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Elucidating Novel Bacterial Targets and Designing Unusual Antimicrobial Peptides: Two Faces of the Same Proteomic Coin

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Abstract

Antibiotics are essential compounds used for the control of bacterial infectious diseases. Resistance to antibiotics has become a worldwide public health problem. Therefore, effective therapy in treating resistant bacteria is essential and, to accomplish this, a comprehensive understanding of mechanisms that trigger drug resistance must be sought. The development of novel pharmacies, here focused on antimicrobial peptides (AMPs), has also been a remarkable challenge. To fill the manifold gaps that remain in clarifying bacterial resistance as well in the discovery of novel peptides with antimicrobial properties, proteomic tools have been pioneeringly used. In this context, this review focuses on novel proteomics techniques, on novel bacterial targets that could be used for drug design and on multiple AMPs found in different organisms. Moreover, the many difficulties and pitfalls in this field are also addressed, to shed some light on the two faces of the same proteomic coin.

Keywords: Antimicrobial peptides; Bacterial resistance; Peptidomics; Proteomics; Drug design

Introduction

The upsurge of resistant and pathogenic infectious bacteria presents an idiosyncratic challenge to medicine. Among such pathogens, the spread of multidrug-resistant 'ESKAPE' organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella* spp., *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) seems to be an enormous task to take on [1]. Indeed, some Gram-negative bacteria strains are resistant to all the currently available antibiotics [2]. The high infectivity of some bacterial strains, in addition to their resistance characteristics, has also raised numerous problems for neonates and elderly people in many parts of the world [3,4].

If on the one hand bacterial strains are becoming more dangerous and lethal each day, on the other, the scientific community is constantly preparing novel antibiotic compounds to control bacterial infectious diseases, and among these compounds are the antimicrobial peptides (AMPs) [5-8]. These compounds create new perspectives in the control of resistant bacteria due to a myriad of functions within a single compound, including bactericidal, fungicidal, anti-virus, immunomodulatory, anti-LPS and antitumor activities [7,9-14]. Antibiotics in general turn off or threaten essential cellular functions, reducing microorganism viability and resistance mechanisms, and thus seem to exploit several possible strategies of drug prevention. In such a dramatic and dynamic relationship between the host and bacterial pathogens, there are multiple genes and proteins involved that could be used in understanding this relationship. However, these proteins can also be seen as targets for the development of novel antibiotics, including peptides that could be designed to dock such molecules. And at this point, proteomics may be a reliable tool that could contribute to drug development with clinical purposes. Although the major types of clinically important resistance mechanisms have been elucidated for a long time, being generally well understood [15,16], bacteria have also created some new strategies to evade novel antibiotics [17,18]. Moreover, proteomics, and especially peptidomics, could be exploited to screen protein and peptide sequences that could be used, after optimization, in the development of novel antibiotics. With these points in mind, this review will focus on the use of original proteomic insights to discover pathogen targets and develop peptides, giving some directions for the next steps that proteomics could take in bacterial control.

Bacterial Resistance and Related Targets: The Heads Side of the Coin

In recent years the bacterial resistance problem has spread across all the continents and has now become a world issue. Bacterial infections caused by resistant strains cause complications in numerous hospitals worldwide, especially in patients compromised by age, disease and treatments with immunity-suppressant medications [19]. The resistance mechanisms include direct antibiotic destruction as occasioned by β-lactamases; target modifications such as the mutation on 30S ribosomal protein RpsL that confers streptomycin resistance; and penetration restriction and/or drug efflux as observed in the linezolid efflux occasioned by the AcrAB–TolC multidrug pump [20,21]. In summary, cases of resistance to all the mainstream antibiotic classes employed in clinical practice have been reported [17,22]. In this context, it is vital to intensify the understanding of resistance mechanisms in order to develop pharmacies with prospective activity against bacterial pathogens. To fill the manifold gaps that remain in our understanding of bacterial resistance, proteomics has been used to elucidate bacterial physiology in response to the presence of antibiotic compounds [17]. In fact, proteomics has advanced to being an essential tool for this research field, due to rapid advances in whole genome sequencing and proteomic technologies [23].

