such as buforin IIb or lactoferricin B (LfcinB) typically disrupt the mitochondria membrane (possibly reflecting evolutionary traces of bacterial origin) and induce apoptosis (143, 156, 157). Other non-oncolytic mechanisms include hyperactivation of phospholipase A2 (158), different apoptose-induction pathways (156), inhibition of protein synthesis (159), overexpression of tumor-suppressing genes or underexpression of oncogenes (160), extensively reviewed in (161).

In contrast, internalization mechanisms proposed for AMPs involve spontaneous lipid-assisted translocation (i.e., by forming a transient pore and leading to the peptide entry during the pore disassembly) (162), and transporter-mediated entry as described for Pro-rich peptides (for instance PR-36 or Bac7), which exploit the inner membrane protein SbmA to efficiently penetrate Gram-negative bacteria bilayers (47, 163, 164). Once AMPs reach the cytoplasm of bacteria, they can interfere with nucleic acid, cell wall and protein synthesis, or with enzymatic activity, to name just a few (extensively reviewed in refs. (164-166)).

Overall, either as a primary mode-of-action or as a complementary mechanism to its membranolytic action, intracellular targeting by AMPs and ACPs may contribute to cell death and should be routinely explored during mechanistic

studies. Considering cationic peptides uniquely as membrane-active peptides may be too oversimplifiyed.

1.2.4 Selectivity of AMPs and ACPs

An essential requirement for any therapeutic agent is selectivity (or lack of toxicity) for its target relative to the host tissue/cells. Ideally, the therapeutic candidate should display high affinity for distinctive and crucial structures or targets. To prevent resistance emergence, these targets should also be practically immutable and common to several (if not all) microbes or tumor cells, which would ensure broad spectrum of activity. Both AMPs and ACPs fulfill these targeting criteria, which makes them appealing therapeutic candidates against cancer and microbial infections. The selectivity of ACPs and AMPs is based on their primary membrane-targeting (168, 169), but additional factors can also contribute.

Fundamental differences exist between microbial, tumor and healthy host cell membranes with respect of architecture, composition and energy, and they determine the preferential toxicity (or the lack thereof) of HDPs against pathogens and tumors. In fact, these membrane variances explain why toxic concentrations against healthy eukaryotic cells are commonly 10-fold higher than effective concentrations against microbes (146, 170). Plus, HDPs display an additional property contributing to their selective action: strategic localization or expression, minimizing interaction with potentially vulnerable host tissues (91). As explained in section 1.2.2, HDPs are constitutively or inducibly-expressed normally on tissues exposed to microbial entrance or by immune cells. This particular regulation regarding release and location entails a spatial-temporal control of peptide action (171).

In addition, several peptides have been shown to change their structure and/or multimeric state upon first cell membrane contact, turning into active structures (described above). This conformational transition at the membrane site also contributes to minimize host-cell toxicity, as peptides remain on their inactive, unstructured conformation until reaching the appropriate target, preventing indiscriminate effects (172).

1.2.5 The leap of AMPs and ACPs into the market

Since the pioneer work in the 1980s, leading to the discovery of cecropins, defensins and magainins (79, 93, 173), more than 8000 potential AMP sequences have been deposited in specialized databases. Approximately 2800 of them have been experimentally validated (the CAMPR3 (174), accessed August 2019). However, despite this promising number of candidates, only a few AMPs have been approved by FDA and/or EMA, mainly for topical applications (e.g., classics such as bacitracin, tyrothricin, gramicidins D and gramicidin S) and less than twenty are under clinical development (175).

Some well-known AMPs, e.g., cecropins, LL-37 and LfcinB have been also described as ACPs, but only a few are enrolled in clinical trials (phases I and II): magainin 2, melittin, fish-derived pardaxin and a hybrid from magainin 3 and bombesin named MG2B (146). The search of ACPs has not been as intense as for AMPs, and only ca. 500 entries are listed on the CancerPPD database (176), accessed in August 2019. None of those have been yet commercialized (this refers strictly to cationic peptides, excluding short (Fab fragment) or the single-domain antibodies from camelids (so-called VHH), which can be also regarded as peptides).

The known limitations of peptide-based pharmaceuticals (non-orally active, limited bioavailability, cost) stand as major hurdles for AMPs and ACPs to leap into the market. Strategies to address these impediments include chemical modifications (i.e., cyclization, peptidomimetics, amino acid substitutions, etc.), delivery systems such as nanoparticles, or conjugation to biopolymers (discussed more in depth on chapter 5). Many successful strategies in this regard use as launching pad a pre-optimized lead, i.e., a privileged bioactive peptide framework found in Nature.

1.3 Borrowing the wisdom of Nature

"Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature—because in her inventions, nothing is lacking—and nothing is superfluous."

Leonardo da Vinci

Time and Nature have been working along to become the best designers ever. They have shaped an extraordinary repertoire of live organisms and elements. During this journey, Nature has conveniently catered to our basic needs, providing us with an arsenal of tools, including the ability to fight against diseases. Naturally-occurring active principles have been used for human therapeutics since early time and continue to represent a gold mine for drug discovery.

1.3.1 Exploding natural sources for drug discovery

About half of the drugs currently used are semi-synthetic or fully synthetic compounds based on pharmacophores found in natural reservoirs (177, 178). In the specific case of anti-infective drugs, about two thirds in the last three decades (1981 – 2010) are Nature-inspired molecules. This trend is even stronger in ACP candidates, with $\sim$ 80 % of Nature-related entities (179).

Ironically, and despite the evident advantages discussed above, use of natural products in drug design programs was sadly neglected in the 1980s – 1990s, as the glamour of emerging new technologies switched drug discovery paradigms in pharmaceutical industry. Focus was particularly placed on quantity and speed, and R&D strategies shifted mainly to the HTS of small-molecules driven by genomics and combinatorial chemistry (180). However, and despite the enormous effort and investment placed on these new methodologies, the outcome has been far more meager than expected and the interest on natural templates for drugs design has been rekindled (181). Also, advances in technologies such as HPLC, MS or NMR (and combinations thereof) in recent years have helped speed up the often laborious tasks of natural product-based drug discovery

(178, 182): successful separation, identification and structural characterization of active molecules in the complex natural mixtures, has led, by modern HTS approaches, to valuable screening libraries of either crude, semi-pure or fully purified natural compounds (183, 184). Refined over hundreds of millions of years of evolutionary pressure, the molecular prototypes found by these approaches embed distinctive, exploitable features, including high chemical diversity, high binding affinities, and often good bioavailability and selectivity (185, 186). For these reasons, natural compounds have also been called "privileged scaffolds", to use as starting point towards the screening of libraries of analogs based on natural parent molecules.

1.3.2 Animal venoms: a wealth of bioactive compounds

Animal venoms are a priceless source of bioactive molecules with high promise in drug discovery (187). Venomics has indeed become a very active research field, with pharmaceutical industry increasingly focusing attention into venom-based drugs (188). Given the astonishing repertoire of structures and functions adopted for self-protection and predation, animal venoms constitute natural libraries of pre-optimized leads with high potency and selectivity for several molecular targets involved in key physiological processes. In general, venoms consist on complex molecular cocktails including mostly protein-based molecules endowed with diverse modes of action. Some toxins affect the nervous system, directly acting on cell-membrane ion channels or disturbing neuronal transmission, while others impair cardiovascular and muscular processes, which interfere with blood coagulation and homeostasis (189).

Venoms have traditionally been used in medicinal chemistry with two main objectives: drug discovery and research tools. Venom sources range from invertebrates such as corals (190), scorpions (191), spiders (192, 193) or ants (194, 195), to vertebrates such as snakes or lizards (196-198). An emblematic venom source are cone snails, from which $\sim$ 100 compounds with therapeutic potential have been identified (199), one of them, the ω-conotoxin MVIIA, developed after slight structural modulation into ziconatide (Prialt®), an analgesic approved by FDA to treat chronic pain. Other FDA-approved venom-related drugs have been isolated from the Gila monster (exenatide –Byetta® and Bydureon®–, used for type 2 diabetes mellitus) or from the

European medicinal leech, whose venom-components have inspired several anticoagulants (bivalirudin –Angiomax®– and lepirudin –Refludan®–), and the anti-thrombotic drug desirudin (Iprivask®). Most of these venom-derived drugs are synthetic replicas (lightly modified vs. native version) or peptidomimetics (more substantially modified vs. native version) of different snake toxins (see Table 1.1).

1.3.3 Harnessing snake venom molecules

Snake venoms (SV) are extremely complex mixtures of bioactive molecules where 90 – 95 % of the dry weight corresponds to peptides and proteins, with lipids, nucleosides, carbohydrates and metal ions making up the rest (200) (Figure 1.7). The proteinaceous portion can be divided into enzymatic and non-enzymatic proteins. The enzyme fraction contains proteins involved in immobilizing the prey and assisting in its digestion such as phospholipases A_2 (PLA_2s), L-amino acid oxidases (L-AAOs), metallo- and serine-proteinases, hyaluronidases or acetylcholinesterases (200, 201). The lower-MW polypeptide (1-10 kDa) fraction includes a whole variety of neuro-, cardio- or myotoxins (Figure 1.7).

While the connection between snakes and medicine dates back to ancient cultures –e.g., traditional Chinese medicine (189)–, systematic investigation of snake-venom components as therapeutics was not properly taken up until a few decades ago. The first hint of the potential of SVs in medicine came in the 1970s with captopril, a first-in-class angiotensin-converting enzyme (ACE) inhibitor for which the starting lead was the bradykinin-potentiating peptide in the venom of the South American rattlesnake *Bothrops jararaca* (202, 203). Similarly, starting leads for other SV-derived drugs (Table 1.1) are mostly peptides and enzymes. For instance, eptifibatide (Integrilin®) is a synthetic peptidomimetic analogs of disintegrin, a small SV-protein acting as a glycoprotein IIb/IIIa inhibitor. Ximelagatran (Exanta®), an anticoagulant acting as thrombin-inhibitor was inspired on the cobra venom, and batroxobin and ancrod are both thrombin-like enzymes belonging to the serine-proteinase family. As shown in Table 1.1, most SV-based medicines approved so far are indicated for cardiovascular syndromes or as diagnostic tools for blood-related abnormalities. On the other hand, new applications currently being investigated in clinical and preclinical trials focus on other areas, such as sclerosis, chronic

Commercial drugs			
Drug (commercial name)	**Source organism**	**Target**	**Therapeutic use**
Captopril (Capoten®)	*Bothrops jararaca*	ACE	Hypertension
Enalapril (Vasotec®)	*Bothrops jararaca*	ACE	Hypertension
Tirofiban (Aggrastat®)	*Echis carinatus*	Glycoprotein IIb/IIIa	Acute coronary syndromes
Eptifibatide (Integrilin®)	*Sistrurus miliarius barbouri*	Glycoprotein IIb/IIIa	Acute coronary syndromes
Batroxobin (Defibrase®)	*Bothrops sp*	Fibrinogen	Infarction / Ischemia / Microcirculation dysfunctions
Platelet gel (Plateltex-	*Bothrops atrox*	Fibrinogen	Platelet-induced tissue-healing
Fibrin sealant (Vivostat®)	*Bothrops moojeni*	Fibrinogen	Autologous fibrin sealant in surgery
Haemocoagulase (Reptilase®)	*Bothrops atrox*	Fibrinogen Factor X / Prothrombin	Hemorrhage
Ximelagatran (Exanta®)*	Cobra venom	Thrombin	Atrial fibrillation / Blood clotting
Ancrod (Viprinex®)*	*Agkistrodon rhodostoma*	Fibrinogen	Heparin-induced thrombocytopenia

Drugs in clinical and pre-clinical trials				
Drug name	**Phase**	**Source organism**	**Target**	**Therapeutic use**
Fibrolase (Alfimeprase)	III	*Agkistrodon contortrix*	Fibrinogen	Stroke and catheter occlusion
Crotoxin	I	*Crotalus durissus terrificus*	Unknown	Cancer
Cenderitide	II	*Dendroaspis angusticeps*	Natriuretic peptide receptor	Heart failure
RPI-MN	I	*Naja atra*	Nicotinic acetylcholine receptor	HIV / Amyotrophic lateral sclerosis / Herpes simplex keratitis
RPI-78M	I/II	*Naja atra*	Nicotinic acetylcholine receptor	Multiple sclerosis / Herpes simplex infections / Adrenomyeloneuropathy
RPI-78	preclinical	*Naja atra*	Nicotinic acetylcholine receptor	Pain / Rheumatoid arthritis
Prohanin	preclinical	*Ophiophagus hannah*	Nitric oxide synthase	Chronic pain
Oxynor	preclinical	*Oxyuranus scutellatus*	Unknown	Wound healing
Natriuretic peptides	preclinical	*Oxyuranus microlepidotus*	Natriuretic peptide receptor	Heart failure
Textilinin-1™	preclinical	*Pseudonaja textilis*	Plasmin	Preoperative bleeding
Vicrostatin	preclinical	Chimeric; *Echis carinatus* / *Agkistrodon contortrix*	Integrin receptor	Cancer
Haempatch™	preclinical	*Pseudonaja textilis*	Prothrombin	Blood loss during vascular trauma
CoVase™	preclinical	*Pseudonaja textilis*	Factor Xa	Hemorrhage
Contortrostatin	preclinical	*Agkistrodon contortrix*	Integrin	Breast cancer

Diagnostic tools			
Product	**Source organism**	**Target**	**Assessment of:**
Protac®	*Agkistrodon contortix*	Protein C activation	Protein C
Reptilase®	*Bothrops jararaca*	Fibrinogen	Fibrinogen
Ecarin clotting time	*Echis carinatus*	Prothrombin	Meizothrombin
Textarin®/Ecarin ratio	*Pseudonaja textilis*	Prothrombin	Lupus anticoagulant
Russell's viper venom-	*Daboia russelii*	Factor X	Factor X
Dilute Russell's Viper	*Daboia russelii*	Factor X, Factor V	Lupus anticoagulant
Taipan Venom Time	*Oxyuranus scutellatus*	Prothrombin	Lupus anticoagulant
Pefakit® APCR Factor V Leiden	*Daboia russelii / Notechis scutatus scutatus*	Factor V / Protein C / Prothrombin	Resistance to activated protein C
Botrocetin®	*Bothrops sp*	Factor VIIIa	von Willebrand Factor

Table 1.1: Snake venom-derived products currently on the market (drugs and diagnostic tools) or in clinical and pre-clinical trials. Information was extracted from (204-209) and updated on the corresponding company website and/or the U.S. National Library of Medicine (https://clinicaltrials.gov/ct2/home). *Withdrawn from the market.

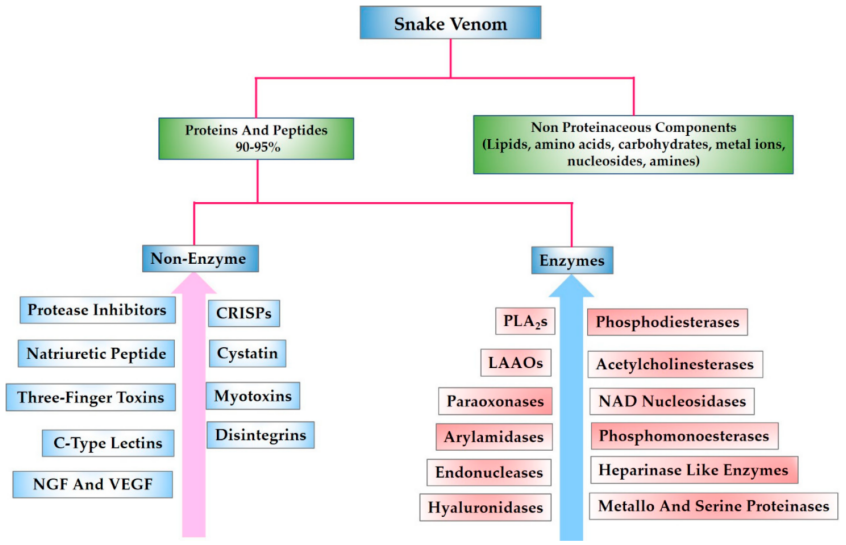


Figure 1.7: Snake venom composition. NFG, nerve growth factor; VEGF, vascular endothelial growth factor; CRISPs, cysteine-rich secretory proteins; PLA_2s, phospholipases A_2; L-AAOs, L-amino acid oxidases. Reprinted from (200).

pain, infections or cancer (Table 1.1), thus revealing the potential of snake toxins to serve as molecular scaffolds in diverse therapeutic fields.

Below, the antimicrobial and antitumoral potential of SVs are described with emphasis on peptide components displaying both activities.

1.3.4 SV-antimicrobial and antitumoral components

The process of envenomation is remarkably associated with a low incidence of microbial infections (210). This is paradoxical if one considers that a snakebite entails the appearance of punctant wounds, and usually takes place on the habitat of snakes, –mud of jungles and forests–, characterized by the presence of pathogenic bacteria. However, the ultimate goal of envenomation is to paralyze the prey prior to its ingestion. Thus, it is reasonable to conclude that SVs contain antibacterial agents that protect both the snake and its prey while feeding (211). Indeed, whole venoms from the *Crotalidae* family have been shown to possess antimicrobial activity against bacteria commonly found

in snake oral cavities, such as *P. aeruginosa* (MIC = 80 – 160 μg/mL) and *Alcaligenes faecalis* (MIC = 5 – 20 μg/mL), but also towards human pathogens such as *S. aureus* (MIC = 5 – 40 μg/mL) and *E. coli* (MIC = 80 – 160 μg/mL) (212). Additional studies demonstrated similar effects of SVs from a large variety of snakes (213), such as those of the *Viperidae* family (i.e., *Agkistrodon rhodostoma*, *Bothrops atrox* and *B. jarraca* are able to inhibit bacterial growth of clinical isolated strains of *Enterococcus faecalis*, *Staphylococcus epidermidis* and *S. aureus* (MICs = 4.5 – 13 μg/mL)) (214). The whole venom of *Bothrops alternatus* displays antimicrobial activity against *E. coli* and *S. aureus* strains at low concentrations (MIC = 0.312 μg/mL and 0.625 – 10 μg/mL, respectively), and *Burkholderia pseudomallei*, which causes melioidosis, is also inhibited by venoms of *Daboia ruselli ruselli* (MIC = 20 μg/mL) and *Pseudechis australis* (MIC = 40 μg/mL) (215).

In parallel, the ability of whole SVs to act against tumor cells was described in the late 1980s, with the discovery of elapid, crotalid and viperid crude venoms acting against melanoma and chondrosarcoma cell lines (216). Venoms from *B. jararaca* and *Crotalus durissus terrificus* also exhibited antitumoral properties, presumably by direct action on tumor cells and by modulating inflammatory responses (217, 218). Recently, the whole venom of *Ophiophagus hannah* demonstrated strong anti-cancer properties *in vitro* against pancreatic tumor cells as well as antiangiogenic ability *in vivo* (219).

Due to the therapeutic potential of SVs, efforts were focused to isolate and characterize the specific active compounds responsible for the antibacterial and antitumoral activity. Examples include enzymes and proteins such as PLA_2s, L-AAOs, metalloproteinases, disintegrins or lectins among others, and in the context of this thesis several SV-derived cationic AMPs and ACPs complete this arsenal of anti-infective and antitumoral components (220, 221).

