Antimicrobial susceptibility testing of antimicrobial peptides requires new and standardized testing structures

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Abstract

The need for optimized as well as standardized test systems of novel antimicrobial peptides (AMPs) was discussed by experts in the field at the International Meeting on Antimicrobial Peptides (IMAP) 2017 and the 2019 Gordon Research Conference (GRC) on Antimicrobial Peptides and a survey related to this topic circulated to participants to collate opinion. The survey included questions ranging from the relevance of susceptibility testing for understanding the mode of action of AMPs, to the importance of optimisation and a degree of standardization of test methods and their clinical relevance. Based on the survey results, suggestions for future improvements in the research field are made.

Key words: antimicrobial peptides, antimicrobial susceptibility testing, standardized test systems, survey results, International Meeting on Antimicrobial Peptides, Gordon Research Conference (GRC) on Antimicrobial Peptides

Introduction

Antimicrobial peptides (AMPs) are a common endogenous defence mechanism against pathogens and occur in all classes of life [1]. AMPs are relatively small peptides (4–50 amino acid residues) that are generally positively charged and often contain an amphipathic conformation with antimicrobial properties. They include various groups as, including cathelicidins, defensins or protegrins. Advances in synthetic biology techniques, chemical synthesis, and structural understanding have led to improvements in the antimicrobial spectrum and tissue compatibility of AMPs [2,3]. Because of their diversity and multiple mechanisms of action, these peptides offer an opportunity to overcome the global health crisis of antimicrobial resistance [4], although despite the promise shown by AMPs, it has been difficult to translate this into clinical approval. AMPs are capable of broadly targeting pathogenic microbes, including bacteria, protozoa, fungi, and viruses [5]. The most commonly described mechanisms of action of AMPs are displayed in Figure 1 and include membranolytic and non-membranotytic mechanisms to inhibit/kill pathogens. For example, when certain AMP reach a threshold concentration and spontaneously insert themselves into pathogen membranes they can lead to different models of pore and ion channel formation, eventually leading to cell lysis and death [6]. Other specific or less common modes of action of AMPs include membrane thickening/thinning [7], septum formation [6], charged lipid clustering [8], nucleic acid targeting [6], anion carriers [9], electroporation [10], non-lytic membrane depolarization [11], and non-bilayer intermediates [12].