In spite of these novel technologies, bacterial resistance has been

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mainly explored by 2-DE-based proteomic approaches focusing on different strains, multiple antibiotics and several stress conditions [17,18,23-29]. Furthermore, label-free quantitative proteomics have also been used to better understand the resistance process. Methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most studied microorganisms that are conspicuous sources of deadly bacterial infections in hospitals, has been also focused by counting-based label-free proteomics [30-34]. In an interesting report, global responses in MRSA and methicillin-susceptible *S. aureus* (MSSA) treated with sub-inhibitory oxacillin doses were studied. Oxacillin induced a modification in MRSA protein expression, with β-lactamase and penicillin-binding protein 2a clearly up-regulated. Moreover, as also previously observed, the presence of an antibiotic leads to a modification in the peptidoglycan biosynthesis pathway and carbohydrate metabolism [17,18,30].

Cationic AMPs resistance

Nevertheless, the examination of bacterial resistance by proteomics tools has not been restricted only to lactams. Bacterial resistance toward AMPs has also been studied, as summarized in Figure 1, because these compounds hold great promise for upcoming years in the field of antibiotic development. In general, AMPs may act directly on bacterial membranes [9-14], and it is known that polymyxins, which are the peptides that are normally commercialized, act on lipid A interaction from phospholipids and lipopolysaccharides (LPSs), competitively displacing Ca^{2+} and Mg^{2+} [9]. In both cases, peptides cause enormous disturbance to the bacterial outer membrane, inflicting damage on cell content and effecting further bacterial death [35]. But the way in which bacteria evade this peptide stress is a very intriguing question. Fehri et al. [36] used proteomic techniques to elucidate the resistance response of *Mycoplasma pulmonis* challenged by the AMPs mellitin and gramicidin D. In this report they establish a clear correlation between antimicrobial resistance and stress reaction, and it was possible to observe an alteration in the expression of genes involved in stress response, such as the hrcA gene, which controls proteinase Lon and DnaK chaperone expression. The same authors also observed an up-regulation in energy metabolism enzymes, probably directed to balancing the increased energy demand [36]. Similar data were obtained by Maria-Neto et al. [18], in a study that also observed the up-regulation of proteins involved in stress response and energy metabolism by using an *Escherichia coli* model with high resistance to magainin. Those authors also observed an improvement in the expression of PNPase, which is responsible for catalyzing mRNA molecules detaching nucleoside diphosphate. This compound stimulates a high energy level in diphosphate bonds. These data suggest that an enhancement in energy metabolism seems to be essential for bacterial resistance.

Furthermore, nitrogen metabolism was also modified, with an up-regulation of glutamine synthetase enzyme, which is capable of activating alterations in the cell wall, such as wall thickness, or of decreasing peptidoglycan cross-linking. Finally and no less importantly, the up-regulation of glucan biosynthesis of protein G was confirmed. This protein is closely involved in cell osmolarity regulation and in cellular envelope organization [18]. Other models also showed that bacterial response to the presence of AMPs is a major combination of stress avoidance and energy metabolism improvement. For example, other authors achieved the same data by using a combo of proteomic tools and real-time PCR in order to study the response of *Vibrio parahaemolyticus* resistance induced by Q6 AMP [37,38].

