Title

Novel drugs against superbugs – preclinical optimizations

Context

Repeated use of antibiotics has given rise to microbial strains that are resistant to these therapeutic methods, making them less effective. This phenomenon, termed antimicrobial resistance, has recently been recognised by the World Health Organisation (WHO) as one of the most pressing global issues of our time, with good reason. The 2014 O'Neill report commissioned by the UK government compiled research by both the RAND Europe Corporation and KPMG, regarding the economic costs of resistance. The review highlighted links between drug-resistant infection and increased length of treatment, reduced chance of recovery, and increased mortality in the host – this in turn leads to greater economic impact in the form of increased cost of treatment, and lost days of labour (O'Neill, 2014). The report went on to estimate that without significant action, the global number of infection-related deaths per annum is likely to reach 10 million by 2050. While the current impact of antimicrobial resistance is certainly problematic, the ultimate scope of the threat lies in the simple fact that so long as we continue to rely on the same tried and tested pharmaceutical methods, the advent of an era where those methods have completely ceased to be effective is certain. Because long-term reliance on these drugs would only worsen the issue of resistance, other methods of treatment proven to be effective against these resistant strains must be considered and investigated, making the evaluation of the potential of antimicrobial peptide (AMP) application more critical than ever.

The term AMP refers to a diverse group of antimicrobial agents present in the innate immune response of many living organisms. Research into these agents has shown much about their therapeutic potential, as evidenced by a 2016 review compiling patents and patent applications related with the therapeutic applications of AMPs (Kosikowska & Lesner, 2016). The review identified that AMPs exert multiple methods of microbicidal action, and are able to do so across a broad spectrum of pathogens, while having high affinity and specificity for their target. This means that AMPs often display relatively low toxicity profiles, particularly when compared to certain currently used antibiotics such as doxorubicin or other anthracycline antibiotics (Kosikowska & Lesner, 2016). AMP activity has numerous benefits when compared to conventional methods, as shown in Table 1. Perhaps their most important advantage, however, is that many drug-resistant pathogen strains remain susceptible to AMP mechanisms of action. This feature highlights AMPs as not only a potentially more efficient therapeutic agent, but a possible asset in the effort to combat the effects of antimicrobial resistance.

Table 1 – Comparison of current antibiotics and AMPs (Marr, et al., 2006)

Property	Conventional antibiotics	Cationic antimicrobial peptides
Spectrum of activity	Bacterial infections (often selective)	Bacterial, fungal and viral infections; septicaemia; and/or inflammation
Uptake	Specific mechanisms	Relatively non-specific: based on charge. Self-promoted uptake
Targets	Usually one dominating target or class of targets (e.g. penicillin-binding proteins, topoisomerases, ribosomes)	Relatively less specific (possibly multiple targets in any given cell)
Resistance rate and mechanism	Resistance development at frequencies of 10^{-7} to 10^{-10}, or after a few passages at sub-MIC. Resistance caused by mechanisms such as reduced uptake or increased efflux, chemical modification or degradation of antibiotic, or altered target	Resistance generally cannot be directly selected. Needs multiple passages on sub-MIC concentrations to induce resistance. Resistance caused by mechanisms such as an impermeable outer membrane or specific proteases (can be overcome by incorporating D-amino acids or backbone alterations)
Additional activities	No	Include anti-endotoxic and/or boosting of innate immunity
Pharmacokinetics	Varies but once per week antimicrobials under development	Short systemic half-life owing to proteolytic degradation
Toxicology	Antibiotics tend to be one of the safest groups of pharmaceuticals	No known topical toxicities; systemic toxicity issues remain undefined
Manufacturing costs	Can be inexpensive (e.g. $0.8 per gram for aminoglycosides)	Expensive ($50–400 per gram)

However, many AMPs are limited by their short plasma half-life. A review compiled in 2013 regarding strategies to increase plasma half-life of peptide drugs highlights that most known peptides composed of naturally occurring amino acids are susceptible to enzymatic degradation, and so exhibit reduced

stability and relatively rapid renal clearance *in vivo* (Kim, et al., 2013). This intrinsic property limits the oral bioavailability of AMPs in their current form, and subsequently their pharmaceutical capability. There are also additional factors that can hinder microbicidal activity *in vivo* that would not be apparent *in vitro.* Certain AMPs have displayed immunomodulatory effects, which would involve host cell interactions that are unlikely to be represented in their interactions with bacterial cell cultures. Similarly, microbicidal activity could be reduced due to interaction with serum proteins that would not be present under *in vitro* conditions. Consideration must also be given to the method of drug administration, which could positively or negatively impact serum concentration of the AMP, and subsequently influence efficacy in a way that *in vitro* testing would not.