1.3.4.1 SV-cathelicidins

OH-CATH: this cathelicidin, identified in the venom gland of the king cobra (*O. hannah*), was the first predicted SV-CATH to be synthesized and experimentally validated as AMP (88) (Table 1.2). OH-CATH exerts strong salt-resistant antibacterial activity against Gram-positive and Gram-negative bacteria (MICs in the 1 – 20 g/mL range) with weak hemolysis (10.8 % hemolysis observed at 200 g/mL) (88, 253). Its mode of action is, apparently, membrane-active and

inhibition of ATP-synthase (254, 255). A collection of analogs was designed by Zhang et al. to study the structure-function relationships of OH-CATH. The four N-terminal amino acid residues are highly responsible for the cytotoxicity towards eukaryotic cells while the 10-residue C-terminal span strongly influences the antimicrobial activity (253). OH-CATH30 and OH-CM6, two downsized versions of the parental peptide, also exhibited potent *in vitro* antibacterial activity by cell membrane disruption and were able to rescue infected mice in a bacteremia model induced by drug-resistant *E. coli* (255, 256).

Source organism	Peptide	Sequence	Lenght
Ophiophagus hannah	OH-CATH	KRFKKFFKKLKNSVKKRAKKFFKKPRVIGVSIPF	34
Bungarus fasciatus	BF-CATH	KRFKKFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	34
	BF-CATH30	KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	30
Naja atra	NA-CATH	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	34
Annulated sea snake	Hc-CATH	KFFKRLLKSVRRAVKKFRKKPRLIGLSTLL	30
Crotalus durissus terrificus, Crotalus durisus cascavella	Crotalicidin	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF	34
Bothrops atrox	Batroxicidin	KRFKKFFKKLKNSVKKRVKKFFRKPRVIGVTFPF	34
Pseudonaja textilis	Pt-CRAMP1	KRFKKFFMKLKKSVKKRVMKFFKKPMVIGVTFPF	34
	Pt-CRAMP2	KRFKKFFRKLKKSVKKRVKKFFKKPRVIGVTIPF	34
Lachesis muta rhombeata	Lachesicidin	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTFPF	34
Bothrops lutzi	Lutzicidin	KRFKKFFKKLKNNVKKRVKKFFRKPRVIGVTIPF	34
Python bivittatus	ΔPb-CATH1	RVKRFKKFFRKIKKGFRKIFKKTKIFIG	28
	Pb-CATH3	HRVKRNGFRKFMRRLKKFFAGG	22
	ΔPb-CATH4	TRSRWRRFIRGAGRFARRYGWRIA	24
	CATHPb1	KRFKKFFRKIKKGFRKIFKKTKIFIGGTIPI	31
	CATHPb2	KRNGFRKFMRRLKKFFAGGGSSIAHIKLH	29
	CATHPb3	KRFQNFFRELEKKFREFFRVYRITIGATIRF	31
	CATHPb4	TRSRWRRFIRGAGRFARRYGWRIALGLVG	29
	CATHPb5	SPPQAMGFPPQVNVEHYIPASYSVAALTVTEEE	33
	CATHPb6	RAAPQRRLRAMARLKKFAEAGGADPDSGGLRARFPER	37

Table 1.2: Experimentally validated SV-CATHs and their properties. Net charge calculated at pH = 7 for free N-terminal and C-terminal peptides. The biological activities described on literature for each peptide was collected and analyzed. Hemolysis (μg/mL) was clasified as high ($HC_{10} \leq 50$), medium ($HC_{10} > 50$ and ≤ 100), or low ($HC_{10} > 100$). Peptides were considered active against microorganisms or tumor cells if their MIC/EC_{50} is < 100 μg/mL.

NA-CATH: this cathelicidin from the venom gland of the Chinese cobra *Naja atra* (88) also demonstrated powerful salt-resistant antimicrobial activity against Gram-positive and Gram-negative bacteria at low concentrations (EC_{50} in the 0.3 – 3 g/mL range) (237-240, 257). NA-CATH is also active against *Mycobacterium*, *Burkholderia thailandensis* (closely related to *B. pseudomallei*, the causing agent of melioidosis) and *Bacillus anthracis* (anthrax causing bacteria) (241-243). The last two strains are of particular relevance due to their potential use as biological weapons.

Net charge	Hemolysis	Activity	Ref
15	Medium	Active against Gram + and Gram - bacteria.	(88, 222, 223)
14	High	Active against Gram + and Gram - bacteria.	(224, 225)
11	High	Active against Gram + and Gram - bacteria, fungi and tumor cells. Activation of innate immunity and antiinflamatory.	(226-236)
15	Low	Active against Gram + and Gram - bacteria.	(237-243)
12	Low	Active against Gram + and Gram - bacteria and fungi. Inactive against tumor cells. Antiinflamatory.	(244, 245)
15	High	Active against Gram +, Gram - bacteria, fungi, parasites and tumor cells. Overall proinflammatory	(223, 246-249)
15	High	Active against Gram +, Gram - bacteria and parasites. Overall proinflammatory	(223, 249, 250)
13	High	Active against Gram + and Gram - bacteria.	(223)
16	n.d.	n.d.	(223)
15	n.d.	n.d.	(223)
15	n.d.	n.d.	(223)
14	Medium	Active against Gram + and Gram - bacteria.	(251)
9.1	Medium or low	Active against Gram + and Gram - bacteria.	(251)
9	Medium	Active against Gram + and Gram - bacteria and tumor cells.	(251)
13	Low	Active against Gram + and Gram - bacteria and fungi. Chemotactic and antiinflammatory.	(252)
9.2	Low	Active against Gram + and Gram - bacteria and fungi. Chemotactic and weakly antiinflammatory.	(252)
6	Low	Inactive against Gram + and Gram - bacteria and fungi. Immunomodulatory inactive.	(252)
9	Medium or high	Active against Gram + and Gram - bacteria and fungi.Weakly antiinflammatory.	(252)
-3.9	Low	Inactive against Gram + and Gram - bacteria and fungi. Immunomodulatory inactive.	(252)
6	Low	Inactive against Gram + and Gram - bacteria and fungi. Weakly antiinflammatory.	(252)

Indeed, *in vivo* studies using wax moth larvae demonstrated that NA-CATH was able to rescue 100 % of waxworms after *B. anthracis* Sterne infection at low peptide concentrations (243). In addition, NA-CATH is not only active against planktonic bacteria, but also able to inhibit biofilm formation (240, 241) while inducing minimal hemolysis (< 2 % at 100 g/mL) (238).

Structurally, NA-CATH folds into a well-defined amphipathic α-helix between residues Phe3 and Lys23 in the presence of TFE. The remaining 11-residue free hydrophobic tail does not present a defined structure but appears to contribute in the interaction with lipid membranes (258). In terms of sequence, NA-CATH contains an imperfectly 11-residue sequence [KR(F/A)KKFFKK(L/P)K] known as ATRA motif, repeated twice inside its full sequence and almost totally shared by other SV-CATHs (compare sequences on Table 1.2). The ATRA motif, when tested isolated, is also active, but the presence of a Pro residue at position 10 dramatically reduces its antibacterial potency, probably by destabilizing its helical structure, while a Phe to Ala change at position 3 does not impair the activity (238).

Du et al. postulated that NA-CATH was able to disrupt bacterial membrane-like liposomes via membrane thinning or transient-pore formation (258), an hypothesis further confirmed *in vitro* by Gupta el al., who described membrane depolarization (and transient-pore formation) of *Mycobacterium smegmatis* after NA-CATH treatment (242). However, Samuel et al. suggested a more detailed mechanism changing from membrane disruption to pore-based lysis depending on liposome lipid composition and phase (259).

BF-CATH: unlike other AMPs predicted as SV-CATHs, the purified peptide from the *Bungarus fasciatus* (banded krait) venom gland is a 30-mer peptide lacking the four N-terminal amino acid residues (Table 1.2), which suggests a different enzymatic processing or post-processing of the 34-residue precursor (236). The expression of BF-CATH is widespread, including stomach, trachea, skin, muscle, heart, kidney, lung, brain, intestine, spleen, liver, ovary and venomous glands (236).

Similar to other SV-CATHs, BF-CATH has random-coil conformation in water solution, but adopts α-helical structure in hydrophobic or membrane-mimic environments, specifically from residues Arg2 to Ala18 (236). BF-CATH presents potent antimicrobial activity including a broad range of standard and clinically isolated drug-resistant Gram-negative and Gram-positive bacteria as

well as saprophytic fungus (236). It partially retains the antibacterial activity in the presence of human serum (260). Interestingly, BF-CATH was less prone to induce bacterial resistance than classical antibiotics such as ciprofloxacin or gentamicin (233). BF-CATH demonstrated to have a membrane disruption antibacterial mechanism (*in vitro* and *in vivo* murine models of *P. aeruginosa* and *Salmonella typhimurium* infections) (233, 260).

In addition, BF-CATH inhibits secretion of pro-inflammatory molecules, such as TNF-α, IL-8, IL-1β, or MCP-1 and $O_2{\cdot}^-$ production induced by *Propionibacterium acnes* (acne vulgaris causing agent) (234). BF-CATH demonstrated *in vivo* prevention of intestinal barrier dysfunction LPS-induced endotoxemia/inflammation in mouse and weaned piglet models (228, 231). In particular, BF-CATH seems to suppress intestinal inflammation and attenuate ulcerative colitis by down-regulating NF-signaling pathway and/or enhancing phagocytosis of immune cells by activating STAT-1 (229, 230). Liu et al. recently demonstrated that pretreatment with BF-CATH ameliorated pneumonia in mice induced by *P. aeruginosa* by effectively activating innate immunity (227). In parallel, BF-CATH also exhibits anticancer activity, inhibiting melanoma cell proliferation *in vivo* and *in vitro* via membrane disruption and DNA-binding, preventing transcription and translation of VEGF gene (232).

Different strategies were addressed to study the structure-activity relationships of BF-CATH and optimize its framework, ranging from size reduction (235, 236), to point mutations (225, 226, 261-263), which showed antimicrobial (both planktonic and biofilm-forming strains), antitumoral, antimalarial and antifungal properties with enhanced properties in some reported cases.

Vipericidins: the discovery of SV-CATHs, named vipericidins, in the venom of South American snakes was described by our lab and motivated this thesis project. The principal features of vipericidins are described in Table 1.2 in order to allow comparisons with other SV-derived peptides but their characterization and further details will be discussed during the next chapters of this thesis.

Other SV-CATHs: SV-CATHs were also predicted/identified in the genome of *Python bivittatus* alternatively by Kim et al. and Cai et al. (see Table 1.2) leading to Pb-CATH1-5 and CATHPb1-6, respectively (251, 252). From the set of Pb-CATHs identified by Kim et al., three of them (Pb-CATH1, Pb-CATH3 and Pb-CATH4) encode mature AMPs that were synthesized and

validated. These demonstrated powerful antibacterial activity against Gram-negative bacteria (MICs from 0.5 to 8 g/mL) (251). Pb-CATH4, for instance, induced bacterial death by toroidal pore formation and displayed low hemolysis and cytotoxicity, as well as considerable stability in serum (251). In parallel, CATHPb1 exhibited protection in mice infected with MRSA and VRSA, via neutrophil-mediated bacteria clearance and immunomodulation throughout MAPKs and NF-pathways (252).

Finally, another cathelicidin-related peptide, Hc-CATH (Table 1.2), was also identified in the genome of the sea snake *Hydrophis cyanocinctus* (annulated sea snake) (244). Unlike the overall preference of the terrestrial snake derived CATHs for Gram-negative bacteria, Hc-CATH displays relatively equivalent activity for both Gram-negative and Gram-positive bacteria both due to membrane permeabilization (244). Hc-CATH has intrinsical structural advantages compared to other CATHs, such as high stability in the presence of salts, temperature and serum proteases, together with low toxicity against eukaryotic cells (244). In a recent study, Carlile et al. demonstrated the anti-inflammatory and bacterial load reduction of Hc-CATH *in vivo*, using wax moth and mouse models of intraperitoneal and respiratory infection induced by *P. aeruginosa*. (245).

1.3.4.2 Defensins

Together with cathelicidins, defensins are a major group of HDPs found in vertebrates and invertebrates exhibiting broad-spectrum activity against bacteria, fungi and enveloped viruses (264). Defensins are cationic, Cys-rich peptides of 3.5 – 6 kDa, with a typical β-sheet-rich fold stabilized by three disulfide bonds. They are divided into three major groups based on length and Cys-pairing: α-, β-, and θ-defensins (265). Although defensins have been deeply studied in mammals, little information is available for those of SV origin. Thirteen β-defensin-like sequences were described in *Bothrops* and *Lachesis* snakes (266, 267) as well as recently in *Colubridae* snakes (268). However, no details regarding their antibacterial or antitumoral potency are available.

1.3.4.3 Crotamine

Crotamine is a 42-mer neuro- and myotoxic peptide initially isolated from the South American rattlesnake (*C. durissus terrificus*), whose anti-infective, antitumoral and cell-penetrating properties have been deeply characterized both *in vitro* and *in vivo* (269). Structurally, crotamine is closely related to β-defensins, displaying an $\alpha_1\beta_1\alpha_2\beta_2$ arrangement, with the whole structure stabilized by three disulfide bonds (270). Crotamine displays modest antibacterial activity (MICs in the 25 – 100 g/mL range against *E. coli* strains) and does not induce hemolysis at high concentrations (no hemolysis observed up to 1024 g/mL) (271). Additional studies revealed antifungal activity of crotamine (12.5 – 50 g/mL) against several fungi of *Candida* spp., including clinically resistant strains (272). The anticancer potential of crotamine has been also studied *in vitro* and *in vivo*, showing selective cytotoxicity against tumor cell lines at low concentration ($\sim$ 5 g/mL) and significant inhibition of tumor growth and increased lifespan in a melanoma mouse model (273, 274).

Different derivatives of crotamine were designed such as CyLoP-1 (cytosol localizing peptide 1) and the NrTPs (nucleolar-targeting peptides), with cytosolic and nucleolar localization respectively, instead of the nuclear distribution pattern of crotamine (275-277).

1.3.4.4 Waprins

Waprins are a family of $\sim$ 50-residue, Cys-rich peptides first isolated in the venom of the *Naja nigricollis* (black-necked spitting cobra), named nawaprin, and subsequently in the venom of the Indian taipan (*Oxyuranus microlepidotus*), named omwaprin (Omw) (278, 279). Omwaprin displays salt-resistant antibacterial activity against Gram-positive bacteria such as *Bacillus megaterium* and *Staphylococcus warneri* (279). Two derivatives, Omw1 and Omw2, also present modest antimicrobial, antifungical and antibiofilm activity at concentrations ranging from 16 – 500 g/mL, presumably by a membrane disruption mechanism with low hemolysis induction within the same concentration range ($\sim$ 10 % hemolysis at 250 – 500 g/mL) (280). Structurally, both parental nawaprin and omwaprin display a complex disc-like shape knotted by four disulfide bonds (278, 281).

1.3.4.5 Other SV-AMPs and -ACPs

Additional SV-proteins and peptides whose principal function is not directly related with bacteria or tumor clearance have demonstrated antimicrobial, antifungal, antmitumoral, and immunomodulatory properties. This is the case of the L-AAO and PLA_2 family of proteins, major components of snake venoms (282, 283). Other SV components displaying such a variety of activities include metalloproteinases (284-286), cardio-, neuro- or myotoxins (287-290), disintegrins (291) or lectins (292-294), among others (295-297). These compounds exert their activity through particular mechanisms of action, including direct toxic action (PLA_2s), free radical generation (L-AAOs), induction of apoptosis (PLA_2s, L-AAOs and metalloproteinases), and anti-angiogenesis (disintegrins and lectins) (295).

References

1. WHO. 2017. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. Geneva: World Health Orgaization;2017 (WHO/EMP/IAU/2017.12). Licence: CC BY-NC-SA 3.0 IGO.

2. O'Neill J. Antimicrobial Resistance: tackling a crisis for the health and wealth of nations. Rev Antimicrobial Resist. 2014.

3. O'Neill J. Tackling drug-resistant infection globally: final report and recommendations. Rev Antimicrobial Resist. 2016.

4. World Bank. 2017. "Drug-Resistant Infections: A Threat to Our Economic Future." Washington, DC: World Bank. License: Creative Commons Attribution CC BY 3.0 IGO.

5. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health. 2014;2:145.

6. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P & T : a peer-reviewed journal for formulary management. 2015;40(4):277-83.

7. Luyt CE, Brechot N, Trouillet JL, Chastre J. Antibiotic stewardship in the intensive care unit. Crit Care. 2014;18(5):480.

8. CDC. 2017. Antibiotic Use in the United States, 2017: Progress and Opportunities. Atlanta, GA: US Department of Health and Human Services, CDC; 2017.

9. Marshall BM, Levy SB. Food animals and antimicrobials: impacts on human health. Clinical microbiology reviews. 2011;24(4):718-33.

10. Bartlett JG, Gilbert DN, Spellberg B. Seven ways to preserve the miracle of antibiotics. Clin Infect Dis. 2013;56(10):1445-50.

11. O'Neill J. Tackling drug-resistant infections globally: an overview of our work. . Rev Antimicrobial Resist. 2016.

12. Gould IM, Bal AM. New antibiotic agents in the pipeline and how they can help overcome microbial resistance. Virulence. 2013;4(2):185-91.

13. Piddock LJV. The crisis of no new antibiotics—what is the way forward? The Lancet Infectious Diseases. 2012;12(3):249-53.

14. Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007;128(6):1037-50.

15. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 2016;4(2).

16. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nature reviews Microbiology. 2015;13(1):42-51.

17. WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics. Available from: https://www.who.int/medicines /publications /global-priority-list-antibiotic-resistant-bacteria/en/, accessed [20 July 2019].

18. Thabit AK, Crandon JL, Nicolau DP. Antimicrobial resistance: impact on clinical and economic outcomes and the need for new antimicrobials. Expert Opin Pharmacother. 2015;16(2):159-77.

19. Nambiar S, Laessig K, Toerner J, Farley J, Cox E. Antibacterial drug development: challenges, recent developments, and future considerations. Clin Pharmacol Ther. 2014;96(2):147-9.

20. Fjell CD, Jenssen H, Hilpert K, Cheung WA, Pante N, Hancock RE, et al. Identification of novel antibacterial peptides by chemoinformatics and machine learning. J Med Chem. 2009;52(7):2006-15.

21. Monserrat-Martinez A, Gambin Y, Sierecki E. Thinking Outside the Bug: Molecular Targets and Strategies to Overcome Antibiotic Resistance. Int J Mol Sci. 2019;20(6).

22. Ghosh C, Sarkar P, Issa R, Haldar J. Alternatives to Conventional Antibiotics in the Era of Antimicrobial Resistance. Trends Microbiol. 2019.