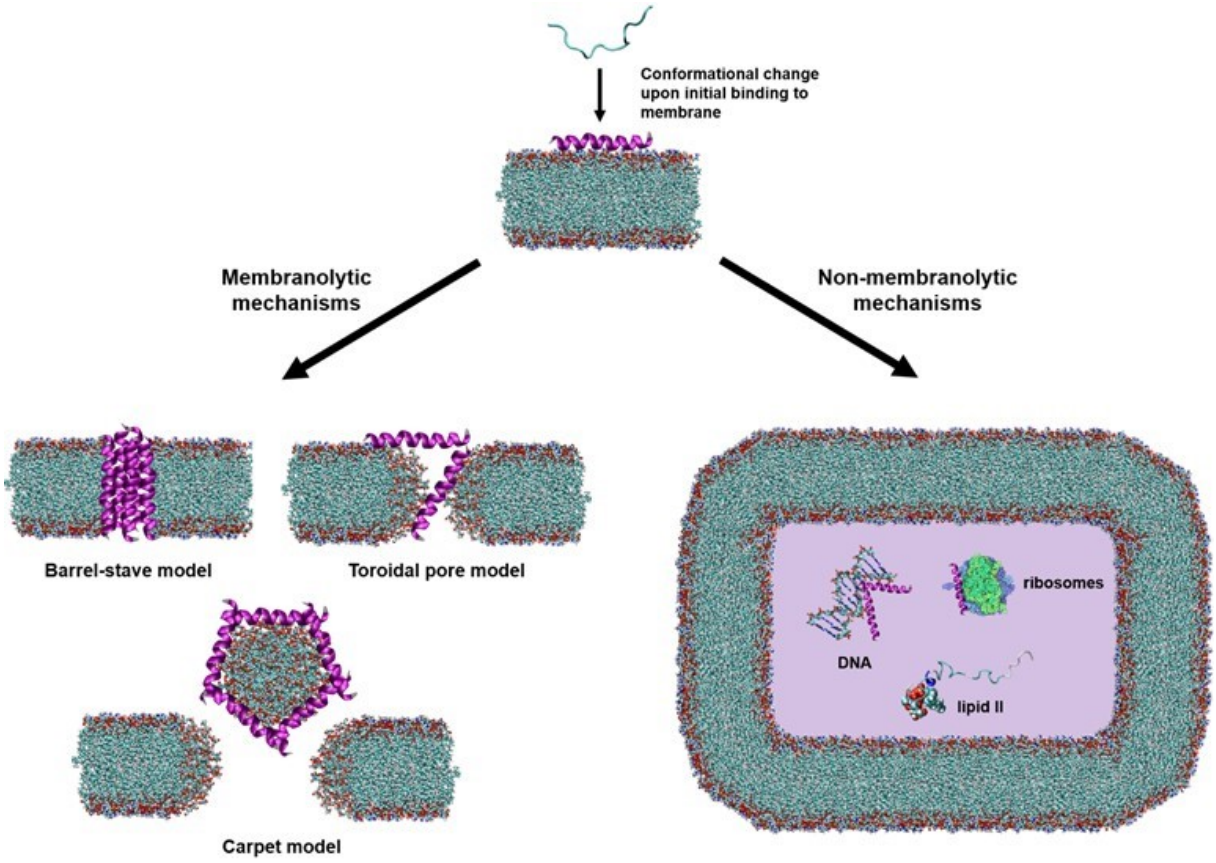

Figure 1. Depiction of selected modes of action of AMPs. Binding to biological membranes changes the conformation of the AMP. The subsequent membranolytic and non-membranolytic mechanisms lead to cell death.

The development of resistance to AMPs does not seem to occur, if at all, at the rate of resistance to conventional antibiotics [13,14]. It is important to be able to accurately assess clinically effective doses and treatment regimens of AMPs, not least to prevent bacterial resistance by underdosing. In order to accurately determine and predict the antimicrobial activity of AMPs, reliable and reproducible susceptibility assays are urgently needed. Susceptibility testing is influenced by many factors as outlined in our recent review [9]. The composition of media used is as important as the pH value, the ionic strength, the presence of proteases and metal ions. In addition, temperature, oxygen content and plasticware play a significant role on the outcome of the testing [15]. Therefore, it is critical to take as many of these factors as possible into

consideration when designing any protocol for susceptibility testing for AMP which can be used as a basis for predicting the *in vivo* activity and clincial efficacy. The need for optimised test systems was discussed by experts in the field at the International Meeting on Antimicrobial Peptides (IMAP) 2017 and the 2019 Gordon Research Conference (GRC) on Antimicrobial Peptides and the different observations made were subsequently collected in a survey, the results of which are reported here.

Methods

The survey was developed in 2019 (see supporting information). The questions covered a variety of topics related to AMPs, ranging from the relevance of susceptibility testing for understanding the mode of action of AMPs, to the importance of optimisation and standardization of test methods and their physiological/clinical relevance. Questions were also asked about possible official recognition of antimicrobial susceptibility testing (AST) for AMPs by organisations including EUCAST (European Committee on Antimicrobial Susceptibility Testing) and CLSI (Clinical and Laboratory Standards Institute), as well as the most appropriate means to describe such methods in publications.

Results

The survey link was opened by invited participants 56 times and 43 responses were collected. From these, 37 completed responses were included in the analysis. A list of statements summarizing the degree of agreement on the closed questions is given in Table 1. The results display a broad consensus that standardized test methods are important and essential for AST of AMPs and that these test methods should reflect the physiological (*i.e.*, *in vivo*) environment in which AMP will have to function as closely as possible. Due to the diverse structures and modes of action of AMPs, the majority of participants did not expect that a single method for testing all peptides can be devised. Interestingly, there was no consensus on the relevance of

standard AST for understanding the biology and potential of AMPs as new therapeutic agents. The majority of responses (81.08%) indicated that currently there is no awareness of the need for new testing methods in the regulatory landscape. Most participants (75.68%) also reported that new susceptibility testing methods are insufficiently described in published peer-reviewed papers.