The observed difference between *in vitro* and *in vivo* AMP activity is undoubtedly a significant contributor to the large discrepancy between the array of peptides listed as potential drug candidates, and those that have undergone successful clinical trials. Therefore, in order to develop effective application of AMP-based therapy, it is clear that these issues must be identified and overcome. It can be observed in nature that the characteristics of a peptide, including activity and susceptibility to enzymatic degradation, can be altered via post-translational modification of the peptide sequence or structure. Studies have subsequently observed multiple synthetic modifications inducing an increase in stability of a peptide; a 2015 review highlights techniques such as the addition of the regulatory protein ubiquitin, or small ubiquitin-like modifiers to an amino acid sequence, as methods that are often associated with an increase in peptide function or stability (Duan & Walther, 2015), although it should be noted that a multitude of possible modifications exist, as exemplified by Figure 1. Additionally, evaluation of AMP interaction with *in vivo* components such as host cells or serum proteins could provide insight into ways by which these factors could be optimised, resulting in an increase in observed *in vivo* efficacy.

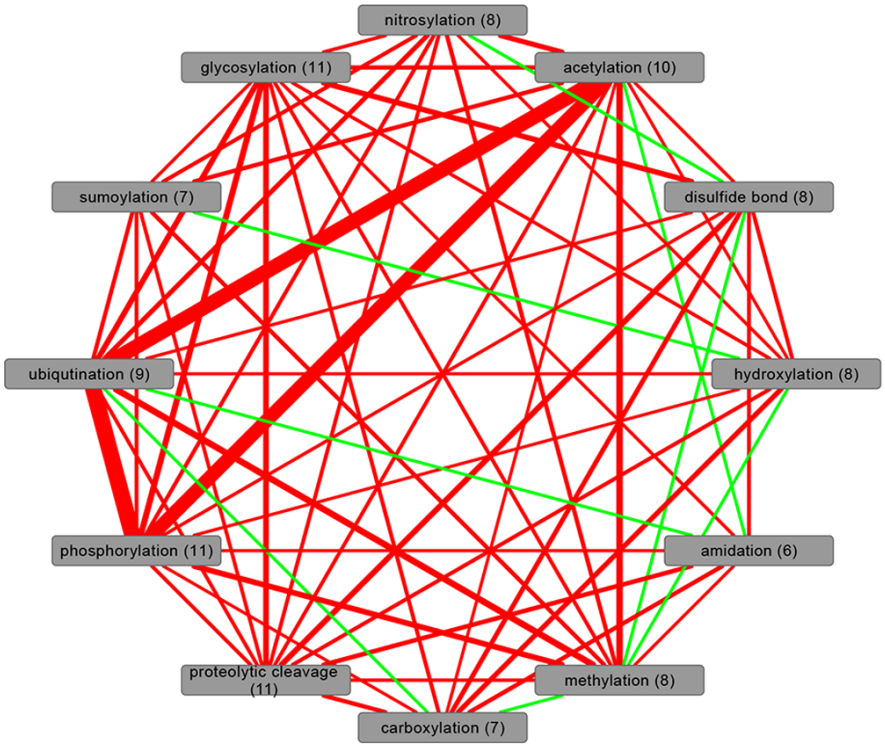

Figure 1 – Network of various peptide modifications found in the Homo Sapiens proteome. Numbers in parentheses refer to number of times said modification exists alongside another. *(Duan & Walther, 2015).*

Should an AMP be discovered that displays activity against a drug-resistant pathogen *in vitro*, yet is also found to have comparatively reduced efficacy *in vivo,* it allows the following hypothesis to be proposed:

A suitable modification can be synthetically administered to the peptide to induce increased efficacy *in vivo*.

The Hilpert group has identified several AMPs that show significant antimicrobial activity against a wide spectrum of multi-drug resistant pathogens, and are now interested in evaluating *in vivo* performance. Suitability of the drug can be assessed by systematically introducing the AMPs to living models of

increasing biological complexity. Factors that negatively impact the expected microbicidal activity of the AMPs can be identified by observing interactions between the AMPs and selected environments – initially cell lines, then waxworms, and lastly mouse models. The peptides can then be modified with the intention of circumventing the observed difficulties. Subsequently, the investigation will be repeated for the revised variants, with the aim of identifying a modification that improves *in vivo* stability without negatively impacting the function of the AMPs. Similarly, different formulation and application methods can be applied and evaluated regarding both wild-type and modified AMPs, allowing a more effective therapeutic strategy to be conceptualised.