23. Rios AC, Moutinho CG, Pinto FC, Del Fiol FS, Jozala A, Chaud MV, et al. Alter-

natives to overcoming bacterial resistances: State-of-the-art. Microbiol Res. 2016;191:51-80.
24. Coates A, Hu Y, Bax R, Page C. The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov. 2002;1(11):895-910.
25. WHO. 2018. Cancer fact sheets. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer, accessed [06 August 2019].
26. Ferlay JE, M. Lam, F. Colombet, M. Mery, L. Piñeros, M. Znaor, A. Soerjomataram, I. Bray, F. Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [06 August 2019]. 2018.
27. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
28. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Pineros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. International journal of cancer. 2019;144(8):1941-53.
29. Wilking NE, Hofmarcher T, Lindgren P, Jönsson B. The burden and direct cost of cancer in Europe (EU-28). Journal of Clinical Oncology. 2016;34(15_suppl):6618-.
30. American Cancer Society Cancer Action Network. 2017. The Costs of Cancer. Available from: https://www.fightcancer.org/. accessed [9 August 2019].
31. Surveillance, Epidemiology, and End Results (SEER) Program (http://www.seer.cancer.gov/) SEER*Stat Database: Incidence - SEER 9 Regs Research Data, Nov 2018 Sub (1975-2016) <Katrina/Rita Population Adjustment> - Linked To County Attributes - Total U.S., 1969-2017 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, released April 2019, based on the November 2018 submission.
32. Cooper M. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. The Development and Causes of Cancer. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9963/.
33. Pitot HC. The molecular biology of carcinogenesis. Cancer. 1993;72(3 Suppl):962-70.
34. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell. 2011;144(5):646-74.
35. Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature. 2006;441(7092):457-62.
36. Sun J, Wei Q, Zhou Y, Wang J, Liu Q, Xu H. A systematic analysis of FDA-approved anticancer drugs. BMC Syst Biol. 2017;11(Suppl 5):87.
37. Kelderman S, Schumacher TN, Haanen JB. Acquired and intrinsic resistance in cancer immunotherapy. Mol Oncol. 2014;8(6):1132-9.
38. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science. 2001;293(5531):876-80.
39. Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. The New England journal of medicine. 2005;352(8):786-92.
40. Tamborini E, Bonadiman L, Greco A, Albertini V, Negri T, Gronchi A, et al. A new mutation in the KIT ATP pocket causes acquired resistance to imatinib in a gastrointestinal stromal tumor patient. Gastroenterology. 2004;127(1):294-9.
41. Sawyers CL. Calculated resistance in cancer. Nature medicine. 2005;11(8):824-5.
42. Perez-Tomas R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. Current medicinal chemistry. 2006;13(16):1859-76.

43. Li J, Jiang E, Wang X, Shangguan AJ, Zhang L, Yu Z. Dormant Cells: The Original Cause of Tumor Recurrence and Metastasis. Cell biochemistry and biophysics. 2015;72(2):317-20.
44. Talukdar S, Bhoopathi P, Emdad L, Das S, Sarkar D, Fisher PB. Dormancy and cancer stem cells: An enigma for cancer therapeutic targeting. Advances in cancer research. 2019;141:43-84.
45. Sierra JM, Fuste E, Rabanal F, Vinuesa T, Vinas M. An overview of antimicrobial peptides and the latest advances in their development. Expert opinion on biological therapy. 2017;17(6):663-76.
46. Ciumac D, Gong H, Hu X, Lu JR. Membrane targeting cationic antimicrobial peptides. J Colloid Interface Sci. 2019;537:163-85.
47. Graf M, Wilson DN. Intracellular Antimicrobial Peptides Targeting the Protein Synthesis Machinery. Adv Exp Med Biol. 2019;1117:73-89.
48. Otvos L, Jr. Immunomodulatory effects of anti-microbial peptides. Acta Microbiol Immunol Hung. 2016;63(3):257-77.
49. Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. 2005;30(7):505-15.
50. Zhang LJ, Gallo RL. Antimicrobial peptides. Curr Biol. 2016;26(1):R14-9.
51. Kim S, Kim SS, Bang YJ, Kim SJ, Lee BJ. In vitro activities of native and designed peptide antibiotics against drug sensitive and resistant tumor cell lines. Peptides. 2003;24(7):945-53.
52. Camilio KA, Berge G, Ravuri CS, Rekdal O, Sveinbjornsson B. Complete regression and systemic protective immune responses obtained in B16 melanomas after treatment with LTX-315. Cancer Immunol Immunother. 2014;63(6):601-13.
53. Hilchie AL, Hoskin DW, Power Coombs MR. Anticancer Activities of Natural and Synthetic Peptides. Adv Exp Med Biol. 2019;1117:131-47.
54. Hansel W, Enright F, Leuschner C. Destruction of breast cancers and their metastases by lytic peptide conjugates in vitro and in vivo. Mol Cell Endocrinol. 2007;260-262:183-9.
55. Johnstone SA, Gelmon K, Mayer LD, Hancock RE, Bally MB. In vitro characterization of the anticancer activity of membrane-active cationic peptides. I. Peptide-mediated cytotoxicity and peptide-enhanced cytotoxic activity of doxorubicin against wild-type and p-glycoprotein over-expressing tumor cell lines. Anti-cancer drug design. 2000;15(2):151-60.
56. Hilchie AL, Sharon AJ, Haney EF, Hoskin DW, Bally MB, Franco OL, et al. Mastoparan is a membranolytic anticancer peptide that works synergistically with gemcitabine in a mouse model of mammary carcinoma. Biochim Biophys Acta. 2016;1858(12):3195-204.
57. Tam JP, Wang S, Wong KH, Tan WL. Antimicrobial Peptides from Plants. Pharmaceuticals. 2015;8(4):711-57.
58. Brogden KA, Ackermann M, McCray PB, Jr., Tack BF. Antimicrobial peptides in animals and their role in host defences. Int J Antimicrob Agents. 2003;22(5):465-78.
59. Yazici A, Ortucu S, Taskin M, Marinelli L. Natural-based Antibiofilm and Antimicrobial Peptides from Microorganisms. Current topics in medicinal chemistry. 2018;18(24):2102-7.
60. Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. Journal of applied microbiology. 2012;113(4):723-36.
61. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415(6870):389-95.
62. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24(12):1551-7.
63. Bastos P, Trindade F, da Costa J, Ferreira

R, Vitorino R. Human Antimicrobial Peptides in Bodily Fluids: Current Knowledge and Therapeutic Perspectives in the Postantibiotic Era. Med Res Rev. 2018;38(1):101-46.
64. Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, et al. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J Biol Chem. 1997;272(24):15258-63.
65. Harder J, Bartels J, Christophers E, Schroder JM. A peptide antibiotic from human skin. Nature. 1997;387(6636):861.
66. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem. 2001;276(8):5707-13.
67. Harder J, Schroder JM. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. J Biol Chem. 2002;277(48):46779-84.
68. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, et al. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. J Biol Chem. 1988;263(16):7472-7.
69. Fleming A, Allison VD. Observations on a Bacteriolytic Substance (âœLysozymeâ) Found in Secretions and Tissues. British journal of experimental pathology. 1922;3(5):252-60.
70. Bals R, Wang X, Zasloff M, Wilson JM. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(16):9541-6.
71. Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell. 1997;88(4):553-60.
72. Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M, et al. Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J Clin Invest. 1998;102(5):874-80.
73. Chan CC, Moser JM, Dyer KD, Percopo CM, Rosenberg HF. Genetic diversity of human RNase 8. BMC Genomics. 2012;13:40.
74. Jones DE, Bevins CL. Defensin-6 mRNA in human Paneth cells: implications for antimicrobial peptides in host defense of the human bowel. FEBS Lett. 1993;315(2):187-92.
75. Cunliffe RN. Alpha-defensins in the gastrointestinal tract. Mol Immunol. 2003;40(7):463-7.
76. Schroeder BO, Ehmann D, Precht JC, Castillo PA, Kuchler R, Berger J, et al. Paneth cell alpha-defensin 6 (HD-6) is an antimicrobial peptide. Mucosal Immunol. 2015;8(3):661-71.
77. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol. 2003;4(3):269-73.
78. Wang G. Human antimicrobial peptides and proteins. Pharmaceuticals. 2014;7(5):545-94.
79. Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI. Primary structures of three human neutrophil defensins. J Clin Invest. 1985;76(4):1436-9.
80. Wilde CG, Griffith JE, Marra MN, Snable JL, Scott RW. Purification and characterization of human neutrophil peptide 4, a novel member of the defensin family. J Biol Chem. 1989;264(19):11200-3.
81. Slifman NR, Loegering DA, McKean DJ, Gleich GJ. Ribonuclease activity associated with human eosinophil-derived neurotoxin and eosinophil cationic protein. J Immunol. 1986;137(9):2913-7.
82. Zanetti M. The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol. 2005;7(2):179-96.
83. Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. The human

antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood. 1997;90(7):2796-803.
84. Frohm Nilsson M, Sandstedt B, Sorensen O, Weber G, Borregaard N, Stahle-Backdahl M. The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect Immun. 1999;67(5):2561-6.
85. Stie J, Jesaitis AV, Lord CI, Gripentrog JM, Taylor RM, Burritt JB, et al. Localization of hCAP-18 on the surface of chemoattractant-stimulated human granulocytes: analysis using two novel hCAP-18-specific monoclonal antibodies. J Leukoc Biol. 2007;82(1):161-72.
86. Stolzenberg ED, Anderson GM, Ackermann MR, Whitlock RH, Zasloff M. Epithelial antibiotic induced in states of disease. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(16):8686-90.
87. Konig E, Bininda-Emonds OR, Shaw C. The diversity and evolution of anuran skin peptides. Peptides. 2015;63:96-117.
88. Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, et al. Identification and characterization of novel reptile cathelicidins from elapid snakes. Peptides. 2008;29(10):1685-91.
89. Tassanakajon A, Somboonwiwat K, Amparyup P. Sequence diversity and evolution of antimicrobial peptides in invertebrates. Developmental and comparative immunology. 2015;48(2):324-41.
90. Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res. 2009;37(Database issue):D933-7.
91. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev. 2003;55(1):27-55.
92. Koehbach J, Craik DJ. The Vast Structural Diversity of Antimicrobial Peptides. Trends Pharmacol Sci. 2019;40(7):517-28.
93. Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proceedings of the National Academy of Sciences of the United States of America. 1987;84(15):5449-53.
94. Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A. The human cathelicidin LL-37–A pore-forming antibacterial peptide and host-cell modulator. Biochim Biophys Acta. 2016;1858(3):546-66.
95. Kokryakov VN, Harwig SS, Panyutich EA, Shevchenko AA, Aleshina GM, Shamova OV, et al. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett. 1993;327(2):231-6.
96. Andersson HS, Figueredo SM, Haugaard-Kedstrom LM, Bengtsson E, Daly NL, Qu X, et al. The alpha-defensin salt-bridge induces backbone stability to facilitate folding and confer proteolytic resistance. Amino Acids. 2012;43(4):1471-83.
97. Hoover DM, Chertov O, Lubkowski J. The structure of human beta-defensin-1: new insights into structural properties of beta-defensins. J Biol Chem. 2001;276(42):39021-6.
98. Cabiaux V, Agerberth B, Johansson J, Homble F, Goormaghtigh E, Ruysschaert JM. Secondary structure and membrane interaction of PR-39, a Pro+Arg-rich antibacterial peptide. European journal of biochemistry. 1994;224(3):1019-27.
99. Rozek A, Friedrich CL, Hancock RE. Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. Biochemistry. 2000;39(51):15765-74.
100. Hsu ST, Breukink E, Tischenko E, Lutters MA, de Kruijff B, Kaptein R, et al. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nature structural & molecular biology. 2004;11(10):963-7.
101. Gonzalez C, Langdon GM, Bruix M, Galvez A, Valdivia E, Maqueda M, et al. Bac-

teriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(21):11221-6.
102. Saether O, Craik DJ, Campbell ID, Sletten K, Juul J, Norman DG. Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. Biochemistry. 1995;34(13):4147-58.
103. Swain SS, Paidesetty SK, Padhy RN. Antibacterial, antifungal and antimycobacterial compounds from cyanobacteria. Biomedicine pharmacotherapy = Biomedecine pharmacotherapie. 2017;90:760-76.
104. Bahar AA, Ren D. Antimicrobial peptides. Pharmaceuticals. 2013;6(12):1543-75.
105. Yeung AT, Gellatly SL, Hancock RE. Multifunctional cationic host defence peptides and their clinical applications. Cell Mol Life Sci. 2011;68(13):2161-76.
106. Gomes B, Augusto MT, Felicio MR, Hollmann A, Franco OL, Goncalves S, et al. Designing improved active peptides for therapeutic approaches against infectious diseases. Biotechnol Adv. 2018;36(2):415-29.
107. Scarsini M, Tomasinsig L, Arzese A, D'Este F, Oro D, Skerlavaj B. Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of Candida species isolated from vaginal infections. Peptides. 2015;71:211-21.
108. Gallo M, Defaus S, Andreu D. 1988-2018: Thirty years of drug smuggling at the nano scale. Challenges and opportunities of cell-penetrating peptides in biomedical research. Archives of biochemistry and biophysics. 2018;661:74-86.
109. Felicio MR, Silva ON, Goncalves S, Santos NC, Franco OL. Peptides with Dual Antimicrobial and Anticancer Activities. Front Chem. 2017;5:5.
110. Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. Biochim Biophys Acta. 2016;1858(5):980-7.
111. Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol. 2000;8(9):402-10.
112. Hilchie AL, Wuerth K, Hancock RE. Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat Chem Biol. 2013;9(12):761-8.
113. Al-Benna S, Shai Y, Jacobsen F, Steinstraesser L. Oncolytic activities of host defense peptides. Int J Mol Sci. 2011;12(11):8027-51.
114. Baxter AA, Lay FT, Poon IKH, Kvansakul M, Hulett MD. Tumor cell membrane-targeting cationic antimicrobial peptides: novel insights into mechanisms of action and therapeutic prospects. Cell Mol Life Sci. 2017;74(20):3809-25.
115. Rosca EVK, J.E.; Rivera, C.G.; Pandey, N.B.; Tamin, A.P.; Popel, A.S. Anti-angiogenic peptides for cancer therapeutics. Curr Pharm Biotechnol. 2011;12(8):1101.
116. Chavakis T, Cines DB, Rhee JS, Liang OD, Schubert U, Hammes HP, et al. Regulation of neovascularization by human neutrophil peptides (alpha-defensins): a link between inflammation and angiogenesis. FASEB J. 2004;18(11):1306-8.
117. Yoo YC, Watanabe S, Watanabe R, Hata K, Shimazaki K, Azuma I. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. Japanese journal of cancer research : Gann. 1997;88(2):184-90.
118. Xu N, Wang YS, Pan WB, Xiao B, Wen YJ, Chen XC, et al. Human alpha-defensin-1 inhibits growth of human lung adenocarcinoma xenograft in nude mice. Molecular cancer therapeutics. 2008;7(6):1588-97.
119. Neundorf I. Antimicrobial and Cell-Penetrating Peptides: How to Understand Two Distinct Functions Despite Similar Physicochemical Properties. Adv Exp Med Biol. 2019;1117:93-109.
120. Epand RM, Epand RF. Domains in bacterial membranes and the action of antimicrobial agents. Mol Biosyst. 2009;5(6):580-7.
121. Mileykovskaya E, Dowhan W. Cardi-

olipin membrane domains in prokaryotes and eukaryotes. Biochim Biophys Acta. 2009;1788(10):2084-91.
122. Malanovic N, Lohner K. Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. Biochim Biophys Acta. 2016;1858(5):936-46.
123. Aurell CA, Wistrom AO. Critical aggregation concentrations of gram-negative bacterial lipopolysaccharides (LPS). Biochem Biophys Res Commun. 1998;253(1):119-23.
124. Alves AC, Ribeiro D, Nunes C, Reis S. Biophysics in cancer: The relevance of drug-membrane interaction studies. Biochim Biophys Acta. 2016;1858(9):2231-44.
125. Casares D, Escriba PV, Rossello CA. Membrane Lipid Composition: Effect on Membrane and Organelle Structure, Function and Compartmentalization and Therapeutic Avenues. Int J Mol Sci. 2019;20(9).
126. Utsugi T, Schroit AJ, Connor J, Bucana CD, Fidler IJ. Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. Cancer research. 1991;51(11):3062-6.
127. Schweizer F. Cationic amphiphilic peptides with cancer-selective toxicity. Eur J Pharmacol. 2009;625(1-3):190-4.
128. Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta. 2008;1778(2):357-75.
129. Sok M, Sentjurc M, Schara M. Membrane fluidity characteristics of human lung cancer. Cancer letters. 1999;139(2):215-20.
130. Domagala W, Koss LG. Surface configuration of human tumor cells obtained by fine needle aspiration biopsy. Scanning electron microscopy. 1980(3):101-8.
131. Travkova OG, Moehwald H, Brezesinski G. The interaction of antimicrobial peptides with membranes. Adv Colloid Interface Sci. 2017.
132. Leite ML, da Cunha NB, Costa FF. Antimicrobial Peptides, Nanotechnology, and Natural Metabolites as Novel Approaches for Cancer Treatment. Pharmacol Ther. 2017.
133. Hancock RE, Bell A. Antibiotic uptake into gram-negative bacteria. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 1988;7(6):713-20.
134. Teixeira V, Feio MJ, Bastos M. Role of lipids in the interaction of antimicrobial peptides with membranes. Prog Lipid Res. 2012;51(2):149-77.
135. Huang HW. Action of antimicrobial peptides: two-state model. Biochemistry. 2000;39(29):8347-52.
136. Melo MN, Ferre R, Castanho MA. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. Nature reviews Microbiology. 2009;7(3):245-50.
137. Oren Z, Lerman JC, Gudmundsson GH, Agerberth B, Shai Y. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. Biochem J. 1999;341 (Pt 3):501-13.
138. Lombardi L, Falanga A, Del Genio V, Galdiero S. A New Hope: Self-Assembling Peptides with Antimicrobial Activity. Pharmaceutics. 2019;11(4).
139. Ros U, Carretero GPB, Paulino J, Crusca E, Jr., Pazos F, Cilli EM, et al. Self-association and folding in membrane determine the mode of action of peptides from the lytic segment of sticholysins. Biochimie. 2019;156:109-17.
140. Gesell J, Zasloff M, Opella SJ. Two-dimensional 1H NMR experiments show that the 23-residue magainin antibiotic peptide is an alpha-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution. Journal of biomolecular NMR. 1997;9(2):127-35.
141. Faust JE, Yang PY, Huang HW. Action of Antimicrobial Peptides on Bacterial and Lipid Membranes: A Direct Comparison. Biophysical journal. 2017;112(8):1663-72.
142. Sani MA, Separovic F. How Membrane-

Active Peptides Get into Lipid Membranes. Acc Chem Res. 2016;49(6):1130-8.
143. Wang L, Dong C, Li X, Han W, Su X. Anticancer potential of bioactive peptides from animal sources (Review). Oncol Rep. 2017;38(2):637-51.
144. Matsuzaki K. Membrane Permeabilization Mechanisms. Adv Exp Med Biol. 2019;1117:9-16.
145. Lee TH, Hofferek V, Separovic F, Reid GE, Aguilar MI. The role of bacterial lipid diversity and membrane properties in modulating antimicrobial peptide activity and drug resistance. Curr Opin Chem Biol. 2019;52:85-92.
146. Deslouches B, Di YP. Antimicrobial peptides with selective antitumor mechanisms: prospect for anticancer applications. Oncotarget. 2017;8(28):46635-51.
147. Matsuzaki K. Magainins as paradigm for the mode of action of pore forming polypeptides. Biochim Biophys Acta. 1998;1376(3):391-400.
148. Pogliano J, Pogliano N, Silverman JA. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol. 2012;194(17):4494-504.
149. Taylor SD, Palmer M. The action mechanism of daptomycin. Bioorg Med Chem. 2016;24(24):6253-68.
150. Xu H, Chen CX, Hu J, Zhou P, Zeng P, Cao CH, et al. Dual modes of antitumor action of an amphiphilic peptide A(9)K. Biomaterials. 2013;34(11):2731-7.
151. Wang C, Tian LL, Li S, Li HB, Zhou Y, Wang H, et al. Rapid cytotoxicity of antimicrobial peptide tempoprin-1CEa in breast cancer cells through membrane destruction and intracellular calcium mechanism. PLoS One. 2013;8(4):e60462.
152. Casteels P, Tempst P. Apidaecin-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity. Biochem Biophys Res Commun. 1994;199(1):339-45.
153. Fuerst JA, Sagulenko E. Protein uptake by bacteria: An endocytosis-like process in the planctomycete Gemmata obscuriglobus. Commun Integr Biol. 2010;3(6):572-5.
154. Wang C, Dong S, Zhang L, Zhao Y, Huang L, Gong X, et al. Cell surface binding, uptaking and anticancer activity of L-K6, a lysine/leucine-rich peptide, on human breast cancer MCF-7 cells. Sci Rep. 2017;7(1):8293.
155. Henriques ST, Huang YH, Chaousis S, Sani MA, Poth AG, Separovic F, et al. The Prototypic Cyclotide Kalata B1 Has a Unique Mechanism of Entering Cells. Chem Biol. 2015;22(8):1087-97.
156. Lee HS, Park CB, Kim JM, Jang SA, Park IY, Kim MS, et al. Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide. Cancer letters. 2008;271(1):47-55.
157. Mader JS, Salsman J, Conrad DM, Hoskin DW. Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Molecular cancer therapeutics. 2005;4(4):612-24.
158. Sharma SV. Melittin-induced hyperactivation of phospholipase A2 activity and calcium influx in ras-transformed cells. Oncogene. 1993;8(4):939-47.
159. Carroll AR, Feng Y, Bowden BF, Coll JC. Studies of Australian Ascidians. 5. Virenamides A-C, New Cytotoxic Linear Peptides from the Colonial Didemnid Ascidian Diplosoma virens. The Journal of organic chemistry. 1996;61(12):4059-61.
160. Ouyang GL, Li QF, Peng XX, Liu QR, Hong SG. Effects of tachyplesin on proliferation and differentiation of human hepatocellular carcinoma SMMC-7721 cells. World J Gastroenterol. 2002;8(6):1053-8.
161. Mulder KC, Lima LA, Miranda VJ, Dias SC, Franco OL. Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides. Front Microbiol. 2013;4:321.
162. Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. FEBS J. 2009;276(22):6483-96.
163. Runti G, Lopez Ruiz Mdel C, Stoilova T,