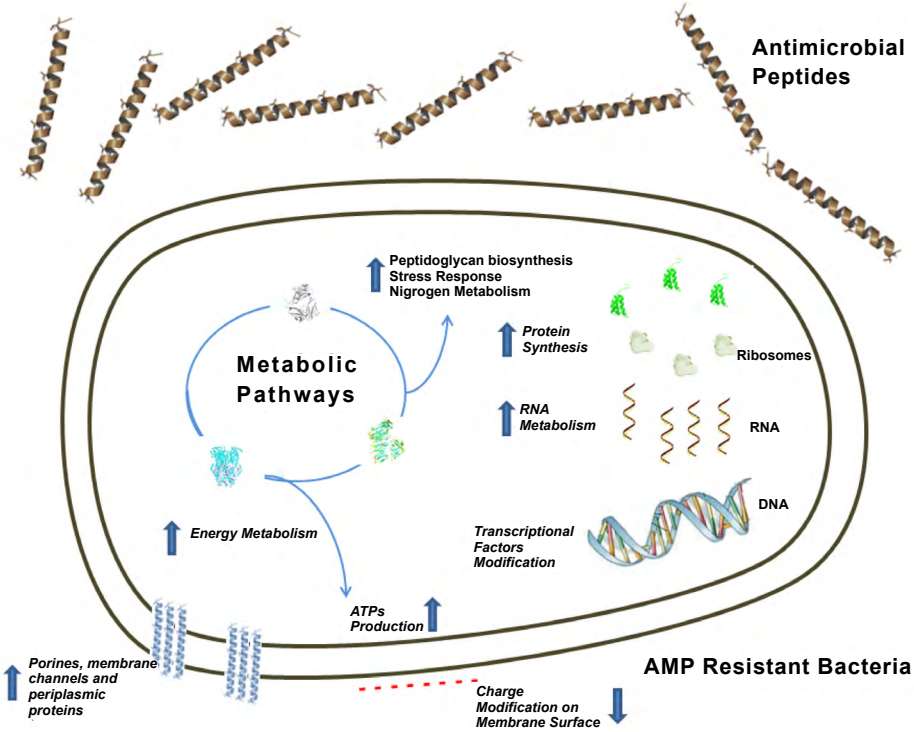

Figure 1: Schematic bacterial cell and the resistance mechanisms to antimicrobial peptides (AMPs) elucidated until now. Arrows pointing upward indicated pathway up-regulation and arrows pointing down indicate pathways down-regulation. Brown helical structures show a poly-alanine antimicrobial peptide Pa-MAP and blue helical structures represent porines and membrane channels. Red dotted line represents charge modification in anionic bacterial cell surface. Metabolic pathways included energy and amino acid synthesis pathways.

Colistin resistance

Among the AMPs, colistin resistance is leading the number of studies. Fernandez-Reyes et al. [39] showed 35 proteins differentially synthesized in a comparison between colistin-resistant and -susceptible *A. baumannii* strains. In resistant strains three outer membrane porins were observed. OMPs (Outer Membrane Proteins) were also found, although colistin does not use porins to cross the outer membrane. The differential porin expression related to polymyxin resistance has also been defined in *Salmonella typhymurium*, *Pseudomonas* spp. and *E. coli* strains [40,41]. Furthermore, a study performed by Fernandez et al. [42] showed the identification of a protein that seems to be involved in the construction of CsuA/B channels in *P. aeruginosa* polymixin-resistant strains. This modification promotes the bacterium's capability of forming biofilms, which have been considered a key virulence factor [43].