When determining whether a modification should be attempted, the amino acid residues of the target peptide must be taken into consideration, as these molecules greatly influence the physical and chemical properties that the peptide will possess. There also lies the possibility that alterations to the structure of a peptide result in a reduction or cessation of activity. Rather than adhering to generalised protocols, experimental methods must be tailored to the individual peptide of interest, to minimise the risk that the peptide sequence fails to respond to the modification at all. This difficulty is multiplied if multiple peptide variants are being investigated – while it is preferable to keep experimental conditions the same between variants, alterations may be necessary in order to accommodate the differences in variant properties.

However, this also serves to highlight the necessity of research regarding novel peptides, where the expected interactions with modification procedures are unknown – if a specific peptide is to ever be put to practical use, these preliminary steps must be taken, and the results recorded for future consultation. The answers to these questions could one day be of great importance to pharmaceutical companies interested in submitting effective, novel drugs for clinical trials.

Methodology

My time estimation for the project is as follows: I will spend the first 2-3 months training in the basic techniques required for toxicity and efficacy studies, followed by further training throughout my PhD for all other required techniques (cellulose peptide synthesis, use of Gait-CAD, etc.). My previous work with [removed] has given me significant experience with on-resin peptide synthesis, which will assist my efforts to refamiliarize with the related techniques. At this stage, I will have the information required to begin *in vivo* testing of the unmodified peptides.

Additionally, the first 1.5-2 years will be spent studying the expected effects of different *in vivo* parameters - human plasma proteins, proteases, human serum and cell lines - on the efficacy of the antimicrobial action of the selected AMPs. Once the possible effects have been identified, it will be possible to design modifications to overcome them. After this planning stage, the best candidates will be tested in *in vivo* conditions. I plan about 3-4 months to write up my thesis.

Initial evaluations

The primary step will be to record performance of the unmodified AMPs in serum and cell lines, and then compare these with the previously determined MIC values in Mueller-Hinton-broth, as this will determine whether further optimisation is necessary and serve as a benchmark when evaluating variant AMP efficacy. The peptides will first be introduced to epithelial cell lines which have been infected with the target organism: multidrug-resistant *Pseudomonas aeruginosa.* AMP efficacy will be evaluated at a concentration range to observe a dose response curve. In the case of low efficacy, peptides will be fluorescence labelled and interaction will be recorded periodically under observation via fluorescence microscopy. The AMPs will then be introduced to biological systems of greater complexity, likely waxworm and mouse models.

Identifying potential modifications

Research will then be undertaken regarding suitable modifications, prioritising modifications typically associated with increased peptide function or stability. Consideration must also be given to probable side effects that modifications may induce; the polarity, configuration and even length of a peptide can impact, for example, its solubility in organic or inorganic solvents, its mechanism of action, and its

capacity and affinity for disulphide bridge formation. These influences will then determine the experimental protocols that will be used to achieve the selected modifications.

Implementation of modifications

Modifications that require alteration to the peptide primary sequence – either to induce certain properties or to facilitate a subsequent reaction step – will necessitate the use of peptide synthesis. The desired AMPs will be produced using solid phase peptide synthesis (SPPS), using resin particles as solid support. The amino acid sequence will subsequently be formed via a series of deprotection-coupling reactions. The use of solid support allows side products remaining after each reaction step to be removed via washing with relative ease, while the desired product remains anchored to the resin. Homogeneity will be determined using reversed phase high performance liquid chromatography (HPLC). The identity of the synthesised compound will then be verified via mass spectrometry. HPLC will also be used to purify the product, which will then be re-analysed to confirm whether the required homogeneity has been obtained.

***In vivo* evaluation of modified peptides**

As with the unmodified peptides, the *in vivo* efficacy of the peptide variants will be systematically assessed, using multiple living models of increasing complexity. This will be done with the aim of identifying the effects of the previous modifications, as well as any complications that have arisen as a direct result of those modifications. To this end, the performance of the peptide variants will be evaluated at identical concentration ranges as those employed in the initial *in vivo* investigations, and data shall be recorded after the same time intervals. Should a relative increase in efficacy not be seen, peptide interaction with the target pathogen may be more thoroughly investigated via microscopy, or the selected modification may be deemed unsuitable. At this stage, it shall also be possible to evaluate methods of administration – AMPs will be delivered intravenously or intramuscularly, to determine the effect this has on exhibited efficacy.