Hussain R, Jennions M, Choudhury HG, et al. Functional characterization of SbmA, a bacterial inner membrane transporter required for importing the antimicrobial peptide Bac7(1-35). J Bacteriol. 2013;195(23):5343-51.
164. Scocchi M, Mardirossian M, Runti G, Benincasa M. Non-Membrane Permeabilizing Modes of Action of Antimicrobial Peptides on Bacteria. Current topics in medicinal chemistry. 2016;16(1):76-88.
165. Le CF, Fang CM, Sekaran SD. Intracellular Targeting Mechanisms by Antimicrobial Peptides. Antimicrob Agents Chemother. 2017;61(4).
166. Shah P, Hsiao FS, Ho YH, Chen CS. The proteome targets of intracellular targeting antimicrobial peptides. Proteomics. 2016;16(8):1225-37.
167. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clinical microbiology reviews. 2006;19(3):491-511.
168. Bobone S, Stella L. Selectivity of Antimicrobial Peptides: A Complex Interplay of Multiple Equilibria. Adv Exp Med Biol. 2019;1117:175-214.
169. Harris F, Dennison SR, Singh J, Phoenix DA. On the selectivity and efficacy of defense peptides with respect to cancer cells. Med Res Rev. 2013;33(1):190-234.
170. Son M, Lee Y, Hwang H, Hyun S, Yu J. Disruption of interactions between hydrophobic residues on nonpolar faces is a key determinant in decreasing hemolysis and increasing antimicrobial activities of alpha-helical amphipathic peptides. ChemMedChem. 2013;8(10):1638-42.
171. Aoki W, Ueda M. Characterization of Antimicrobial Peptides toward the Development of Novel Antibiotics. Pharmaceuticals. 2013;6(8):1055-81.
172. Chen L, Patrone N, Liang JF. Peptide self-assembly on cell membranes to induce cell lysis. Biomacromolecules. 2012;13(10):3327-33.
173. Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature. 1981;292(5820):246-8.
174. Waghu FH, Barai RS, Gurung P, Idicula-Thomas S. CAMPR3: a database on sequences, structures and signatures of antimicrobial peptides. Nucleic Acids Res. 2015.
175. Costa F, Teixeira C, Gomes P, Martins MCL. Clinical Application of AMPs. Adv Exp Med Biol. 2019;1117:281-98.
176. Tyagi A, Tuknait A, Anand P, Gupta S, Sharma M, Mathur D, et al. CancerPPD: a database of anticancer peptides and proteins. Nucleic Acids Res. 2015;43(Database issue):D837-43.
177. Paterson I, Anderson EA. Chemistry. The renaissance of natural products as drug candidates. Science. 2005;310(5747):451-3.
178. Koehn FE, Carter GT. The evolving role of natural products in drug discovery. Nat Rev Drug Discov. 2005;4(3):206-20.
179. Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. Chemical reviews. 2009;109(7):3012-43.
180. Macarron R. Critical review of the role of HTS in drug discovery. Drug discovery today. 2006;11(7-8):277-9.
181. Butler MS. The role of natural product chemistry in drug discovery. J Nat Prod. 2004;67(12):2141-53.
182. Li JW, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? Science. 2009;325(5937):161-5.
183. Harvey AL. Natural products in drug discovery. Drug discovery today. 2008;13(19-20):894-901.
184. Koehn FE. High impact technologies for natural products screening. Progress in drug research Fortschritte der Arzneimittelforschung Progres des recherches pharmaceutiques. 2008;65:175, 7-210.
185. Clardy J, Walsh C. Lessons from natural molecules. Nature. 2004;432(7019):829-37.
186. Ganesan A. The impact of natural products upon modern drug discovery. Curr Opin Chem Biol. 2008;12(3):306-17.

187. Escoubas P, King GF. Venomics as a drug discovery platform. Expert Rev Proteomics. 2009;6(3):221-4.
188. King GF, Coaker H. The future of venoms-based drug discovery: an interview with Glenn King. Future Med Chem. 2014;6(15):1613-5.
189. Calvete JJ. Venomics: digging into the evolution of venomous systems and learning to twist nature to fight pathology. J Proteomics. 2009;72(2):121-6.
190. Leal MC, Calado R, Sheridan C, Alimonti A, Osinga R. Coral aquaculture to support drug discovery. Trends in biotechnology. 2013;31(10):555-61.
191. Ortiz E, Gurrola GB, Schwartz EF, Possani LD. Scorpion venom components as potential candidates for drug development. Toxicon. 2015;93:125-35.
192. Escoubas P, Bosmans F. Spider peptide toxins as leads for drug development. Expert opinion on drug discovery. 2007;2(6):823-35.
193. Pineda SS, Undheim EA, Rupasinghe DB, Ikonomopoulou MP, King GF. Spider venomics: implications for drug discovery. Future Med Chem. 2014;6(15):1699-714.
194. Aili SR, Touchard A, Escoubas P, Padula MP, Orivel J, Dejean A, et al. Diversity of peptide toxins from stinging ant venoms. Toxicon. 2014;92:166-78.
195. Touchard A, Aili SR, Fox EG, Escoubas P, Orivel J, Nicholson GM, et al. The Biochemical Toxin Arsenal from Ant Venoms. Toxins (Basel). 2016;8(1).
196. Harvey AL. Toxins and drug discovery. Toxicon. 2014;92:193-200.
197. Reeks TA, Fry BG, Alewood PF. Privileged frameworks from snake venom. Cell Mol Life Sci. 2015;72(10):1939-58.
198. Chan YS, Cheung RC, Xia L, Wong JH, Ng TB, Chan WY. Snake venom toxins: toxicity and medicinal applications. Appl Microbiol Biotechnol. 2016;100(14):6165-81.
199. Essack M, Bajic VB, Archer JA. Conotoxins that confer therapeutic possibilities. Mar Drugs. 2012;10(6):1244-65.
200. Munawar A, Ali S, Akrem A, Betzel C. Snake Venom Peptides: Tools of Biodiscovery. Toxins. 2018;10(11):474.
201. Simoes-Silva R, Alfonso J, Gomez A, Holanda RJ, Sobrinho JC, Zaqueo KD, et al. Snake Venom, A Natural Library of New Potential Therapeutic Molecules: Challenges and Current Perspectives. Curr Pharm Biotechnol. 2018;19(4):308-35.
202. Ondetti MA, Rubin B, Cushman DW. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. Science. 1977;196(4288):441-4.
203. Camargo AC, Ianzer D, Guerreiro JR, Serrano SM. Bradykinin-potentiating peptides: beyond captopril. Toxicon. 2012;59(4):516-23.
204. Francischetti IMB, Reyes Gil M. Chapter 164 - Diagnostic Use of Venoms. In: Shaz BH, Hillyer CD, Reyes Gil M, editors. Transfusion Medicine and Hemostasis (Third Edition): Elsevier; 2019. p. 969-75.
205. Koh DC, Armugam A, Jeyaseelan K. Snake venom components and their applications in biomedicine. Cell Mol Life Sci. 2006;63(24):3030-41.
206. Takacs Z, Nathan S. Animal Venoms in Medicine. In: Wexler P, editor. Encyclopedia of Toxicology (Third Edition). Oxford: Academic Press; 2014. p. 252-9.
207. King GF. Venoms as a platform for human drugs: translating toxins into therapeutics. Expert opinion on biological therapy. 2011;11(11):1469-84.
208. Fox JW, Serrano SM. Approaching the golden age of natural product pharmaceuticals from venom libraries: an overview of toxins and toxin-derivatives currently involved in therapeutic or diagnostic applications. Current pharmaceutical design. 2007;13(28):2927-34.
209. Vonk FJ, Jackson K, Doley R, Madaras F, Mirtschin PJ, Vidal N. Snake venom: From fieldwork to the clinic: Recent insights into snake biology, together with new technology allowing high-throughput screening of venom, bring new hope for drug discovery.

Bioessays. 2011;33(4):269-79.
210. Carr A, Schultz J. Prospective evaluation of the incidence of wound infection in rattlesnake envenomation in dogs. Journal of veterinary emergency and critical care (San Antonio, Tex : 2001). 2015;25(4):546-51.
211. de Lima DC, Alvarez Abreu P, de Freitas CC, Santos DO, Borges RO, Dos Santos TC, et al. Snake Venom: Any Clue for Antibiotics and CAM? Evidence-based complementary and alternative medicine : eCAM. 2005;2(1):39-47.
212. Talan DA, Citron DM, Overturf GD, Singer B, Froman P, Goldstein EJ. Antibacterial activity of crotalid venoms against oral snake flora and other clinical bacteria. The Journal of infectious diseases. 1991;164(1):195-8.
213. Perumal Samy R, Gopalakrishnakone P, Ho B, Chow VT. Purification, characterization and bactericidal activities of basic phospholipase A2 from the venom of Agkistrodon halys (Chinese pallas). Biochimie. 2008;90(9):1372-88.
214. Ferreira SH. A bradykinin-potentiatinf factor (BPF) present in the venom of Bothrops jararaca. Br J Pharmacol Chemother. 1965;24:163-9.
215. Perumal Samy R, Pachiappan A, Gopalakrishnakone P, Thwin MM, Hian YE, Chow VT, et al. In vitro antimicrobial activity of natural toxins and animal venoms tested against Burkholderia pseudomallei. BMC infectious diseases. 2006;6:100.
216. Chaim-Matyas A, Ovadia M. Cytotoxic activity of various snake venoms on melanoma, B16F10 and chondrosarcoma. Life Sci. 1987;40(16):1601-7.
217. da Silva RJ, da Silva MG, Vilela LC, Fecchio D. Antitumor effect of Bothrops jararaca venom. Mediators Inflamm. 2002;11(2):99-104.
218. Gomes A, Bhattacharjee P, Mishra R, Biswas AK, Dasgupta SC, Giri B. Anticancer potential of animal venoms and toxins. Indian J Exp Biol. 2010;48(2):93-103.
219. Kerkkamp H, Bagowski C, Kool J, van Soolingen B, Vonk FJ, Vlecken D. Whole snake venoms: Cytotoxic, anti-metastatic and antiangiogenic properties. Toxicon. 2018;150:39-49.
220. de Oliveira Junior NG, e Silva Cardoso MH, Franco OL. Snake venoms: attractive antimicrobial proteinaceous compounds for therapeutic purposes. Cell Mol Life Sci. 2013;70(24):4645-58.
221. Ma R, Mahadevappa R, Kwok HF. Venom-based peptide therapy: insights into anti-cancer mechanism. Oncotarget. 2017;8(59):100908-30.
222. Zhang Y, Zhao H, Yu GY, Liu XD, Shen JH, Lee WH. Structure-function relationship of king cobra cathelicidin. Peptides. 2010;31(8):1488-93.
223. Falcao CB, de La Torre BG, Perez-Peinado C, Barron AE, Andreu D, Radis-Baptista G. Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers. Amino Acids. 2014;46(11):2561-71.
224. Tajbakhsh M, Karimi A, Tohidpour A, Abbasi N, Fallah F, Akhavan MM. The antimicrobial potential of a new derivative of cathelicidin from Bungarus fasciatus against methicillin-resistant Staphylococcus aureus. Journal of microbiology (Seoul, Korea). 2018;56(2):128-37.
225. Tajbakhsh M, Akhavan MM, Fallah F, Karimi A. A Recombinant Snake Cathelicidin Derivative Peptide: Antibiofilm Properties and Expression in Escherichia coli. Biomolecules. 2018;8(4).
226. Jin L, Bai X, Luan N, Yao H, Zhang Z, Liu W, et al. A Designed Tryptophan- and Lysine/Arginine-Rich Antimicrobial Peptide with Therapeutic Potential for Clinical Antibiotic-Resistant Candida albicans Vaginitis. J Med Chem. 2016;59(5):1791-9.
227. Liu C, Qi J, Shan B, Gao R, Gao F, Xie H, et al. Pretreatment with cathelicidin-BF ameliorates Pseudomonas aeruginosa pneumonia in mice by enhancing NETosis and the autophagy of recruited neutrophils and macrophages. Int Immunopharmacol.

2018;65:382-91.
228. Zhang H, Zhang B, Zhang X, Wang X, Wu K, Guan Q. Effects of cathelicidin-derived peptide from reptiles on lipopolysaccharide-induced intestinal inflammation in weaned piglets. Vet Immunol Immunopathol. 2017;192:41-53.
229. Zhang H, Xia X, Han F, Jiang Q, Rong Y, Song D, et al. Cathelicidin-BF, a Novel Antimicrobial Peptide from Bungarus fasciatus, Attenuates Disease in a Dextran Sulfate Sodium Model of Colitis. Mol Pharm. 2015;12(5):1648-61.
230. Yi H, Yu C, Zhang H, Song D, Jiang D, Du H, et al. Cathelicidin-BF suppresses intestinal inflammation by inhibiting the nuclear factor-kappaB signaling pathway and enhancing the phagocytosis of immune cells via STAT-1 in weanling piglets. Int Immunopharmacol. 2015;28(1):61-9.
231. Song D, Zong X, Zhang H, Wang T, Yi H, Luan C, et al. Antimicrobial peptide Cathelicidin-BF prevents intestinal barrier dysfunction in a mouse model of endotoxemia. Int Immunopharmacol. 2015;25(1):141-7.
232. Wang H, Ke M, Tian Y, Wang J, Li B, Wang Y, et al. BF-30 selectively inhibits melanoma cell proliferation via cytoplasmic membrane permeabilization and DNA-binding in vitro and in B16F10-bearing mice. Eur J Pharmacol. 2013;707(1-3):1-10.
233. Zhou H, Dou J, Wang J, Chen L, Wang H, Zhou W, et al. The antibacterial activity of BF-30 in vitro and in infected burned rats is through interference with cytoplasmic membrane integrity. Peptides. 2011;32(6):1131-8.
234. Wang Y, Zhang Z, Chen L, Guang H, Li Z, Yang H, et al. Cathelicidin-BF, a snake cathelicidin-derived antimicrobial peptide, could be an excellent therapeutic agent for acne vulgaris. PLoS One. 2011;6(7):e22120.
235. Chen W, Yang B, Zhou H, Sun L, Dou J, Qian H, et al. Structure-activity relationships of a snake cathelicidin-related peptide, BF-15. Peptides. 2011;32(12):2497-503.
236. Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, et al. Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. PLoS One. 2008;3(9):e3217.
237. Amer LS, Bishop BM, van Hoek ML. Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against Francisella. Biochem Biophys Res Commun. 2010;396(2):246-51.
238. de Latour FA, Amer LS, Papanstasiou EA, Bishop BM, van Hoek ML. Antimicrobial activity of the Naja atra cathelicidin and related small peptides. Biochem Biophys Res Commun. 2010;396(4):825-30.
239. Dean SN, Bishop BM, van Hoek ML. Susceptibility of Pseudomonas aeruginosa Biofilm to Alpha-Helical Peptides: D-enantiomer of LL-37. Front Microbiol. 2011;2:128.
240. Dean SN, Bishop BM, van Hoek ML. Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against Staphylococcus aureus. BMC Microbiol. 2011;11:114.
241. Blower RJ, Barksdale SM, van Hoek ML. Snake Cathelicidin NA-CATH and Smaller Helical Antimicrobial Peptides Are Effective against Burkholderia thailandensis. PLoS Negl Trop Dis. 2015;9(7):e0003862.
242. Gupta K, Singh S, van Hoek ML. Short, Synthetic Cationic Peptides Have Antibacterial Activity against Mycobacterium smegmatis by Forming Pores in Membrane and Synergizing with Antibiotics. Antibiotics (Basel). 2015;4(3):358-78.
243. Blower RJ, Popov SG, van Hoek ML. Cathelicidin peptide rescues G. mellonella infected with B. anthracis. Virulence. 2017:1-7.
244. Wei L, Gao J, Zhang S, Wu S, Xie Z, Ling G, et al. Identification and Characterization of the First Cathelicidin from Sea Snakes with Potent Antimicrobial and Anti-inflammatory Activity and Special Mechanism. J Biol Chem. 2015;290(27):16633-52.
245. Carlile SR, Shiels J, Kerrigan L, Delaney R, Megaw J, Gilmore BF, et al. Sea snake cathelicidin (Hc-cath) exerts a protective effect in mouse models of lung inflammation