It is important to remember that the central question seems to involve how colistin-resistant bacteria modify the LPS binding site on the outer membrane, preventing the antibiotic from entering the periplasmic space. This antibiotic attachment could be primarily affected by the combination of additional palmitic acid units, leading to an improvement in LPS hydrophobicity. Additionally, structural modifications can also occur by esterification of the lipid A phosphate group, causing a decreased negative charge [44]. The reduction of anionic charges seems to be an important issue in bacterial resistance to cationic AMPs. For example, Gram-positive bacteria can resist cationic AMPs by adjusting their anionic teichoic acids following amalgamation of D-alanyl residues to neutralize their superficial charge. This electrochemical modification modifies the barrier properties of Streptococcus cell walls and protects the bacterial membrane by decreasing the AMP penetration [45]. Furthermore, other genes and proteins have also been related to resistance to colistin. In a proteomic study of *S. typhymurium* strains resistant to colistin, none of the enzymes involved in LPS modification was identified, while PhoP, a LPS phosphorylation system component, was observed in the resistant strain [46]. Moreover, in colistin-resistant strains, a small periplasmic protein (OsmY), which is used as stress marker, was also located, and it is hypothesized that OsmY may compete with colistin, thus circumventing bacterial cell death [47,48]. Many other proteins have been found in colistin-resistant bacteria, including signal peptidases involved in cell invasion [48], and also multiple ribosomal proteins [39]. Fernandez et al. [42] suggested that in *A. baumannii* colistin-resistant strains, an up-regulation of the ribosomal protein S2 happens, which assists in periplasmic molecular translocation. This process could reduce the activity of the chaperone system, causing a loss in protein folding and deterioration in the overall protein synthesis.

In addition to colistin, heterogeneous vancomycin-intermediate *S. aureus* (hVISA), normally associated with clinical treatment failure, was also evaluated by proteomic techniques, since the hVISA resistance mechanism had not been fully clarified. For that, comparative proteomic analysis of two pairs of isogenic vancomycin-susceptible *S. aureus* (VSSA) and hVISA strains showed five up-regulated proteins (IsaA, MsrA2, Asp23, GpmA, and AhpC) but after further analyses, including real-time PCR, only the increased expression of isaA, a transglycosilase involved in peptidoglycan cleavage, may be related to hVISA resistance [49,50].

The challenge of multi-resistance understanding

Despite the exciting contributions of proteomics, it seems that the resistance process involves something much more complex than one or two proteins. Such complexity clearly makes it difficult to use a proteomic approach to find the holy grail of resistance, as occurred in the discovery of lactamase years ago. In this context, the understanding of multi-resistance to different classes of antibiotics has also been studied, but only conflicting data have been found [51]. Some authors noticed some equal protein targets related to resistance against different antibiotics. For example, the low abundance of two components of respiratory nitrate reductase (Nar) was validated in the presence of streptomycin, gentamicine, ceftazidime, tetracycline and nalidixic acid-resistant *Escherichia coli* strains by using gel-based proteomics and Western blot [52]. These data suggest that a low abundance of Nar seems to be essential for *E. coli* in resistance to aminoglycoside and cephalosporin antibiotics. Other authors also proposed that carbohydrate, lipid and amino acid metabolic pathways have an important role in the *E. coli* resistance process [17,19]. Multi-drug-resistant *A. baumannii* was also compared in large-scale 2D LC/MS/MS-based quantitative proteomics with drug-sensitive strains [53]. Interestingly, twenty percent of the expressed proteome was modified at least two-fold between the compared strains, including proteins related to resistance mechanisms, alteration of xenobiotics or drug transportation. Moreover porins, stress-response-related proteins, OMPs (Outer Membrane Proteins), secretion-related proteins, transporters, cell wall- and expolysaccharide-related proteins and lipoproteins were also detected, showing an extremely complex panorama [53].

All those data have allowed us to only scratch the surface of knowledge about resistance processes (Figure 1), since all those pathways could be a reflection of antibiotic therapy stress and, in spite of information provided for the understanding of bacterial physiology, the AMPs studied so far may not good candidates for drug development. Moreover, AMP mechanisms of action have not been fully elucidated. So it is still necessary to consider new routes to clarify the mechanisms established by these microorganisms to avoid the activity of antimicrobial molecules. Little is known about the metabolic and structural cellular modifications that start the resistance process. More wide-ranging studies must be performed by using multiple omics tools with the aim of explicating the processes that occur in sub-cellular structures, and in the near future we will probably see a more reliable map of strategies that bacteria use to evade AMP action. Such studies will unquestionably lead to the main targets and also to the development of new pharmacies to combat pathogens of clinical importance, providing unusual and extraordinary alternatives for the treatment of patients with chronic infections.