The importance of standardised AST methods for AMPs, adapted to physiological conditions was again emphasized in the open questions of the survey (table S1). Aspects of susceptibility testing method development and its dissemination were listed as being important. In addition to the method's requirement to mimic the physiological environment, the importance of having a method that is simple, cheap, robust, reproducible, and automatable was noted. Otherwise, routine testing of large sample quantities (pathogens &/or AMPs) would not be feasible. Key factors for *in vitro* testing include pH, ion and bicarbonate concentration, and the presence of serum in the medium. Storage, dilution, structure and mechanism of action should be considered on the peptide side, as well as whether the biological matrix used (*e.g.*, growth medium, blood, serum, etc.) has an inhibitory effect on the tested peptides. Stability, purity and cytotoxicity must also be taken into account when working with peptides. Besides the peptides, the pathogens must also be considered. The inoculum density, the growth phase and conditions as well as biofilms, non-growing microorganisms, persister cells, small colony variants and spores are important factors. In general, the site of infection, the polymicrobial nature of infections, the presence of immune cells as well as the potential immune status of donors and possible synergies should be considered and also reflected by *in vitro* systems. Possible development of resistance to AMPs should also be assessed. Some participants thought that simulating a local environment by *in vitro* systems in which the peptide will later be applied (*e.g.*, topically to the skin, intravenously or nebulised into the lungs) is more important than a general standardized *in vitro* method for a peptide. It would be beneficial to test conventional antibiotics, as well as AMPs, in a physiological relevant new media or test system in order to obtain reference values.

For AMP AST methods to be more comprehensive and the values derived more comparable between labs and existing AST methods, many survey respondents stated that they would like to see AST methods for AMP described better and in more detail in publications. It is recommended that publications with incomplete descriptions of methodologies should not be cited. As an alternative resource to share/access information, the establishment of a website for publication of test methods or special journals for methods would be welcomed. The inclusion of comprehensive and clear AST protocols for AMPs in the CLSI guidelines was also proposed.

Conclusion

The research and development of AMPs as new potential classes of antimicrobial is thriving and expanding on a global basis. Optimised and where possible, standardised susceptibility testing methods for these promising antimicrobial candidates are needed to more accurately predict their therapeutic potential, as well as to enable direct comparisons among different AMPs. Due to the diversity of AMP structures and modes of action and the different physiological environments in which they will be applied as therapies, a range of defined test methods will be necessary. Close cooperation with relevant institutions including CLSI, EUCAST, USCAST, regulatory bodies and International Organization for Standardization (ISO) will be important going forward for the recognition and adoption of these new methods.

Supporting Information Available

The supporting information includes the survey design and the list of statements that were given to the open questions by the participants of the survey (Table S1). This information is available free of charge on the ACS Publications website.

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Table 1. Results of a survey among participants of IMAP (International Meeting on Antimicrobial Peptides) 2017 and 2019 Gordon Research Conference on Antimicrobial Peptides on the requirements for antimicrobial susceptibility testing (AST) of antimicrobial peptides (AMPs)/host defence peptides (HDPs).

Statement	yes	no	# of answers	% agreeing
1.1 Standard antimicrobial susceptibility testing (AST) methods (CLSI/EUCAST) are relevant for understanding of the basic biology (e. g. screening, mechanism of action) of AMP/HDP	15	21	37	40.54
1.2. Standard antimicrobial susceptibility testing (AST) methods (CLSI/EUCAST) are relevant for understanding of AMP/HDP as potential new therapeutic molecules (i.e. efficacy)	16	20	37	43.24
2. There is awareness of the need of the changing regulatory landscape with respect to proof of efficacy of non-traditional antimicrobials (FDA Workshop, EMA Concept paper, Tripartite meetings of FDA, EMA & PMDA, etc.)	7	28	37	18.92
3. Standardized methods are needed to test the antimicrobial activities of AMP/HDP, similar to the CLSI/EUCAST approved standards	33	0	37	89.19
4.1 Standardized methods are essential for peptide AST	34	2	37	91.89
4.2 One method or one set of methods can be relevant to a spectrum of AMP/HDP with diverse structures, functions, mechanisms of action etc.	13	20	37	35.14
4.3 A more specific framework should be developed from a set of methods targeted and bespoke to different types of peptides based on their structure, mechanism of action, etc.	28	5	37	75.68
5.1 AST conditions should better reflect the physiological environment in which AMP/HDP function	33	3	37	89.19
5.2 More physiologically relevant *in vitro* methods that more closely replicate *in vivo* conditions are important in facilitating prediction of *in vivo* outcomes and to replace, reduce & refine (three R's) essential *in vivo* animal experiments	32	2	37	86.49
6.1 Descriptions of novel/alternative methods for testing antimicrobial activity of AMP/HDP are sufficiently described in publications (for example, is detail limited by authors or editorial decisions, etc.)	9	22	37	24.32

Colour code	
Agreement	>80%
Agreement	70-80%
Agreement	30-70%
Agreement	0-30%