and infection. Sci Rep. 2019;9(1):6071.
246. Falcao CB, Perez-Peinado C, de la Torre BG, Mayol X, Zamora-Carreras H, Jimenez MA, et al. Structural Dissection of Crotalicidin, a Rattlesnake Venom Cathelicidin, Retrieves a Fragment with Antimicrobial and Antitumor Activity. J Med Chem. 2015;58(21):8553-63.
247. Cavalcante CS, Falcao CB, Fontenelle RO, Andreu D, Radis-Baptista G. Antifungal activity of Ctn[15-34], the C-terminal peptide fragment of crotalicidin, a rattlesnake venom gland cathelicidin. The Journal of antibiotics. 2016;70(3):231-7.
248. Bandeira ICJ, Bandeira-Lima D, Mello CP, Pereira TP, De Menezes R, Sampaio TL, et al. Antichagasic effect of crotalicidin, a cathelicidin-like vipericidin, found in Crotalus durissus terrificus rattlesnake's venom gland. Parasitology. 2017:1-6.
249. Oliveira-Junior NG, Freire MS, Almeida JA, Rezende TMB, Franco OL. Antimicrobial and proinflammatory effects of two vipericidins. Cytokine. 2018;111:309-16.
250. Mello CP, Lima DB, Menezes RR, Bandeira IC, Tessarolo LD, Sampaio TL, et al. Evaluation of the antichagasic activity of batroxicidin, a cathelicidin-related antimicrobial peptide found in Bothrops atrox venom gland. Toxicon. 2017;130:56-62.
251. Kim D, Soundrarajan N, Lee J, Cho HS, Choi M, Cha SY, et al. Genome wide analysis of the antimicrobial peptides in Python bivittatus and characterization of cathelicidins with potent antimicrobial activity and low cytotoxicity. Antimicrob Agents Chemother. 2017.
252. Cai S, Qiao X, Feng L, Shi N, Wang H, Yang H, et al. Python Cathelicidin CATHPb1 Protects against Multidrug-Resistant Staphylococcal Infections by Antimicrobial-Immunomodulatory Duality. J Med Chem. 2018;61(5):2075-86.
253. Zhang Y, Zhao H, Yu GY, Liu XD, Shen JH, Lee WH, et al. Structure-function relationship of king cobra cathelicidin. Peptides. 2010;31(8):1488-93.
254. Azim S, McDowell D, Cartagena A, Rodriguez R, Laughlin TF, Ahmad Z. Venom peptides cathelicidin and lycotoxin cause strong inhibition of Escherichia coli ATP synthase. Int J Biol Macromol. 2016;87:246-51.
255. Li SA, Lee WH, Zhang Y. Efficacy of OH-CATH30 and its analogs against drug-resistant bacteria in vitro and in mouse models. Antimicrob Agents Chemother. 2012;56(6):3309-17.
256. Zhao F, Lan XQ, Du Y, Chen PY, Zhao J, Zhao F, et al. King cobra peptide OH-CATH30 as a potential candidate drug through clinic drug-resistant isolates. Zool Res. 2018;39(2):87-96.
257. Juba M, Porter D, Dean S, Gillmor S, Bishop B. Characterization and performance of short cationic antimicrobial peptide isomers. Biopolymers. 2013;100(4):387-401.
258. Du H, Samuel RL, Massiah MA, Gillmor SD. The structure and behavior of the NA-CATH antimicrobial peptide with liposomes. Biochim Biophys Acta. 2015;1848(10 Pt A):2394-405.
259. Samuel R, Gillmor S. Membrane phase characteristics control NA-CATH activity. Biochim Biophys Acta. 2016;1858(9):1974-82.
260. Xia X, Zhang L, Wang Y. The antimicrobial peptide cathelicidin-BF could be a potential therapeutic for Salmonella typhimurium infection. Microbiol Res. 2015;171:45-51.
261. Hao Q, Wang H, Wang J, Dou J, Zhang M, Zhou W, et al. Effective antimicrobial activity of Cbf-K16 and Cbf-A7 A13 against NDM-1-carrying Escherichia coli by DNA binding after penetrating the cytoplasmic membrane in vitro. J Pept Sci. 2013;19(3):173-80.
262. Tian Y, Wang H, Li B, Ke M, Wang J, Dou J, et al. The cathelicidin-BF Lys16 mutant Cbf-K16 selectively inhibits non-small cell lung cancer proliferation in vitro. Oncol Rep. 2013;30(5):2502-10.
263. Fang Y, He X, Zhang P, Shen C, Mwangi J, Xu C, et al. In Vitro and In Vivo Antimalarial Activity of LZ1, a Peptide Derived

from Snake Cathelicidin. Toxins (Basel). 2019;11(7).
264. De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnology letters. 2005;27(18):1337-47.
265. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3(9):710-20.
266. Correa PG, Oguiura N. Phylogenetic analysis of beta-defensin-like genes of Bothrops, Crotalus and Lachesis snakes. Toxicon. 2013;69:65-74.
267. van Hoek ML. Antimicrobial peptides in reptiles. Pharmaceuticals. 2014;7(6):723-53.
268. de Oliveira YS, Correa PG, Oguiura N. Beta-defensin genes of the Colubridae snakes Phalotris mertensi, Thamnodynastes hypoconia, and T. strigatus. Toxicon. 2018;146:124-8.
269. Kerkis I, Hayashi MA, Prieto da Silva AR, Pereira A, De Sa Junior PL, Zaharenko AJ, et al. State of the art in the studies on crotamine, a cell penetrating peptide from South American rattlesnake. BioMed research international. 2014;2014:675985.
270. Coronado MA, Gabdulkhakov A, Georgieva D, Sankaran B, Murakami MT, Arni RK, et al. Structure of the polypeptide crotamine from the Brazilian rattlesnake Crotalus durissus terrificus. Acta crystallographica Section D, Biological crystallography. 2013;69(Pt 10):1958-64.
271. Oguiura N, Boni-Mitake M, Affonso R, Zhang G. In vitro antibacterial and hemolytic activities of crotamine, a small basic myotoxin from rattlesnake Crotalus durissus. The Journal of antibiotics. 2011;64(4):327-31.
272. Yamane ES, Bizerra FC, Oliveira EB, Moreira JT, Rajabi M, Nunes GL, et al. Unraveling the antifungal activity of a South American rattlesnake toxin crotamine. Biochimie. 2013;95(2):231-40.
273. Pereira A, Kerkis A, Hayashi MA, Pereira AS, Silva FS, Oliveira EB, et al. Crotamine toxicity and efficacy in mouse models of melanoma. Expert Opin Investig Drugs. 2011;20(9):1189-200.
274. Nascimento FD, Sancey L, Pereira A, Rome C, Oliveira V, Oliveira EB, et al. The natural cell-penetrating peptide crotamine targets tumor tissue in vivo and triggers a lethal calcium-dependent pathway in cultured cells. Mol Pharm. 2012;9(2):211-21.
275. Ponnappan N, Budagavi DP, Chugh A. CyLoP-1: Membrane-active peptide with cell-penetrating and antimicrobial properties. Biochim Biophys Acta. 2017;1859(2):167-76.
276. Radis-Baptista G, de la Torre BG, Andreu D. Insights into the uptake mechanism of NrTP, a cell-penetrating peptide preferentially targeting the nucleolus of tumour cells. Chem Biol Drug Des. 2012;79(6):907-15.
277. Rodrigues M, Andreu D, Santos NC. Uptake and cellular distribution of nucleolar targeting peptides (NrTPs) in different cell types. Biopolymers. 2015;104(2):101-9.
278. Torres AM, Wong HY, Desai M, Moochhala S, Kuchel PW, Kini RM. Identification of a novel family of proteins in snake venoms. Purification and structural characterization of nawaprin from Naja nigricollis snake venom. J Biol Chem. 2003;278(41):40097-104.
279. Nair DG, Fry BG, Alewood P, Kumar PP, Kini RM. Antimicrobial activity of omwaprin, a new member of the waprin family of snake venom proteins. Biochem J. 2007;402(1):93-104.
280. Thankappan B, Angayarkanni J. Biological characterization of omw1 and omw2: antimicrobial peptides derived from omwaprin. 3 Biotech. 2019;9(8):295.
281. Banigan JR, Mandal K, Sawaya MR, Thammavongsa V, Hendrickx AP, Schneewind O, et al. Determination of the X-ray structure of the snake venom protein omwaprin by total chemical synthesis and racemic protein crystallography. Protein Sci. 2010;19(10):1840-9.
282. Izidoro LF, Sobrinho JC, Mendes MM, Costa TR, Grabner AN, Rodrigues VM, et al. Snake venom L-amino acid oxidases: trends in pharmacology and biochemistry. BioMed

research international. 2014;2014:196754.
283. Almeida JR, Palacios ALV, Patino RSP, Mendes B, Teixeira CAS, Gomes P, et al. Harnessing snake venom phospholipases A2 to novel approaches for overcoming antibiotic resistance. Drug Dev Res. 2019;80(1):68-85.
284. Modahl CM, Frietze S, Mackessy SP. Transcriptome-facilitated proteomic characterization of rear-fanged snake venoms reveal abundant metalloproteinases with enhanced activity. J Proteomics. 2018;187:223-34.
285. Samy RP, Gopalakrishnakone P, Chow VT, Ho B. Viper metalloproteinase (Agkistrodon halys pallas) with antimicrobial activity against multi-drug resistant human pathogens. J Cell Physiol. 2008;216(1):54-68.
286. Markland FS, Jr., Swenson S. Snake venom metalloproteinases. Toxicon. 2013;62:3-18.
287. Sala A, Cabassi CS, Santospirito D, Polverini E, Flisi S, Cavirani S, et al. Novel Naja atra cardiotoxin 1 (CTX-1) derived antimicrobial peptides with broad spectrum activity. PLoS One. 2018;13(1):e0190778.
288. Samy RP, Stiles BG, Chinnathambi A, Zayed ME, Alharbi SA, Franco OL, et al. Viperatoxin-II: A novel viper venom protein as an effective bactericidal agent. FEBS Open Bio. 2015;5:928-41.
289. Sampaio SC, Hyslop S, Fontes MR, Prado-Franceschi J, Zambelli VO, Magro AJ, et al. Crotoxin: novel activities for a classic beta-neurotoxin. Toxicon. 2010;55(6):1045-60.
290. Samy RP, Kandasamy M, Gopalakrishnakone P, Stiles BG, Rowan EG, Becker D, et al. Wound healing activity and mechanisms of action of an antibacterial protein from the venom of the eastern diamondback rattlesnake (Crotalus adamanteus). PLoS One. 2014;9(2):e80199.
291. Allane D, Oussedik-Oumehdi H, Harrat Z, Seve M, Laraba-Djebari F. Isolation and characterization of an anti-leishmanial disintegrin from Cerastes cerastes venom. J Biochem Mol Toxicol. 2018;32(2).
292. Sulca MA, Remuzgo C, Cardenas J, Kiyota S, Cheng E, Bemquerer MP, et al. Venom of the Peruvian snake Bothriopsis oligolepis: Detection of antibacterial activity and involvement of proteolytic enzymes and C-type lectins in growth inhibition of Staphylococcus aureus. Toxicon. 2017;134:30-40.
293. Nolte S, de Castro Damasio D, Barea AC, Gomes J, Magalhaes A, Mello Zischler LF, et al. BJcuL, a lectin purified from Bothrops jararacussu venom, induces apoptosis in human gastric carcinoma cells accompanied by inhibition of cell adhesion and actin cytoskeleton disassembly. Toxicon. 2012;59(1):81-5.
294. Nunes Edos S, de Souza MA, Vaz AF, Santana GM, Gomes FS, Coelho LC, et al. Purification of a lectin with antibacterial activity from Bothrops leucurus snake venom. Comp Biochem Physiol B Biochem Mol Biol. 2011;159(1):57-63.
295. Calderon LA, Sobrinho JC, Zaqueo KD, de Moura AA, Grabner AN, Mazzi MV, et al. Antitumoral activity of snake venom proteins: new trends in cancer therapy. BioMed research international. 2014;2014:203639.
296. Gomes VM, Carvalho AO, Da Cunha M, Keller MN, Bloch C, Jr., Deolindo P, et al. Purification and characterization of a novel peptide with antifungal activity from Bothrops jararaca venom. Toxicon. 2005;45(7):817-27.
297. Sarzaeem A, Zare Mirakabadi A, Moradhaseli S, Morovvati H, Lotfi M. Cytotoxic effect of ICD-85 (venom-derived peptides) on HeLa cancer cell line and normal LK cells using MTT Assay. Arch Iran Med. 2012;15(11):696-701.

2

FROM VENOMS TO PEPTIDE DRUGS

This chapter describes the discovery of vipericidins, a new family of SV-derived cathelicidins.

Contents

2.1 Introduction

2.1.1 Tools to discover natural bioactive peptides

The identification and/or prediction of naturally-occurring bioactive peptides is an increasingly valuable research area. Current approaches for bioactive peptide discovery are either empirical or computational, both methodologies often integrated (1). The empirical approach involves isolation and identification of peptides by means of HPLC and MS directly from available natural sources or after pre-processing (e.g., proteolysis or fermentation) of proteic fractions in the initial mixture (2). For its part, the computational, bioinformatics-driven approach relies on *in silico* screening of -omic information (proteomic, transcriptomic, genomic) available in databases to predict bioactive peptides based on distinctive features, such as evolutionary conservation, specific amino-acid composition or molecular descriptors (3-5).

Specifically, alignment methods have been extensively used for identification of unannotated AMPs (6). For instance, the common cysteine knot motif of cyclotides, which connects their three disulfide bonds into highly knotted topologies, has been used for the identification of new members of this highly stable peptide family found in plants (7). Other conserved patterns have helped to identify new members of known families, such as cathelicidins or defensins (8). However, this approach deals with the general low sequence homology of AMPs, which makes it difficult to find preserved sequence motifs for the routinely implementation of local alignments in the peptide discovery pipeline. Alternatives such as machine learning methods able to recognize patterns based on various descriptors (composition, physicochemical properties or structural characteristics of amino acid residues) rather than sequence specifications have been successfully implemented (9, 10). In fact, different machine learning-based AMP prediction servers are available: CAMPR3 (11), AntiBP2 (12), and others have been developed for the analysis of whole protein databases to decrypt antimicrobial stretches (13-15). This toolbox has been extensively used to effectively identify AMPs and other membrane-active peptides from a vast variety of natural sources such as scorpion venom, snake toxins or the rumen bacterial metagenome (16-19).

In sum, nowadays we have access to a plethora of databases and bioinformatic tools to predict bioactive peptide leads in general (20), and membrane-active (e.g., AMPs, ACPs and CPPs) (11, 21, 22) in particular.

2.1.2 Cathelicidins

Cathelicidins are a family of structurally diverse bioactive peptides with antimicrobial, anticancer and immunomodulatory functions acting as effector molecules of the innate immune system (23, 24). Members of the cathelicidin family possess a unifying feature in their precursor form, namely a highly homologous pre- and pro-region comprising the N-terminal signal peptide motif and the cathelin-like domain, respectively (25, 26) (Figure 2.1). In contrast, the C-terminal domain that encodes for the mature bioactive peptide is structurally quite diverse in amino acid sequence among species (Figure 2.1) (25, 26). While most mature cathelicidins are linear, 25-35 residue-long, amphipathic α-helix peptides, some family members (e.g., protegrins) are smaller, 12-18 residue peptides displaying β-hairpin structures stabilized by disulfide bonds. Others consist of sequences enriched in specific amino acids such as Trp-rich indolicidin (27). Despite these conformational and composition variabilities, most cathelicidins share certain physicochemical properties, including general positive charge and amphipathicity.

Cathelicidins have been found in humans (i.e., LL-37) but also in other mammals like cows, pigs, rabbits or mice (28-33), and non-mammalian vertebrates like fish, birds or reptiles (34-36). Zhao et al. described in 2008 for the first time the identification of cathelicidins cloned from venom gland cDNA libraries of elapid snakes (35) and further studies confirmed their antitumor, anti-infective and immunomodulatory properties as well as that of their derived analogs (37-46).

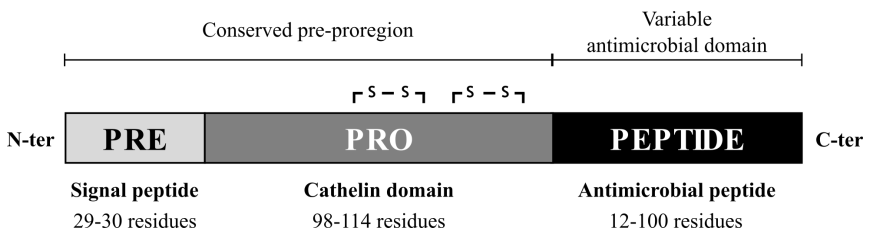


Figure 2.1: Schematic representation of the precursor structure of cathelicidins. Adapted from (27, 47).

On the basis of the accessible techniques to discover bioactive peptides (for instance aligning methods) and the sequence characteristics for the cathelicidin family outlined on this chapter introduction, this project was launched with the initial goal of screening South American snake venoms for novel cathelicidin-related AMPs identification.

References

1. Daliri EB, Lee BH, Oh DH. Current trends and perspectives of bioactive peptides. Crit Rev Food Sci Nutr. 2018;58(13):2273-84.

2. Li-Chan ECY. Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. Current Opinion in Food Science. 2015;1:28-37.

3. Yan L, Yan Y, Liu H, Lv Q. Stepwise identification of potent antimicrobial peptides from human genome. Biosystems. 2013;113(1):1-8.

4. Khaldi N. Bioinformatics approaches for identifying new therapeutic bioactive peptides in food. Functional Foods in Health and Disease. 2012;2(10):325-38.

5. Tu M, Cheng S, Lu W, Du M. Advancement and prospects of bioinformatics analysis for studying bioactive peptides from food-derived protein: Sequence, structure, and functions. TrAC Trends in Analytical Chemistry. 2018;105:7-17.

6. Porto WF, Pires AS, Franco OL. Computational tools for exploring sequence databases as a resource for antimicrobial peptides. Biotechnol Adv. 2017;35(3):337-49.

7. Mulvenna JP, Mylne JS, Bharathi R, Burton RA, Shirley NJ, Fincher GB, et al. Discovery of cyclotide-like protein sequences in graminaceous crop plants: ancestral precursors of circular proteins? Plant Cell. 2006;18(9):2134-44.

8. De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnology letters. 2005;27(18):1337-47.

9. Liu S, Fan L, Sun J, Lao X, Zheng H. Computational resources and tools for antimicrobial peptides. J Pept Sci. 2017;23(1):4-12.

10. Dziuba B, Dziuba M. New milk protein-derived peptides with potential antimicrobial activity: an approach based on bioinformatic studies. Int J Mol Sci. 2014;15(8):14531-45.

11. Waghu FH, Barai RS, Gurung P, Idicula-Thomas S. CAMPR3: a database on sequences, structures and signatures of antimicrobial peptides. Nucleic Acids Res. 2015.

12. Lata S, Mishra NK, Raghava GP. AntiBP2: improved version of antibacterial peptide prediction. BMC Bioinformatics. 2010;11 Suppl 1:S19.
13. Torrent M, Di Tommaso P, Pulido D, Nogues MV, Notredame C, Boix E, et al. AMPA: an automated web server for prediction of protein antimicrobial regions. Bioinformatics. 2012;28(1):130-1.
14. Brand GD, Magalhaes MT, Tinoco ML, Aragao FJ, Nicoli J, Kelly SM, et al. Probing protein sequences as sources for encrypted antimicrobial peptides. PLoS One. 2012;7(9):e45848.
15. Niarchou A, Alexandridou A, Athanasiadis E, Spyrou G. C-PAmP: large scale analysis and database construction containing high scoring computationally predicted antimicrobial peptides for all the available plant species. PLoS One. 2013;8(11):e79728.
16. Guilhelmelli F, Vilela N, Smidt KS, de Oliveira MA, da Cunha Morales Alvares A, Rigonatto MC, et al. Activity of Scorpion Venom-Derived Antifungal Peptides against Planktonic Cells of Candida spp. and Cryptococcus neoformans and Candida albicans Biofilms. Front Microbiol. 2016;7:1844.
17. Sala A, Cabassi CS, Santospirito D, Polverini E, Flisi S, Cavirani S, et al. Novel Naja atra cardiotoxin 1 (CTX-1) derived antimicrobial peptides with broad spectrum activity. PLoS One. 2018;13(1):e0190778.
18. Oyama LB, Girdwood SE, Cookson AR, Fernandez-Fuentes N, Prive F, Vallin HE, et al. The rumen microbiome: an underexplored resource for novel antimicrobial discovery. NPJ biofilms and microbiomes. 2017;3:33.
19. Jorge P, Lourenco A, Pereira MO. New trends in peptide-based anti-biofilm strategies: a review of recent achievements and bioinformatic approaches. Biofouling. 2012;28(10):1033-61.
20. Minkiewicz P, Dziuba J, Iwaniak A, Dziuba M, Darewicz M. BIOPEP database and other programs for processing bioactive peptide sequences. Journal of AOAC International. 2008;91(4):965-80.
21. Tyagi A, Tuknait A, Anand P, Gupta S, Sharma M, Mathur D, et al. CancerPPD: a database of anticancer peptides and proteins. Nucleic Acids Res. 2015;43(Database issue):D837-43.
22. Gautam A, Singh H, Tyagi A, Chaudhary K, Kumar R, Kapoor P, et al. CPPsite: a curated database of cell penetrating peptides. Database (Oxford). 2012;2012:bas015.
23. Wong JH, Ye XJ, Ng TB. Cathelicidins: peptides with antimicrobial, immunomodulatory, anti-inflammatory, angiogenic, anticancer and procancer activities. Curr Protein Pept Sci. 2013;14(6):504-14.
24. Agier J, Efenberger M, Brzezinska-Blaszczyk E. Cathelicidin impact on inflammatory cells. Cent Eur J Immunol. 2015;40(2):225-35.
25. Braff MH, Hawkins MA, Di Nardo A, Lopez-Garcia B, Howell MD, Wong C, et al. Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. J Immunol. 2005;174(7):4271-8.
26. Tomasinsig L, Zanetti M. The cathelicidins–structure, function and evolution. Curr Protein Pept Sci. 2005;6(1):23-34.
27. Gennaro R, Zanetti M. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers. 2000;55(1):31-49.
28. Das H, Sharma B, Kumar A. Cloning and characterization of novel cathelicidin cDNA sequence of Bubalus bubalis homologous to Bos taurus cathelicidin-4. DNA sequence : the journal of DNA sequencing and mapping. 2006;17(6):407-14.
29. Brogden KA, Kalfa VC, Ackermann MR, Palmquist DE, McCray PB, Jr., Tack BF. The ovine cathelicidin SMAP29 kills ovine respiratory pathogens in vitro and in an ovine model of pulmonary infection. Antimicrob Agents Chemother. 2001;45(1):331-4.
30. Zhao C, Nguyen T, Boo LM, Hong T,

Espiritu C, Orlov D, et al. RL-37, an alpha-helical antimicrobial peptide of the rhesus monkey. Antimicrob Agents Chemother. 2001;45(10):2695-702.