Antimicrobial Peptide Discovery and Design: The Tails Side of the Coin

If on the one hand the discovery of bacterial targets for antibiotic development has improved in recent years, on the other the discovery of AMPs by omics is just beginning. In spite of the enormous databanks created by proteomics and peptidomics, there are some limitations to those techniques. First, the work with native plants and animals has been extremely limited due to incomplete or inexistent reference databanks [54]. This clearly reduces the peptide matches in the database, decreasing the possibility of finding small peptides. Moreover, since predicting AMPs has been an enormous and unresolved task in the last decade, the search has been limited to peptides with known patterns including, specifically, folds and conserved Cys-Cys bond arrangements [55,56]. Success in finding AMPs has also been hindered by limited protein

detection, especially for peptides at lower concentrations and small molecular sizes. Both problems have restricted the discovery of AMPs by using 2D-gel based methods. Nevertheless, despite such pitfalls, the screening of AMPs continues to focus on a variety of organisms and a number of research groups are involved (Figure 2).

Amphibians

One of the key targets in sorting AMPs using peptidomics has recently been to study amphibian secretions, due to the relative ease with which they are extracted and also thanks to the large amounts peptides found. Amphibians also produce a wide variety of skin defensive compounds in response to different abiotic and biotic factors for their everyday survival, which makes them interesting study models [57]. Peptidomic investigation has led to the categorization of multiple AMPs in norepinephrine-stimulated skin secretions of three species of frogs from the Ranidae family, including *Lithobates forreri*, *Hylarana luctuosa*, and *Hylarana signata* [58]. Different AMP classes were observed among the three species, including ranatuerins, brevinins, temporins, esculentins and palustrins. In addition to the clear biotechnological potential of these peptides, the data were also used to better understand the phylogenetic relations between the three species. The same group also explored Merlin's clawed frog *Pseudhymenochirus merlini* from the Pipidae family by similar methods [59], showing peptides belonging to the hymenochirin and pseudhymenochirin families. Additionally, dermaseptin-like AMPs were also found in *Pachymedusa dacnicolor*. In addition to AMPs, post-translationally modified peptides with anti-infectivity capability such as bradykinins were also detected by MS in this same species, showing potent antibacterial activity against various pathogenic bacteria [60].

Indeed, AMP diversity in skin secretions is extremely high. In the case of the torrent frog, *Amolops jingdongensis*, 31 antimicrobial peptides belonging to nine different families were identified, including brevinins, odorranains, esculentins, temporins, amolopins and ranacyclins, as well two novel classes named jingdongins -1 and -2 [61]. Both were synthesized and their antimicrobial activities confirmed, clearly indicating the screening potential of the peptidomics technique. Furthermore, 29 different antimicrobial peptide precursors were characterized from the skin of *Hylarana spinulosa*, which have produced 23 mature AMPs pertaining to 12 different families.

In order to confirm the AMPs in the skin tissue and improve peptidome coverage, a combination of liquid chromatography with tandem mass spectrometry and gas-phase fractionation analysis was used. The antibacterial activities of peptides were further confirmed [62]. In fact, the combination of multiple tools for screening novel AMPs has definitively improved the number of goals in this field, especially due to coverage enhancement [62]. In this case, the mishmash of multiple omics tools such as proteome, peptidome, transcriptome and genome allow researchers to improve the detection from dozens to hundreds or thousands of AMPs. In the case of amphibian secretions, those techniques were successfully combined. Genomics and peptidomics were used in studying an array of anti-infection AMPs from the skin of the frog *Odorrana grahami*. From an individual skin, 372 cDNA AMPs sequences were described, encoding more than 100 AMPs.

This specific contribution almost triples the number of currently reported amphibian AMPs. Furthermore, diversification patterns suggest that point mutations as well as insertions, deletions and shuffling of oligonucleotide sequences were mainly responsible for this remarkable diversity [63]. In addition to an astonishing number of

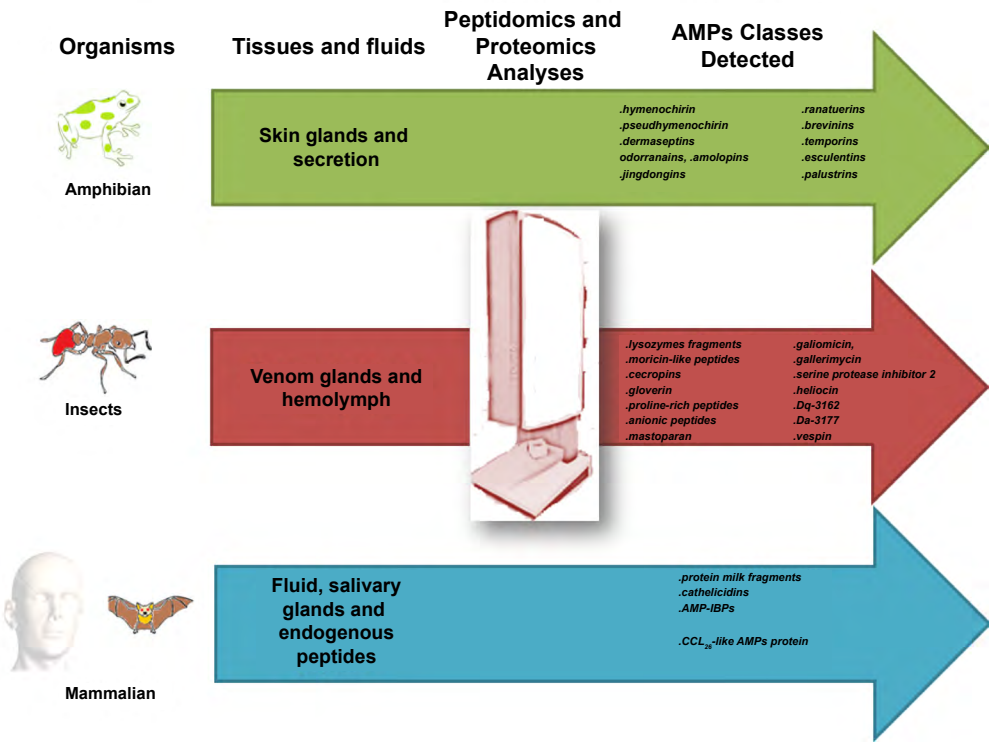

Figure 2: Antimicrobial peptides isolated in different groups of organisms by using proteomics and peptidomics. On the left side all classes were described. Green arrow corresponds to amphibians, brown arrow to insects and blue arrow to mammals. A MALDI ToF is represented in the centre of figure.

AMPs that could be explored as pharmacies in the future, this specific study produced a novel hypothesis in the animal defense process, casting doubt on the generally held opinion that only two or three dozen different AMPs are able to protect an amphibian.

The antimicrobial mechanisms of peptides selected were also examined, showing that they could exert their bactericidal functions by different strategies including peeling off the cell walls, making lamellar mesosome-like structures, yielding pores and inducing DNA condensation [63]. Years later [64], a strategy for peptide structural characterization, including the integration of shotgun cDNAs cloning encoding peptide precursors, deduction of supposed bioactive AMP structures and validation of these structures using tandem MS/MS sequencing were performed. These techniques led to the elucidation of the primary structures of nigrocin-2 homologues from skin secretions of four species of Chinese Odorrana frogs. Synthetic AMPs were challenged against bacteria and antimicrobial activities were confirmed.