31. Nagaoka I, Tsutsumi-Ishii Y, Yomogida S, Yamashita T. Isolation of cDNA encoding guinea pig neutrophil cationic antibacterial polypeptide of 11 kDa (CAP11) and evaluation of CAP11 mRNA expression during neutrophil maturation. J Biol Chem. 1997;272(36):22742-50.

32. Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, et al. Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. J Biol Chem. 1997;272(20):13088-93.

33. Termen S, Tollin M, Olsson B, Svenberg T, Agerberth B, Gudmundsson GH. Phylogeny, processing and expression of the rat cathelicidin rCRAMP: a model for innate antimicrobial peptides. Cell Mol Life Sci. 2003;60(3):536-49.

34. Xiao Y, Cai Y, Bommineni YR, Fernando SC, Prakash O, Gilliland SE, et al. Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. J Biol Chem. 2006;281(5):2858-67.

35. Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, et al. Identification and characterization of novel reptile cathelicidins from elapid snakes. Peptides. 2008;29(10):1685-91.

36. Uzzell T, Stolzenberg ED, Shinnar AE, Zasloff M. Hagfish intestinal antimicrobial peptides are ancient cathelicidins. Peptides. 2003;24(11):1655-67.

37. Amer LS, Bishop BM, van Hoek ML. Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against Francisella. Biochem Biophys Res Commun. 2010;396(2):246-51.

38. Dean SN, Bishop BM, van Hoek ML. Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against Staphylococcus aureus. BMC Microbiol. 2011;11:114.

39. Gupta K, Singh S, van Hoek ML. Short, Synthetic Cationic Peptides Have Antibacterial Activity against Mycobacterium smegmatis by Forming Pores in Membrane and Synergizing with Antibiotics. Antibiotics (Basel). 2015;4(3):358-78.

40. Blower RJ, Popov SG, van Hoek ML. Cathelicidin peptide rescues G. mellonella infected with B. anthracis. Virulence. 2017:1-7.

41. Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, et al. Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. PLoS One. 2008;3(9):e3217.

42. Wang H, Ke M, Tian Y, Wang J, Li B, Wang Y, et al. BF-30 selectively inhibits melanoma cell proliferation via cytoplasmic membrane permeabilization and DNA-binding in vitro and in B16F10-bearing mice. Eur J Pharmacol. 2013;707(1-3):1-10.

43. Song D, Zong X, Zhang H, Wang T, Yi H, Luan C, et al. Antimicrobial peptide Cathelicidin-BF prevents intestinal barrier dysfunction in a mouse model of endotoxemia. Int Immunopharmacol. 2015;25(1):141-7.

44. Yi H, Yu C, Zhang H, Song D, Jiang D, Du H, et al. Cathelicidin-BF suppresses intestinal inflammation by inhibiting the nuclear factor-kappaB signaling pathway and enhancing the phagocytosis of immune cells via STAT-1 in weanling piglets. Int Immunopharmacol. 2015;28(1):61-9.

45. Li SA, Lee WH, Zhang Y. Efficacy of OH-CATH30 and its analogs against drug-resistant bacteria in vitro and in mouse models. Antimicrob Agents Chemother. 2012;56(6):3309-17.

46. Zhao F, Lan XQ, Du Y, Chen PY, Zhao J, Zhao F, et al. King cobra peptide OH-CATH30 as a potential candidate drug through clinic drug-resistant isolates. Zool Res. 2018;39(2):87-96.

47. Shinnar AE, Butler KL, Park HJ. Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. Bioorg Chem. 2003;31(6):425-36.

2.2 Article I

Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers.

Synopsis

This work describes the initial step of this thesis project, namely the identification of a new family of peptides, named vipericidins, in the venom gland of different South American rattlesnakes by means of PCR homology screening of cDNA libraries and gene sequencing. Identified sequences were examined *in silico* for antimicrobial sequence prediction, and the putative peptides were synthesized and experimentally validated as bacterial-selective AMPs.

Article I

Falcao CB, de La Torre BG, Pérez-Peinado C, Barron AE, Andreu D, Rádis-Baptista G. Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers. Amino acids. 2014;46(11):2561–71. DOI: 10.1007/s00726-014-1801-4

3

PEPTIDE OPTIMIZATION

This chapter describes the structural dissection of crotalicidin (Ctn) into a small, structurally and functionally optimized fragment.

Contents

3.1 Introduction

3.1.1 Peptide size optimization

Sequence reduction is a common approach in bioactive peptide optimization, given the possibility of lower production costs, improved synthesis yields and faster production time. Several strategies have been applied to define the minimal motifs or pharmacophores of bioactive peptides, ranging from structural approaches to sequence-based dissection.

The laboratory where the current thesis was conducted has contributed many examples of structure-guided substantial size reductions such as the downsizing of crotamine into nucleolar-targeting peptides (NrTPs) (1) or the simplification of the cysteine-rich thionin from *Pyrularia pubera* (PpTH) (2). In the first instance, structural analysis of crotamine revealed close spatial proximity between the N-terminal α-helix and the C-terminal endpiece. Such proximity was successfully exploited to design a drastically downsized, 14-residue version of the native peptide, named NrTP, which possessed strong cell membrane translocating properties (1). Similarly, the cysteine-rich PpTH was dissected into various shortened versions subsequently tested for *in vitro* anti-infective activity. The 26-residue central antiparallel double helix fully preserved the parental activity (45 % size reduction) and entailed a simplified synthesis workflow (2).

If the peptide structure remains unknown, sequence-based approaches such as Ala or Lys scan often prove enlightening. Sequentially downsizing at both N- and C-termini, resulting in, e.g., single point mutation or truncated analog libraries, often allows to define key amino acid residues or minimal stretches that contribute to the peptide activity. For instance, Simonsen et al. conducted an Ala scan in a prototypic cyclotide by systematically replacing each of its 23 amino acid residues with Ala, revealing a specific cluster responsible for the insecticidal and hemolytic activity, separated from its the membrane binding domain (3). In an attempt to define the minimal length preserving antibacterial activity of the cecropin A-melittin hybrid peptide, Andreu et al. designed a peptide library by sequentially truncating melittin at both termini, achieving a

size reduction of 40 % of the 26-residue parental hybrid peptide to shortened 15-residue peptides with similar or even better antibacterial activity (4).

Other similarly inspired strategies have been implemented to accomplish peptide size reduction, such as the proteolytic digestion of peptides into downsized fragments retaining the desired biological effect, or even displaying enhanced properties in some reported cases (5-7). In fact, this strategy to generate bioactive peptides and/or proteolytic fragments can be found in nature, as occurs for buforin I, an AMP that results from pepsin-mediated cleavage of the cytoplasmic histone H2A (5), or as for cathelicidins.

3.1.2 Cathelicidin proteolytic processing

Cathelicidins remain stored in specific granules as pre-pro-peptide forms. Upon cell activation, the sequence processing occurs and the mature, active form is secreted (8). Peptide processing entails the cleavage of the signal peptide motif by the universal signal peptidases and other optional cleavages: C-terminal processing by the peptidylglycin α-amidating monooxygenase may occurs and the cathelin domain is cleaved by different species-dependent enzymes, such as porcine and bovine neutrophil elastase, the proteinase 3 from humans, and presumably some prohormone convertases from the hagfish (9).

Although LL-37 is the most commonly studied mature human cathelicidin (encoded by a single gene, named hCAP18), differential proteolytic strategies may be performed, yielding diverse mature active antimicrobial peptides. For instance, once the gene product is secreted into the skin surface it may be further processed into new small peptides different from LL-37 (10) (Figure 3.1). Interestingly, these distinctive proteolytic-processed pieces display enhanced *in vitro* antimicrobial action against skin-infective agents (*S. aureus* and *Candida albicans*) but lose the immunostimulatory effect, compared to LL-37 (10). Similarly, the same human propeptide derived from the hCAP18 gene and stored in the seminal plasma may suffer an alternative cleavage by gastricsin, rendering an alternative 38-residue AMP when incubated at pH values of the vagina (11).

Both examples demonstrate how different proteolytic processing routes may yield distinct or even enhanced products and illustrate how nature strategies

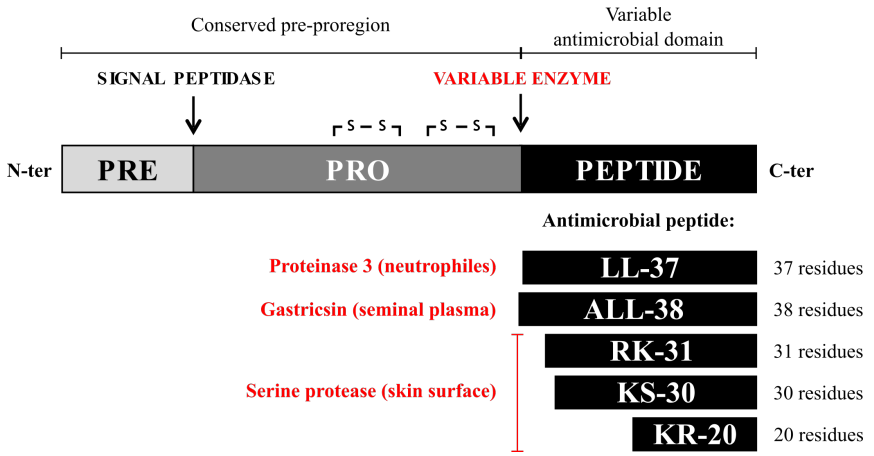


Figure 3.1: Proteolytic processing of the human cathelicidin h-CAP18 into different active fragments. Apart of its processing into the mature LL-37, the alternative proccesing of the prepropeptide form may yield different active mature peptides. Adapted from (9, 12).

adapt to produce specific agents for particular situations optimizing "space" in the genetic code.

Based on the cathelicidin proteolytic processing alternatives disclosed herein and the previous illustration of how different structural and sequence-based strategies may contribute to design optimized, shortened peptides, we proposed a rational sequence optimization approach for Ctn involving its excision by specific enzymes into small active fragments preserving the main activity of the parental peptide.

References

1. Radis-Baptista G, de la Torre BG, Andreu D. A novel cell-penetrating peptide sequence derived by structural minimization of a snake toxin exhibits preferential nucleolar localization. J Med Chem. 2008;51(22):7041-4.

2. Vila-Perello M, Sanchez-Vallet A, Garcia-Olmedo F, Molina A, Andreu D. Structural dissection of a highly knotted peptide reveals minimal motif with antimicrobial activity. J Biol Chem. 2005;280(2):1661-8.

3. Simonsen SM, Sando L, Rosengren KJ, Wang CK, Colgrave ML, Daly NL, et al. Alanine scanning mutagenesis of the prototypic cyclotide reveals a cluster of residues essential for bioactivity. J Biol Chem. 2008;283(15):9805-13.

4. Andreu D, Ubach J, Boman A, Wahlin B, Wade D, Merrifield RB, et al. Shortened

cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. FEBS Lett. 1992;296(2):190-4.
5. Kim HS, Yoon H, Minn I, Park CB, Lee WT, Zasloff M, et al. Pepsin-Mediated Processing of the Cytoplasmic Histone H2A to Strong Antimicrobial Peptide Buforin I. The Journal of Immunology. 2000;165(6):3268-74.
6. Ulvatne H, Vorland LH. Bactericidal kinetics of 3 lactoferricins against Staphylococcus aureus and Escherichia coli. Scandinavian journal of infectious diseases. 2001;33(7):507-11.
7. Sieprawska-Lupa M, Mydel P, Krawczyk K, Wojcik K, Puklo M, Lupa B, et al. Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases. Antimicrob Agents Chemother. 2004;48(12):4673-9.
8. Kosciuczuk EM, Lisowski P, Jarczak J, Strzalkowska N, Jozwik A, Horbanczuk J, et al. Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep. 2012;39(12):10957-70.
9. Shinnar AE, Butler KL, Park HJ. Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. Bioorg Chem. 2003;31(6):425-36.
10. Murakami M, Lopez-Garcia B, Braff M, Dorschner RA, Gallo RL. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J Immunol. 2004;172(5):3070-7.
11. Sorensen OE, Gram L, Johnsen AH, Andersson E, Bangsboll S, Tjabringa GS, et al. Processing of seminal plasma hCAP-18 to ALL-38 by gastricsin: a novel mechanism of generating antimicrobial peptides in vagina. J Biol Chem. 2003;278(31):28540-6.

3.2 Article II

Structural dissection of crotalicidin, a rattlesnake venom cathelicidin, retrieves a fragment with antimicrobial and antitumor activity.

Synopsis

For this work, we selected crotalicidin (Ctn) from the list of identified vipericidins (see article I) and *in silico* dissected the peptide with sequence optimization purposes. We retrieved a fragment, Ctn[15-34], with promising antitumoral and antibacterial activity and additional enhanced selectivity and serum stability. In parallel, we structurally characterized peptides by nuclear magnetic resonance.

Article II

Falcao CB, Pérez-Peinado C, de la Torre BG, Mayol X, Zamora-Carreras H, Jiménez MA, et al. Structural Dissection of Crotalicidin, a Rattlesnake Venom Cathelicidin, Retrieves a Fragment with Antimicrobial and Antitumor Activity. Journal of medicinal chemistry. 2015;58(21):8553–63. DOI: 10.1021/acs.jmedchem.5b01142

4

INSIGHTS INTO MECHANISMS

This chapter describes the antimicrobial and antitumoral mechanistic studies of crotalicidin (Ctn) and Ctn[15-34], with special emphasis on their action at the membrane level.

Contents

4.1 Introduction

4.1.1 Tools to study membrane-active peptides

Within the broad family of membrane-active peptides, characterized by their action on the lipid membranes of the target organisms, AMPs (with their often additional performance as ACPs, CPP or even anti-viral peptides) are particularly relevant (1-3).

The great interest and variety of applications of AMPs, ACPs, etc., has fostered the development of biophysical methodologies to study the peptide-lipid interactions governing the membrane-active peptide mechanism. Such techniques range from the use of spectroscopic probes at cellular level to membrane-mimetic systems models (4). Table 4.1, extracted from (5), illustrates this point.

Throughout this chapter, we aimed to study the mechanism of action of membrane-active peptides. In particular, article III deals with the study of the events occurring at the membrane level during the bactericidal action of Ctn and Ctn[15-34]. The set of biophysical techniques used included flow cytometry, confocal microscopy and atomic force microscopy as well as bacterial-mimicking membrane models.

Selected recent illustrative papers on the application of biophysical techniques directly in bacteria to study the action of AMP				
Technique	**AMP**	**Bacterium**	**Short description**	**Ref.**
AFM and zeta-potential light scattering	pepR and BP100	*E. coli*	Zeta-potential measurements of live bacterial cells in the presence of AMP. At MIC, the surface of the cells becomes neutral, and the membranes collapse. Quantitative surface roughness analyses were performed.	(6)
FLIM	AlexaFluor-430-K-14	*E. coli*	Direct imaging of the action of the lytic action of AMP in living bacterial cells.	(7)
AFM	R-BP100 and RW-BP100	*E. coli* and *S. aureus*	Antibacterial efficiency follows the affinity for bacterial membrane and is mainly driven by electrostatic interactions.	(8)
AFM, zeta-potential light scattering, flow cytometry, fluorescence spectroscopy, and confocal imaging	Sub3	*E. coli* and *S. aureus*	Sub3 targets the anionic outer membrane of Gram-negative bacteria by electrostatic attraction, permeates the outer membrane, and translocates the inner membrane to reach intracellular targets. A similar mechanism is proposed for Gram-positive, with lipoteichoic acids replacing the liposaccharides as electrostatic attractors of AMP	(9)
Steady-sate and time-resolved fluorescence spectroscopy	PMAP-23	*E. coli*	The number of AMP required to kill a bacterium was estimated.	(10)
CD	Magainin 2 and cecropin A	*E. coli*	Study of AMP conformational changes upon bacterial binding.	(11)
Flow cytometry/membrane potential-sensitive dyes; AFM (imaging and force spectroscopy), and zetapotential light scattering	rBPI21	*E. coli* and *S. aureus*	Surface perturbation on cells is followed by lysis. rBPI21 has a binding pocket that may participate on the binding to Gram-negative bacteria.	(12)
Time-resolved FACS	pepR	*E. coli*	Describes and analyzes the kinetics of bacterial AMP-induced permeabilization. Quantitative kinetic parameters on AMP binding to bacterial membrane, cooperativity, and permeabilization are retrieved.	(13)

Table 4.1: Biophysical techniques applied to the study of AMP mechanisms at the bacterial membrane level. Table modified from (5). Abbreviations correspond to: AFM, atomic force microscopy; FLIM, fluorescence-lifetime imaging microscopy; MIC, minimal inhibitory concentration; CD, circular dichroism; FACS, fluorescence-activated cell sorting.

References

1. Sani MA, Separovic F. How Membrane-Active Peptides Get into Lipid Membranes. Acc Chem Res. 2016;49(6):1130-8.

2. Riedl S, Zweytick D, Lohner K. Membrane-active host defense peptides–challenges and perspectives for the development of novel anticancer drugs. Chem Phys Lipids. 2011;164(8):766-81.

3. Raghuraman H, Chattopadhyay A. Melittin: a membrane-active peptide with diverse functions. Biosci Rep. 2007;27(4-5):189-223.

4. Henriques ST, Melo MN, Castanho MA. How to address CPP and AMP translocation? Methods to detect and quantify peptide internalization in vitro and in vivo (Review). Mol Membr Biol. 2007;24(3):173-84.

5. Freire JM, Gaspar D, Veiga AS, Castanho MA. Shifting gear in antimicrobial and anticancer peptides biophysical studies: from vesicles to cells. J Pept Sci. 2015;21(3):178-85.