Insects

But not only amphibian secretions have been explored; insects have been also focused in this field (Figure 2). A very comprehensive AMP repertoire from the wax moth *Galleria mellonella* hemolymph was investigated by using LC/MS, showing 18 AMPs including lysozyme fragments, moricin-like peptides, cecropins, gloverin, proline-rich peptides, anionic peptides, galiomicin, gallerimycin, serine protease inhibitor 2 and heliocin-like peptide [65]. Another example of successful AMP screenings is the insect venoms, in spite of noxious effects inflicted by them, which also show high peptide content and less complicated extraction procedures. Furthermore, venoms are also found in several animals with no genetic relations, which improved the possibility of finding really unusual AMPs. In this context, ants from the Dinoponera genus were explored by complementary mass spectrometric approaches. In addition to proteomic characterizations, two AMPs, called Dq-3162 and Da-3177, were identified and evaluated, showing wide-ranging antimicrobial activity [66]. Moreover, a combination of genomics and peptidomics was used to explore the venom gland of the wasp *Vespa tropica,* allowing the identification of nine different AMPs classified as mastoparan and vespid chemotactic peptides [67].

Mammals

Finally and no less importantly, mammals have also been evaluated for their abilities to produce AMPs, by proteomic tools (Figure 2). One example was a vampirome study [68]. Vampire bats are famous for being the only mammals that strictly feed on fresh blood. Since their saliva has been associated with anticoagulants, this secretion is an obvious target for drug screening. With this aim, the submandibular salivary glands of *Desmodus rotundus* were evaluated by transcriptomic analyses and, surprisingly, in addition to anticoagulant and vasodilator peptides, members of the TSG-6 (anti-inflammatory), antigen 5/CRISP and CCL28-like AMP protein families were also sequenced. Proteome analysis by nano LC-MS/MS confirmed the data [68].

Another mammalian fluid focused by AMP screening was milk. Milk is conventionally considered a perfect source of the basic essential nutrients required by newborns. A thorough examination in the last decade revealed that milk represents a more efficient ensemble of components that benefit infants and mothers, which included host-protection and also AMP synthesis. Peptidomics was used to analyze human milk, yielding an extensive protein array showing over 300 milk peptides yielded by larger protein milk components including β-casein [69]. Since a wide number of observed peptides showed significant sequence overlap with AMPs, antibacterial assays were performed, showing that milk peptide mixtures were able to inhibit bacterial development. Furthermore, goats' milk was also evaluated in order to better understand the proteins synthesized in response to intramammary challenge with bacterial LPS, which elicited strong animal immune responses [70]. Early 2-DE-based proteomics evaluation revealed few modifications in the expression of casein and plasma protein serum albumin, which are known to leak into milk during coliform mastitis in dairy cattle. Moreover, peptides were sequenced using nano-flow liquid chromatography coupled with tandem MS and, despite the notable presence of casein proteins and β-lactoglobulin, AMPs from the cathelicidn family was also observed [70]. These data suggest that milk proteins contain AMPs, providing a selective advantage through evolution by protecting the mother's mammary gland and her nursing offspring from infection.

Endogenous peptides

Rather than just protein degradation artifacts, endogenous peptides have been established to be important bioactive molecules acting as neurotransmitters, hormones, and antimicrobial agents. Since AMPs have common properties such as cationicity, amphiphilicity and helical structures, peptidomics have been also used to detect endogenous AMPs [71]. In a peptidomic survey of endogenous peptides an unusual intramolecular disulfide-linked 22-residue amidated peptide was identified [72]. This peptide, named AMP-IBP5 (antimicrobial peptide derived from insulin-like growth factor-binding protein 5), showed antimicrobial activity against several microorganisms tested at lower concentrations. Another practical application of this idea on AMP selection was applied by Sasaki et al. [73], using electron transfer dissociation (ETD) technology as well as collision-induced dissociation (CID), to identify endogenous peptides derived from secretory granules of a human endocrine cellular line. ETD provided more widespread fragmentation, leading to the identification of peptides that are not touched by CID. Among such extra peptides, a novel AMP from the neurosecretory protein VGF was identified, demonstrating once more the importance of different and integrated techniques.

Conclusive Remarks and Prospects

Nowadays, proteomic tools have accomplished noteworthy progress in the characterization of proteins involved in mechanisms of bacterial resistance as well as in the discovery of AMPs. On both sides, proteomics have been an amazing tool providing reliable contributions. However, the approach focused here is just at its beginning, since there are too many problems to be solved in order to provide a real contribution on drug development for combating infectious diseases.

On the bacterial resistance side, each antibiotic has a distinct protein expression profile, which clearly makes it difficult to construct a single database of proteins involved in the resistance process. At this point it is extremely difficult to choose an ultimate technique that could riddle the bacterial resistance. Every technique presents their benefits and pitfalls. For example 2DE are extremely important for protein panorama visualization, including the comparisons between protein patterns. Nevertheless this technique show limited resolution of proteins and peptides at lower concentrations. This limitation could be solved by LC-MS and quantitative label free techniques, which are able to improve the detection of such proteins. Nor can all such modifications be easily detected by proteomic techniques, due to

technical limitations that may be solved by using more sensitive MS techniques, such as Nano-UPLC, as well in combination with novel pyro-sequencing techniques applied to genomics and transcriptomics [74]. The current capability of ultra-sequencing associated with novel proteomic techniques may help to attain a deeper understanding of the molecular resistance mechanism, leading to novel targets that could be used for discovering unusual compounds from chemical libraries.

On the other side, the discovery of AMPs by proteomics tools has been a real challenge. Until now only a few samples have really explored, and many of them have focused on secretions such as venoms or amphibian skins due to the sample properties [63,66]. Although some initial trials have been performed with more astringent tissues such as flowers [14], any success has so far been obtained by using proteomics techniques. There are still thousands of organisms to be explored, including marine organisms, extremophiles and several others that showed the ability to produce AMPs by conventional technologies; for them, proteomics techniques must be improved and they must be integrated with transcriptomics techniques [75,76]. Although tandem mass spectrometry (MS/MS) combined with bioinformatics tools has permitted rapid and systematic protein identification based on peptide-to-spectrum matches, it has been an enormous task to obtain accurate identification of endogenous peptides, such as peptide hormones, neuropeptides, peptide pheromones and AMPs. Since these peptides are processed at sites that are problematic to predict reliably, the pursuit of their MS/MS spectra in sequence databanks needs to be performed without proteinase setting. Furthermore, many endogenous peptides have various post-translational modifications, making it essential to take these into account in database exploration. In order to fill those gaps, a novel MS/MS spectrum search tool has been developed for highly accurate identification of endogenous peptides by merging two diverse fragmentation approaches, including collision-induced dissociation and electron transfer dissociation, being very effective in discriminating correct peptide identifications from false hits [77]. At the moment this method is being applied to elucidating neuropeptides extracted from mouse pituitary tumor cells, but searching for AMPs by these methods seems to be a promising direction.

Different surfactants have also been explored in order to improve peptide ionization, which can be a real challenge in complex mixtures [78], and in the near future this may be applied to AMP selection. Additionally, for easy AMP detection the primary structures of such compounds must be better understood [54,56]. On this specific point more knowledge is needed, since several AMPs are quite promiscuous, being able to act in different ways under different conditions but with almost identical sequences [79]. This specific property could make it difficult to predict the function by proteomics, although MS techniques have been applied as a reliable and effective method for high-throughput AMP screening [13], and more studies must be performed in order to clarify structure-functional relations.

In summary, in spite of enormous efforts, we are just starting to understand bacterial resistance and AMP development by using proteomics, and great advances can be expected over the next years in this field. Those studies will soon be vital to control lethal bacterial pathogens and decrease the severe damage produced.

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