6. Alves CS, Melo MN, Franquelim HG, Ferre R, Planas M, Feliu L, et al. Escherichia coli cell surface perturbation and disruption induced by antimicrobial peptides BP100 and pepR. J Biol Chem. 2010;285(36):27536-44.

7. Gee ML, Burton M, Grevis-James A, Hossain MA, McArthur S, Palombo EA, et al. Imaging the action of antimicrobial peptides on living bacterial cells. Sci Rep. 2013;3:1557.

8. Torcato IM, Huang YH, Franquelim HG, Gaspar D, Craik DJ, Castanho MA, et al. Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. Biochim Biophys Acta. 2013;1828(3):944-55.

9. Torcato IM, Huang YH, Franquelim HG, Gaspar DD, Craik DJ, Castanho MA, et al. The antimicrobial activity of Sub3 is dependent on membrane binding and cell-penetrating ability. Chembiochem. 2013;14(15):2013-22.

10. Roversi D, Luca V, Aureli S, Park Y, Mangoni ML, Stella L. How many antimicrobial peptide molecules kill a bacterium? The case of PMAP-23. ACS Chem Biol. 2014;9(9):2003-7.

11. Avitabile C, D'Andrea LD, Romanelli A. Circular Dichroism studies on the interactions of antimicrobial peptides with bacterial cells. Sci Rep. 2014;4:4293.

12. Domingues MM, Silva PM, Franquelim HG, Carvalho FA, Castanho MA, Santos NC. Antimicrobial protein rBPI21-induced surface changes on Gram-negative and Gram-positive bacteria. Nanomedicine. 2014;10(3):543-51.

13. Freire JM, Gaspar D, de la Torre BG, Veiga AS, Andreu D, Castanho MA. Monitoring antibacterial permeabilization in real time using time-resolved flow cytometry. Biochim Biophys Acta. 2015;1848(2):554-60.

4.2 Article III

Mechanisms of bacterial membrane permeabilization by crotalicidin (Ctn) and its fragment Ctn(15-34), antimicrobial peptides from rattlesnake venom.

Synopsis

In this study, we employed a set of accurate biophysical techniques to characterize with high-resolution the spatial-temporal events occurring during Ctn and Ctn[15-34] action on the bacterial membrane.

Article III

Pérez-Peinado C, Dias SA, Domingues MM, Benfield AH, Freire JM, Rádis-Baptista G, et al. Mechanisms of bacterial membrane permeabilization by crotalicidin (Ctn) and its fragment Ctn(15–34), antimicrobial peptides from rattlesnake venom. The Journal of biological chemistry. 2018;293(5):1536–49. DOI: 10.1074/jbc.RA117.000125

4.3 Introduction

4.3.1 Tools for peptide intracellular targeting studies

In the introduction to article III we have referred to biophysical techniques commonly employed to study membrane-active peptides. A similar approach has been chosen to study the oncolytic mechanism of action of Ctn and Ctn[15-34] in article IV. However, as mentioned in the general introduction, AMPs and ACPs may also additionally act by inhibition/interference with internal targets. Studies reporting non-lytic mechanisms of AMPs and ACPs have increased in recent years, but despite the increasing effort on such modes of action, non-lytic features are not systematically characterized to an extent comparable with lytic mechanisms, often only a single aspect being taken into consideration (1). A few representative examples exploring the inhibition of key biological metabolic routes of ACPs beyond the membranolytic action are included below to illustrate this idea.

Mader et al. showed that LfcinB induces apoptosis and dissipation of the mitochondria membrane potential in leukemia cells. Further studies were addressed to determine the role of caspases as well as the involvement of mitochondria targeting in its apoptotic activity. Results allowed to conclude that LfcinB triggers the mitochondrial pathway of apoptosis, and that generation of ROS and caspase-2 activity are required to induce mitochondrial damage (2).

Arachnid-derived gomesin peptides have been shown to reduce viability and proliferation of melanoma cells. In an attempt to study the underlying antiproliferative mechanisms, signaling cascades known to drive proliferation of melanoma cells such us the Hippo, AKT and MAPK pathways were investigated, finally determining that gomesin peptides inhibit the MAPK pathway and stimulate the Hippo and p53/p21 axis (3).

Cheng et al. also detected tumor cell viability decrease induced by the cationic peptide GW-H1, accompanied by apoptosis induction and loss of the mitochondrial membrane potential (4). To gain insights into the apoptotic mechanism, western blot analyses were run for evaluating the caspase-dependent apoptotic

pathway. Additionally, two-dimensional gel electrophoresis of cell lysates incubated with GW-H1 at different times followed by LC-MS/MS identification of up-/down-regulated proteins was performed.

As concluded from the above examples, tools to address the study of internal structures/molecular pathways/targets linked to cationic peptide-driven cellular death usually rely on techniques limited to explore a set of pre-defined proteins or routes, or are circumscribed to specific organelles (i.e., plasma membrane, mitochondria), but without a broad coverage and usually lacking large-scale studies. In this regard, biochemical affinity purification methods possibly provide the most direct approach to finding targeted molecules (5); their coupling to MS identification provides a valuable tool to screening without preconceptions the ample landscape of possible AMP/ACP targets.

During the course of the project described in article V, we sought to complement the oncolytic studies of Ctn and Ctn[15-34] with a large-scale methodology such as affinity purification followed by proteomic identification, in order to explore potential intracellular interactors of both peptides ultimately related with their killing mechanism.

References

1. Scocchi M, Mardirossian M, Runti G, Benincasa M. Non-Membrane Permeabilizing Modes of Action of Antimicrobial Peptides on Bacteria. Current topics in medicinal chemistry. 2016;16(1):76-88.

2. Mader JS, Salsman J, Conrad DM, Hoskin DW. Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Molecular cancer therapeutics. 2005;4(4):612-24.

3. Ikonomopoulou MP, Fernandez-Rojo MA, Pineda SS, Cabezas-Sainz P, Winnen B, Morales RAV, et al. Gomesin inhibits melanoma growth by manipulating key signaling cascades that control cell death and proliferation. Sci Rep. 2018;8(1):11519.

4. Chen YL, Li JH, Yu CY, Lin CJ, Chiu PH, Chen PW, et al. Novel cationic antimicrobial peptide GW-H1 induced caspase-dependent apoptosis of hepatocellular carcinoma cell lines. Peptides. 2012;36(2):257-65.

5. Schenone M, Dancik V, Wagner BK, Clemons PA. Target identification and mechanism of action in chemical biology and drug discovery. Nat Chem Biol. 2013;9(4):232-40.

4.4 Article IV

Selective tumor cell attack by crotalicidin (Ctn) and its fragment Ctn[15-34]: insights into their dual membranolytic and intracellular targeting mechanism.

Synopsis

This work aims to characterize the antitumoral mechanism of Ctn and Ctn[15-34] as well as their activity impairment by serum proteases. Combining proteomics, flow cytometry and confocal microscopy assays, we explored both the membranolytic and intracellular targeting mechanisms using a leukemia cell line as a proof of concept.

Article IV

Pérez-Peinado C, Valle J, Freire JM, Andreu D. Tumor Cell Attack by Crotalicidin (Ctn) and Its Fragment Ctn[15–34]: Insights into Their Dual Membranolytic and Intracellular Targeting Mechanism. ACS chemical biology. 2020;15(11):2945–57. DOI: 10.1021/acschembio.0c00596

5

PROTEOLYTIC SENSITIVITY

This chapter explores the underlying features influencing peptide stability in human serum.

Contents

5.1 Introduction

5.1.1 Proteolytic susceptibility of peptides

The feasibility of peptide therapeutics is very often hampered by poor bioavailability, as peptide degradation by endogenous proteases results in generally short *in vivo* lifetimes (1). Proteolysis can occurs at multiple locations: gastrointestinal lumen, intestinal brush border, enterocytes, hepatocytes, antigen-presenting cells and plasma. In such a scenario peptide oral delivery is usually non-viable and even parenteral administration often requires high dosages (2). In view of this limitations, developing protease-resistant versions is a must if therapeutic peptides must live up to their promise.

5.1.2 Intrinsic features for proteolytic protection in Nature

Virtually all peptides are, by definition, vulnerable to proteolysis. However, some naturally-occurring peptides are found to display partial or even full intrinsic resistance to enzymatic digestion. Protection towards protease action is achieved by several strategies including the presence of D-amino acids in the sequence (3, 4), folding into highly knotted tertiary structures (5), post-translational modifications (PTM) (6), self-assembly (7) and/or arresting by circulating proteins (8, 9). Next, we discuss some noteworthy examples.

In bombins, amphibian AMPs, post-translational chirality inversion of one L-amino acid into its D-enantiomer occurs. Epimerization enhances activity and increases lifetime in serum (3). D-amino acids have also been found in AMPs from bacteria (bacteriocins) (4) and animal venoms (10).

Many natural cyclic AMPs are also highly resistant toward protease action due to their knotted conformations (5). Three main cyclic patterns have been found in natural AMPs, contributing to their packaging into compact structures: i) backbone to backbone, e.g., head-to-tail fusion such as in the cyclotides (11); ii) backbone to side chain, as found in lasso peptides (12); and iii) side chain

to side chain, usually via disulfide bridges, as displayed by defensins and quite a few other AMPs (13).

Additional post-translational strategies increasing peptide stability have also been described. Thus, C-terminal amidation is very common in natural AMPs while N-terminal acetylation, though not so widespread, also prevents exoprotease action (6, 14). Several additional PMTs such as lipidation, halogenation, glycosylation, phosphorylation or sulfation have been found also in natural peptides, improving both stability and bioactivity (6).

Peptide self-assembly into super-structures is only rarely observed in Nature, but mutations or modifications of the natural template may endow peptides with self-aggregating properties and resulting lower susceptibility to proteases (7). In particular, dimerization almost invariably decreases protease vulnerability relative to the monomer, and strategies have been proposed in this regard (15).

Finally, although AMP binding to serum carrier proteins protecting peptides from enzymatic action has not been explored in depth, a few examples with synthetic analogs demonstrate that AMPs may bind to serum proteins, including albumin, lipoproteins or the α-1 acid glycoprotein, which improves the lifetime in serum (8, 16-18).

5.1.3 Peptide engineering towards proteolytic protection

Aside from a few privileged frameworks with intrinsic resistance to proteases, most natural bioactive peptides of therapeutic interest are linear and highly prone to degradation. Thus, modifications at different levels (peptide backbone, amino acid side-chains or at a higher structural order), frequently inspired in Nature, are usually attempted to overcome this main limitation, in the transition from early natural lead to therapeutic peptide (19). Single point amino acid replacement by non-labile units (e.g., D-amino acids, β-amino acids, methylated amino acids, tetrasubstituted, e.g., Aib, or unusual amino acids), different types of cyclization, N- and C-terminal blocking (acetylation, pyroglutamate, C-amidation, glycosylation and so on), peptidomimetics (e.g., peptoids) or conjugation to biopolymers (e.g., PEGylation, PASylation, HESylation), are

some of the common strategies, reviewed in (20-23).

5.1.4 Tools to explore proteolytic susceptibility of peptides

The current available toolbox for *in vitro* early evaluation of protease susceptibility is poor, limited and lacks universal standardized assays. Present methodologies include (i) *in silico* prediction of peptide degradation by specific enzymes, (ii) *in vitro* evaluation by HPLC and MS of reaction with either isolated enzymes or biological matrices, (iii) *in vitro* assessment of biological activity upon serum addition or pre-incubation, or (iv) *in vivo* pharmacokinetics/dynamics studies (24-26). Unfortunately, none of these techniques provide reliable insights, i.e., generalizable *rules*, of the molecular features that regulate peptide behavior in biological fluids. This still unachieved goal of pre-determining which enzymes and/or non-enzymatic proteins are likely to interact with a therapeutic peptide in biological fluids would greatly contribute to scaffold optimization in order to achieve long lifetimes.

Based on a literature revision, we addressed this last chapter with two main goals; i) establishing general protocols for *in vitro* evaluation of peptide stability, thus contributing new methodologies to the currently available toolbox, and ii) applying such methodologies to our case in study.

References

1. Werner HM, Cabalteja CC, Horne WS. Peptide Backbone Composition and Protease Susceptibility: Impact of Modification Type, Position, and Tandem Substitution. Chembiochem. 2015;17(8):712-8.

2. Weinstock MT, Francis JN, Redman JS, Kay MS. Protease-resistant peptide design-empowering nature's fragile warriors against HIV. Biopolymers. 2012;98(5):431-42.

3. Simmaco M, Kreil G, Barra D. Bombinins, antimicrobial peptides from Bombina species. Biochim Biophys Acta. 2009;1788(8):1551-5.

4. Cotter PD, O'Connor PM, Draper LA, Lawton EM, Deegan LH, Hill C, et al. Posttranslational conversion of L-serines to D-alanines is vital for optimal production and activity of the lantibiotic lacticin 3147. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(51):18584-9.

5. Namjoshi S, Benson HA. Cyclic peptides as potential therapeutic agents for skin disorders. Biopolymers. 2010;94(5):673-80.

6. Wang G. Post-translational Modifications of Natural Antimicrobial Peptides and Strategies for Peptide Engineering. Curr Biotechnol. 2012;1(1):72-9.

7. Tian X, Sun F, Zhou XR, Luo SZ, Chen L.

Role of peptide self-assembly in antimicrobial peptides. J Pept Sci. 2015;21(7):530-9.
8. Sivertsen A, Brandsdal BO, Svendsen JS, Andersen JH, Svenson J. Short cationic antimicrobial peptides bind to human alpha-1 acid glycoprotein with no implications for the in vitro bioactivity. J Mol Recognit. 2013;26(10):461-9.
9. Rimac H, Debeljak Z, Bojic M, Miller L. Displacement of Drugs from Human Serum Albumin: From Molecular Interactions to Clinical Significance. Current medicinal chemistry. 2017;24(18):1930-47.
10. Kreil G. D-amino acids in animal peptides. Annu Rev Biochem. 1997;66:337-45.
11. Craik DJ, Du J. Cyclotides as drug design scaffolds. Curr Opin Chem Biol. 2017;38:8-16.
12. Hegemann JD, Zimmermann M, Xie X, Marahiel MA. Lasso peptides: an intriguing class of bacterial natural products. Acc Chem Res. 2015;48(7):1909-19.
13. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3(9):710-20.
14. Mura M, Wang J, Zhou Y, Pinna M, Zvelindovsky AV, Dennison SR, et al. The effect of amidation on the behaviour of antimicrobial peptides. European biophysics journal : EBJ. 2016;45(3):195-207.
15. Lorenzon EN, Piccoli JP, Santos-Filho NA, Cilli EM. Dimerization of Antimicrobial Peptides: A Promising Strategy to Enhance Antimicrobial Peptide Activity. Protein and peptide letters. 2019;26(2):98-107.
16. Svenson J, Brandsdal BO, Stensen W, Svendsen JS. Albumin binding of short cationic antimicrobial micropeptides and its influence on the in vitro bactericidal effect. J Med Chem. 2007;50(14):3334-9.
17. Sivertsen A, Isaksson J, Leiros HK, Svenson J, Svendsen JS, Brandsdal BO. Synthetic cationic antimicrobial peptides bind with their hydrophobic parts to drug site II of human serum albumin. BMC Struct Biol. 2014;14:4.
18. Sorensen O, Bratt T, Johnsen AH, Madsen MT, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma. J Biol Chem. 1999;274(32):22445-51.
19. Erak M, Bellmann-Sickert K, Els-Heindl S, Beck-Sickinger AG. Peptide chemistry toolbox - Transforming natural peptides into peptide therapeutics. Bioorg Med Chem. 2018;26(10):2759-65.
20. Yin N. Enhancing the Oral Bioavailability of Peptide Drugs by using Chemical Modification and Other Approaches. Medicinal Chemistry. 2014;4(12):763-9.
21. Molchanova N, Hansen PR, Franzyk H. Advances in Development of Antimicrobial Peptidomimetics as Potential Drugs. Molecules. 2017;22(9).
22. Goodwin D, Simerska P, Toth I. Peptides as therapeutics with enhanced bioactivity. Current medicinal chemistry. 2012;19(26):4451-61.
23. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug discovery today. 2010;15(1-2):40-56.
24. Gasteiger E, Hoogland C, Gattiker A, Duvaud Se, Wilkins MR, Appel RD, et al. Protein Identification and Analysis Tools on the ExPASy Server. In: Walker JM, editor. The Proteomics Protocols Handbook. Totowa, NJ: Humana Press; 2005. p. 571-607.
25. Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, et al. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol. 1999;112:531-52.
26. Jenssen H, Aspmo SI. Serum stability of peptides. Methods Mol Biol. 2008;494:177-86.

5.2 Article V

Structural determinants conferring unusual long life in human serum to rattlesnake-derived antimicrobial peptide Ctn[15-34]

Synopsis

This paper evaluates the structural contributions (e.g., peptide sequence arrangement, terminal modification and self-aggregation) of Ctn[15-34] to its unusual prolonged lifespan in serum as well as the impact of serum addition on its bactericidal activity.

Article V

Pérez-Peinado C, Dias SA, Mendonça DA, Castanho MAR., Veiga AS, Andreu D. Structural determinants conferring unusual long life in human serum to rattlesnake-derived antimicrobial peptide Ctn[15-34]. Journal of peptide science. 2019;25(8):e3195–n/a. DOI: 10.1002/psc.3195

5.3 Introduction

The previous introductory section (related to article V) included the general aspects and considerations about peptide proteolysis, protease-resistance mechanisms and general tools to address it. It highlighted the limitations of current methodologies for in-depth studies of peptide proteolytic susceptibility/resistance issues and their behavior/interaction with biological matrixes and their components. In the next article (article VI), we applied proteomics to explore peptide partners in the biological matrix, specifically using as a proof of concept human serum, the principal acellular component of blood, with plenty of protein components to interfere with the therapeutic activity of peptides when administered by intravenous route.

5.4 Article VI

Decoding the human serum interactome of snake-derived antimicrobial peptide Ctn[15-34]: Toward an explanation for unusually long half-life

Synopsis

In this work we studied the potential serum interactors of Ctn[15-34], ultimately acting as peptide arresting proteins conferring protection against serum proteases. Using an affinity purification/mass spectrometry approach combined with surface plasmon resonance, we screened and validated peptide-protein interactions possibly affecting the peptide degradation behavior in serum. We additionally retrieved valuable information from the proteomic analysis pointing out to a putative immunomodulatory role of Ctn[15-34].

Article VI

Pérez-Peinado C, Defaus S, Sans-Comerma L, Valle J, Andreu D. Decoding the human serum interactome of snake-derived antimicrobial peptide Ctn[15-34]: Toward an explanation for unusually long half-life. Journal of proteomics. 2019;204:103372–. DOI: 10.1016/j.jprot.2019.04.022

6

ACHIEVEMENTS, CONCLUSIONS AND FUTURE PERSPECTIVES

The final chapter summarizes the main achievements and conclusions of the present thesis and includes a compendium of challenges and ideas for future work.

Contents

6.1 Achievements

Each chapter of the present thesis has dealt with diverse questions matching the initial objectives of our project. They can be summarized as follows: (i) can we find bioactive peptides in snake venoms with therapeutic properties of interest?; (ii) could we apply these natural peptides to treat microbial infections and cancer?; (iii) can they be optimized to become more efficient and stable?; (iv) how do these peptides work?; (v) are they stable enough against proteases to really achieve any therapeutic effect?; (vi) what determines the proteolytic susceptibility of peptides and how can we address it?

To answer these questions, six full papers have been presented in the preceding sections, with an appropriate discussion. In closing, I would like to devote a final section to briefly integrating the lessons learned so far and to evaluating them as a whole.

6.1.1 Value and impact of the discovery of vipericidins

This thesis started with the initial goal of identifying CATH-related peptides in the venoms of South America snakes. In this regard, we identified six novel CATHs: four of them described for the first time from pit vipers (collectively named vipericidins) and two new CATHs from an elapid snake, to add to the three previously known elapid CATHs (**article I**). Since that initial discovery, 12 additional works related to vipericidins have been published (including articles of this thesis). The most substantial part of this studies, involving Ctn and Ctn[15-34], is summarized on Figure 6.1 and recapitulated below. Studies focused on other vipericidins, for instance batroxicidin, the 34-mer peptide in the venom gland of *B. atrox*, have found antimicrobial and anti-parasitic effect against *Trypanosoma cruzi*, the causing agent of Chagas' disease, and also an overall proinflammatory profile (1, 2).

As summarized in Figure 6.1 and in line with our work, colleagues from Brazil focused on additional therapeutic applications of Ctn and Ctn[15-34]. For instance, Cavalcante et al. described their activity against opportunistic yeast and dermatophytes, alone or in combination with conventional antifungals.

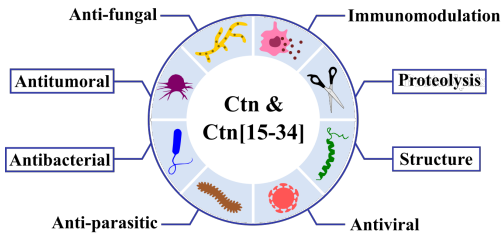


Figure 6.1: Summary of relevant information currently available for Ctn and Ctn[15-34]. Significant biological activities and additional key features provided throughout this thesis (encircled) and other related works are depicted on this figure.

Although the Ctn[15-34] fragment lost activity against dermatophytes, it became more active against pathogenic yeasts, such as several *Candida* species (MICs $\sim 5 - 10$ M) and synergized with amphotericin B (3). In particular, the fragment acts through *C. albicans* membrane permeabilization and necrosis (4). Like batroxicidin, Ctn showed a predominant proinflammatory profile and was also able to induce necrosis of all developmental forms of *T. cruzi* at low M range. Instead, Ctn[15-34] only preserved the activity against the trypomastigote form of *T. cruzi* (2, 5). The fragment also showed antiviral activity against infectious myonecrosis virus, an epizootic agent that threatens the shrimp production in Brazil and for which so far no treatment exists (6).

Insights into the functionality of structural domains of SV-CATH were presented by Oliveira-Júnior et al., using as a model Ctn[15-34]. As discussed in **article I**, SV-CATHs contain an anionic region between the cathelin domain and the mature AMP domain not usually found in other cathelicidins. By including a Glu decapeptide in the N-term of Ctn[15-34] to generate a "pro-peptide" model, Oliveira-Júnior et al. confirmed that this acidic moiety prevents peptide antimicrobial activity (preventing also peptide action before mature peptide release) and contributes to a more helical conformation (7).

6.1.2 Lessons and achievements of the optimization strategy

One of our initial goals was to identify the minimal active motif of the native peptide candidate and, from there, to obtain a shortened, optimized version. To this end, we dissected Ctn into two fragments, based on an *in silico* proteolytic

		Expressed/calculated as:	Ctn	Ctn[15-34]
Therapeutic effect	Bacterial activity	MIC, *E. coli* ATCC 25922	0.78	3.13
	Antitumoral activity	EC_{50}, U937 cells	0.47	18.77
	Antifungal activity	MIC, *C. albicans* CI from urine	40	10
Toxicity	Anti-parasitic	IC_{50}, T. *cuzi,* trypomastigote	0.22	9.5
	Hemolysis	HC_{50}	> 400	> 400
	Healthy cells toxicity	TC_{50}, PBMC	7.8	130
Selectivity	Selectivity for bacteria	TC_{50}, PBMC / MIC, *E. coli* ATCC 25922	10	42
	Selectivity for tumor cells	TC_{50}, PBMC / EC_{50}, U937 cells	16.6	6.9
	Selectivity for fungi	TC_{50}, PBMC / MIC, *C. albicans* CI from urine	0.195	13
	Selectivity for parasites	TC_{50}, PBMC / IC_{50}, *T. cuzi* , trypomastigote	35.5	13.7
Proteolysis	Proteolysis	$t_{1/2}$, estimated by HPLC	71	770
	Activity impairment by proteolysis (bacteria)	MIC, 12 h serum pre-incubation, *E. coli* ATCC 25922	-	100
	Activity impairment by proteolysis (tumor)	IC_{50}, 12 h serum pre-incubation, U937 cells	0.9	84
Others	Synthesis yield	#	8.7	25
	Costs of production	*	590	360

Table 6.1: Summary of relevant properties of Ctn and Ctn[15-34]. MIC, minimal inhibitory concentration; EC_{50}, half effective concentration; HC_{50}, half hemolytic concentration; TC_{50}, half-toxic concentration; (all values expressed in μM). $t_{1/2}$, half-life; (expressed in min). #Calculated as a percentage of the expected mg divided by the obtained mg. Price in for 5 mg of 90 % pure product, synthesized by SPPS. Data from current rates of the UPF peptide synthesis core facility (considerably lower than those applied by custom synthesis European companies).

approach (**article II**). The dissection resulted into a totally inactive fragment, Ctn[1-14], and the active fragment Ctn[15-34]. Initially, this result was somewhat puzzling because it was contrary to conventional wisdom, according to which AMPs tend to be amphipathic helices, which was precisely the case with Ctn[1-14]. Our findings were very conclusive to the contrary, in this particular instance, showing the only relative value of *in silico* tools for prediction of antimicrobial regions. In contrast, and despite its overall disordered structure, Ctn[15-34] defied predictions and showed remarkably interesting properties.

In retrospect, the obvious question that we may ask is: did we succeed or not? The answer is, to some extent, yes. Table 6.1 may help clarify this point.

In respect to its therapeutic activity and selectivity, Ctn possesses overall lower effective concentrations (against bacteria, parasites and tumor cells) but also displays higher toxicity. If we compare both peptides in terms of selectivity, Ctn[15-34] is more efficient on discerning bacterial and fungi cells from healthy eukaryotic cells while Ctn displays a higher selectivity ratio for tested tumor cells and parasites. Such disparity does not have a clear explanation, but it might be ultimately related to the specific membrane molecules targeted by each peptide.

The impact of downsizing on the general mode of action was practically insignificant. Ctn[15-34] preserved the overall membrane-disruptive ability of Ctn against bacterial and tumor cells (**articles III and IV**). Nevertheless, slight differences on their permeabilization kinetics were detected. In addition, the enriched cellular processes retrieved from the proteomic study of peptide interaction with tumor cell lysates (**article IV**) showed shared pathways shared by Ctn and Ctn[15-34], but also some exclusive routes for each peptide. These slight differences have been addressed in depth on each paper. In short, differences found may be due to the variability of charge, amphipathic conformation and structure between both peptides.

From a manufacturing perspective, we can consider the downsizing into Ctn[15-34] a success (Table 6.1). The 40 % reduction in size entails a significant reduction in costs, production time and synthetic efficacy. More significantly, the half-life in human serum was greatly improved in the shortened version (Table 6.1), a beneficial quality attribute as a therapeutic drug candidate. In particular, **articles V and VI** dealt with the specific determinants of Ctn[15-34] half-life and both suggest that this peptide still maintains antibacterial activity up to 12 h in presence of human serum. Similar results were obtained for its antitumoral activity, but interestingly the parental peptide showed almost the same ability to induce tumor cell death after 12 h pre-incubation with serum.

Altogether, we can consider the rational dissection of Ctn to give Ctn[15-34] a success. Alternative or additional optimization strategies should be considered in order to improve its selectivity for tumor cells and parasites. Additional *in vivo* pharmacokinetic experiments will also clarify on the stability and bioavailability issues (see future perspectives in the next pages). In summary, a take-home message can be drawn from this dissection exercise: peptide size

reduction based on proteolytic studies can contribute to the rational design and optimization of initial lead candidates.

6.2 Conclusions

This work allowed us to conclude that:

1. Vipericidins are a new family of peptides with cathelicidin structure present in snakes of the *Viperidae* family. Cathelicidin-related peptides were isolated for the first time from pit vipers by means of venom gland cDNA libraries screening and sequencing. The alignment of vipericidins with other snake-derived cathelicidins from elapid snakes demonstrated a remarkable conservation among sequences encoding for mature peptides. It also revealed the presence of a distinctive anionic region intercalated between the cathelin and the mature peptide domain, not present in other vertebrate cathelicidins. Phylogenetic analysis nested these reptilian cathelicidins within a larger group including those from ungulate mammals, marsupials, turtles, birds and primitive rodents.

2. Synthetic replicas of mature vipericidins were developed and functionally characterized as AMPs and/or ACPs. Two vipericidins (Ctn and batroxicidin) were efficiently synthesized by SPPS and confirmed as antibacterial agents, showing preference for Gram-negative strains. Ctn also induces strong decrease in tumor cell viability. Their therapeutic activity in the low M range, together with their low ability to induce hemolysis, portray them as attractive prototypes for new therapeutic agents, particularly crotalicidin (Ctn).

3. In membrane-like environments, Ctn displays a well-defined helix at residues 3-21 followed by an unstructured C-terminal stretch. These findings provide valuable clues in terms of structure-activity relationships. The structure analysis of Ctn by CD and NMR of Ctn suggests an overall random coil conformation in aqueous solution that shifts to a highly helical organization when enclosed in micelles resembling biological membranes. Results also point out to a key role of the N-terminal amphipathic helix in the initial interaction and recruitment into biological membranes. In contrast, the C-terminal, hydrophobic disordered stretch holds a multidisciplinary role: it may act as an anchor to insert within membranes once the peptide is located in bacterial or tumor cell surfaces and also serves as an interaction domain with protein carriers in serum.

4. Dissection of Ctn at Val14 retrieved an helical N-terminal inactive fragment, Ctn[1-14], and an structurally disorganized C-terminal fragment, Ctn[15-34], that not only retains the main activity of Ctn but also shows some enhanced properties. Ctn[15-34] presents a higher half-life in human serum, less hemolytic effect, size reduction and hence easier/lower-costs of production than Ctn. In addition, this fragment retains, to a reasonable extent, the antimicrobial behavior against Gram-negative strains of Ctn and its activity against some tumor cells such as myeloid leukemia cell line U937. Ctn[15-34] also presents enhanced selectivity for bacteria, while Ctn shows better selectivity for tumor cells.

5. Ctn and Ctn[15-34] induce bacterial death by disrupting the membrane. Using *E.coli* strain as bacterial model and applying a set of high-resolution techniques, we confirmed the membranolytic ability of both peptides and characterized/monitored the mechanism steps. The membranolytic process includes: i) initial recruitment by electrostatic interactions with anionic components (i.e., lipopolysaccharide, phosphatidylserine and cardiolipin); ii) peptide accumulation up to an effective threshold concentration; iii) final membrane destabilization and induction of cell death with ensuing leakage of cytoplasmic content.

6. Ctn and Ctn[15-34] attack tumor cells by a plausible dual mechanisms, combining membrane disruption and inhibition of key intracellular processes. Despite slight differences, overall Ctn and Ctn[15-34] are internalized by endocytosis and locate preferentially at the tumor cell membrane and nucleus. Cell death occurs by permeabilization of the membrane and/or interference with vital processes such as metabolism of proteins and DNA, cell cycle, signal transduction and/or programmed cell death.

7. Ctn and Ctn[15-34] appear to exert complementary immunomodulatory properties. The AP-MS studies used to explore intracellular tumoral targets as well as serum interactors identified a set of proteins functionally related to immune-system processes. The role of these proteins and pathways in the context of Ctn and Ctn[15-34] bioactivity remains to be elucidated in future studies.

8. Ctn and Ctn[15-34] can exert their therapeutic activity in the blood stream after intravenous administration without rapid suppression of activity by serum proteases. Our studies on protease-rich

fluids ruled out oral administration of Ctn[15-34] but support an intravenous route of administration. Bioactivity determination in serum suggests that peptides retain activity during a large enough interval to exert therapeutic effect before complete degradation.

9. Structural determinants and binding to serum proteins explain the unusual prolonged half-life in serum of Ctn[15-34]. Despite being a linear peptide *a priori* susceptible of rapid clearance by proteases, Ctn[15-34] in fact shows a ca. 12 h half-life in human serum. C-terminal amidation and the constitutive sequence layout mentioned above in #3 are crucial for its slow degradation. Five serum carriers (two members of the albumin family, two apolipoproteins and a globulin) with peptide-arresting potential were identified and validated as Ctn[15-34] interactors, presumably at the C-terminal hydrophobic stretch.

10. The proteolytic proneness of peptides is governed by diverse factors, often overlooked in proteolytic studies. Key events such as peptide aggregation or protein binding may be crucial to modulate protease activity against peptides. Structural determinants intrinsic to the peptide framework also play a role. In this regard, this thesis provides a set of methodologies to address such issues that ultimately may contribute to design peptide replicas with desired and optimized lifespan in proteolytic-rich fluids.

11. AP-MS analysis must be take into consideration in studying the therapeutic potential of peptides. Affinity purification followed by mass spectrometry identification has been used to explore tumor intracellular targets of Ctn and Ctn[15-34] as well as to investigate serum interactors modulating Ctn[15-34] half-life. The methodology allowed us to gain important insights into the molecular effectors responsible for bioactivity.

6.3 Future perspectives

Research is a continuous dynamic process. Completion of this work has unveiled a miscellanea of additional ideas and experiments that must unfortunately be left for future endeavors. They can be gathered into two main groups:

i) Extending the present results:

1. Exploring alternative mechanisms of action contributing to the bactericidal action. The membranolytic activity of Ctn and Ctn[15-34] was evaluated, but alternative killing mechanisms should be investigated. Additional screening by proteomic and/or transcriptomic experiments, for instance, might shed light on those proteins/pathways modulated by peptides and ultimately inducing bacterial death.

2. The membranolytic mechanism of Ctn and Ctn[15-34] may be further investigated. The specific orientation/location of peptides on/inside the membrane and their specific mode of membrane destabilization could be explored by high resolution molecular techniques such as molecular dynamic simulations, by super-resolution light microscopy or (cryogenic) electron microscopy.

3. The endocytic routes used by Ctn and Ctn[15-34] to enter into tumor cells must be further characterized. While we obtained some insights into the specific internalization route into tumor cells using specific endocytic inhibitors, increasing evidence of their lack of specificity suggest the need for additional approaches to unequivocally define a route of entry, such as knocking down of critical endocytic proteins.

4. Additional analysis and validation of peptide interaction with retrieved tumor intracellular proteins are required. The AP-MS approach used to investigate Ctn and Ctn[15-34] intracellular targets was used to get initial insights into putative pathways altered by peptides and should be considered as a screening tool rather than a way to identify individual targets. Due to the large number of proteins retrieved and the difficulties foreseen to validate the contribution of each protein to the antitumoral activity, complementary approaches should be used as well. One must keep in mind that the AP-MS experiments were conducted on cell lysate incubated with peptides,

a setup that does not represent physiological conditions. Other methodologies such as transcriptomics or 2D gel electrophoresis of protein extracts from tumor cells incubated with peptides would be desirable to delimit the set of putative targets.

5. The immunomodulatory role of Ctn and Ctn[15-34] should be explored in depth. Some clues were indirectly obtained throughout our work suggesting a immunomodulatory role of Ctn and Ctn[15-34]. Specific proteins were revealed by proteomic studies to possibly be involved in such activity. Still, it would be of particular interest to further define the specific immunomodulatory role of Ctn and Ctn[15-34] and explore the molecular pathways/effectors already retrieved by the AP-MS assays.

ii) Suggestions for future work:

6. The spectrum of action of Ctn and Ctn[15-34] may be further analyzed. Additional activities of peptides can be further explored, for instance their ability to fight biofilm-forming bacteria or to act against solid tumors.

7. Microbial resistance to Ctn and Ctn[15-34] should be evaluated. Although both peptides, due to their mode of action, are expected to induce less bacterial and/or tumor cell resistances than conventional antibiotic drugs, experimental confirmation to this extent is recommendable.

8. *In vivo* studies are required. Effectiveness in animals (i.e., bacteremia and leukemia models), immunogenicity, safety and toxicity of Ctn and Ctn[15-34], as well as their pharmacokinetics and biodistribution *in vivo* are necessary.

9. Additional peptide optimizations may be considered. Although we have quite successfully optimized the sequence of Ctn and apparently defined a minimal motif preserving its bioactivity, one can not exclude that further structural manipulation can bring forth unexpected benefits. Reengineering to improve peptide selectivity for pathogenic rather than commensal bacteria remains an attractive challenge.

10. Alternative drug delivery strategies (peptide encapsulation). In the event that *in vivo* studies of the intravenous administration of free Ctn or Ctn[15-34] do not match our *in vitro* findings of protease resistance, peptide encapsulation (e.g., liposomes or nanoparticles) would be an appealing route to explore.

References

1. Mello CP, Lima DB, Menezes RR, Bandeira IC, Tessarolo LD, Sampaio TL, et al. Evaluation of the antichagasic activity of batroxicidin, a cathelicidin-related antimicrobial peptide found in Bothrops atrox venom gland. Toxicon. 2017;130:56-62.

2. Oliveira-Junior NG, Freire MS, Almeida JA, Rezende TMB, Franco OL. Antimicrobial and proinflammatory effects of two vipericidins. Cytokine. 2018;111:309-16.

3. Cavalcante CS, Falcao CB, Fontenelle RO, Andreu D, Radis-Baptista G. Anti-fungal activity of Ctn[15-34], the C-terminal peptide fragment of crotalicidin, a rattlesnake venom gland cathelicidin. The Journal of antibiotics. 2016;70(3):231-7.

4. Cavalcante CSP, de Aguiar FLL, Fontenelle ROS, de Menezes R, Martins AMC, Falcao CB, et al. Insights into the candidacidal mechanism of Ctn[15-34] - a carboxyl-terminal, crotalicidin-derived peptide related to cathelicidins. Journal of medical microbiology. 2018;67(1):129-38.

5. Bandeira ICJ, Bandeira-Lima D, Mello CP, Pereira TP, De Menezes R, Sampaio TL, et al. Antichagasic effect of crotalicidin, a cathelicidin-like vipericidin, found in Crotalus durissus terrificus rattlesnake's venom gland. Parasitology. 2017:1-6.

6. Vieira-Girao PRN, Falcao CB, Rocha I, Lucena HMR, Costa FHF, Radis-Baptista G. Antiviral Activity of Ctn[15-34], A Cathelicidin-Derived Eicosapeptide, Against Infectious Myonecrosis Virus in Litopenaeus vannamei Primary Hemocyte Cultures. Food Environ Virol. 2017;9(3):277-86.

7. Junior NGO, Cardoso MH, Candido ES, van den Broek D, de Lange N, Velikova N, et al. An acidic model pro-peptide affects the secondary structure, membrane interactions and antimicrobial activity of a crotalicidin fragment. Sci Rep. 2018;8(1):11127.

A

Appendix

Contents:

A.1 Appendix I

New potent membrane-targeting antibacterial peptides from viral capsid proteins.

Dias SA, Freire JM, Pérez-Peinado C, Domingues MM, Gaspar D, Vale N, et al. New Potent Membrane-Targeting Antibacterial Peptides from Viral Capsid Proteins. Frontiers in microbiology. 2017;8:775–775. DOI: 10.3389/fmicb.2017.00775