Antimicrobial Peptides: Agents of Public Health

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We often think of toxins, such as the cholera toxin discovered by Koch, as being the insidious chemical agents that microbes use against higher organisms. Many would be surprised to hear that higher organisms, from plants to man, also produce microbicidal toxins. These 'toxins' are peptides with considerable sequence variation, formed by cleavage of precursor proteins. Collectively known as 'antimicrobial peptides', they comprise one component of innate immunity, and have been helping to manage a balance with the microbial world for a very long time.

Man vs Microbe

The prevailing paradigm of humankind's contact with the microbial biome is one of escalating chemical warfare. The war was largely one of attrition until the discovery of penicillin in 1928, and its widespread usage by 1944. Streptomycin followed soon thereafter, and when numerous other antibiotics were available by 1965, the imminent triumph of mankind over infectious diseases seemed manifest. While bacterial resistance to these agents in clinical settings was recognised, in 1952 Joshua and Esther Lederberg had already identified streptomycin-resistant *E. coli* in cultures that had never been exposed to the agent (1). Echoes of this discovery continue today, with the relative ease of isolating soil bacteria not only resistant to almost every type of antibiotic, but that can actually live on these antibiotics as a sole carbon source (2), even if these 'resistance' mechanisms are not (yet) clinically relevant (3,4). Bacterial defences to mankind's newfound arsenal clearly already existed, and selection for these resistant bacteria via wanton application of antibiotics throughout the subsequent decades brought a prophetic twist to University of Chicago historian William McNeill's observations from 1975 (5): *"Newly acquired skills made humanity increasingly capable of transforming the balance of nature in unforeseen and far reaching ways. Accordingly, the disease liability of emerging humankind also began to change dramatically."*

History is written by the victors, however, and so the proud anthropocentric storyline of man's victory over microbial infection with antibiotics is difficult to rewrite within the collective consciousness, and calls to update mankind's arsenal (6) have yet to be earnestly heeded.

Meanwhile, an alternate, more 'yin yang' view of the relationship between humankind and microbes has been gaining momentum, perhaps beginning with McNeill's historical perspective. This view holds that man should acknowledge the necessity of managing a balance with the microbial world, rather than trying to eradicate it with cluster bomb weapons. One of the more fluid memes within this view, in addition to man's responsibility for destabilising his balance with the microbial biome through misuse of antibiotics, is the realisation of the importance of one's natural gastrointestinal biome to digestion and health. Perversely, these two themes may be very much related, as evidence suggests that one of the highest concentrations of broad-spectrum antibiotic resistance factors may be in the human gut, and this may be where cross-strain transmission of these factors occurs (7-9).

Antimicrobial Peptides

Bacteria are generally of mutual benefit, often living symbiotically with higher organisms, and are not a problem provided that they are not introduced into the wrong places. In this context, the relatively recent discovery of antimicrobial peptides is particularly intriguing.

That higher organisms reply to microbial insult by producing a plethora of antimicrobial peptides suggests the chemical warfare with microbes may be more naturally balanced than we previously appreciated. Indeed, contemporary literature is replete with reports of antimicrobial peptides, as they are found in just about every otherwise microbe-friendly place one thinks to look. Human defensins and other peptides are expressed in external and internal endothelial tissues, and by some leukocytes (10,11); flies and other insects do not lead the cleanest lifestyle, have no known adaptive immune systems, but have well-evolved innate immunity comprised significantly of antimicrobial defensin and cecropin peptides (12,13); frogs necessarily live in warm, damp habitats, and produce the most extensively catalogued array of antimicrobial peptides identified (14,15); and even plants produce peptides to protect both themselves and their seeds (16).

These antimicrobial peptides constitute a major part of all higher organisms' systems of innate immunity (17). They are typically cationic peptides of less than 50 amino acids length (often much less); are usually either structured by virtue of disulfide bonds, or are unstructured until bound to a target; and are generally amphipathic, when structured. Their mode of antimicrobial activity varies – some need to traverse into the bacterial cytoplasm, but many appear to directly target and compromise the integrity of the bacterial membrane. Those that specifically target the membrane do so at least partly based on charge, as the bacterial membrane carries a net-negative charge in the outer leaflet, whilst eukaryotic membranes are negatively charged only on the inner bilayer leaflet.

There are several canonical mechanisms for membrane disruption: the barrel-stave pore, whereby peptides pack against each other like the slats of a barrel, and against lipid tails on the outer perimeter; the toroidal pore, where both peptides and lipid head groups line a doughnut-hole shaped pore; and the carpet-mechanism, where lipids act as a detergent to dissolve the membrane (**Fig. 1**). In some sense the toroidal pore and the carpet mechanism are related, as the local topology of peptide and lipid is similar. The difference may be simply based on whether the lower energy

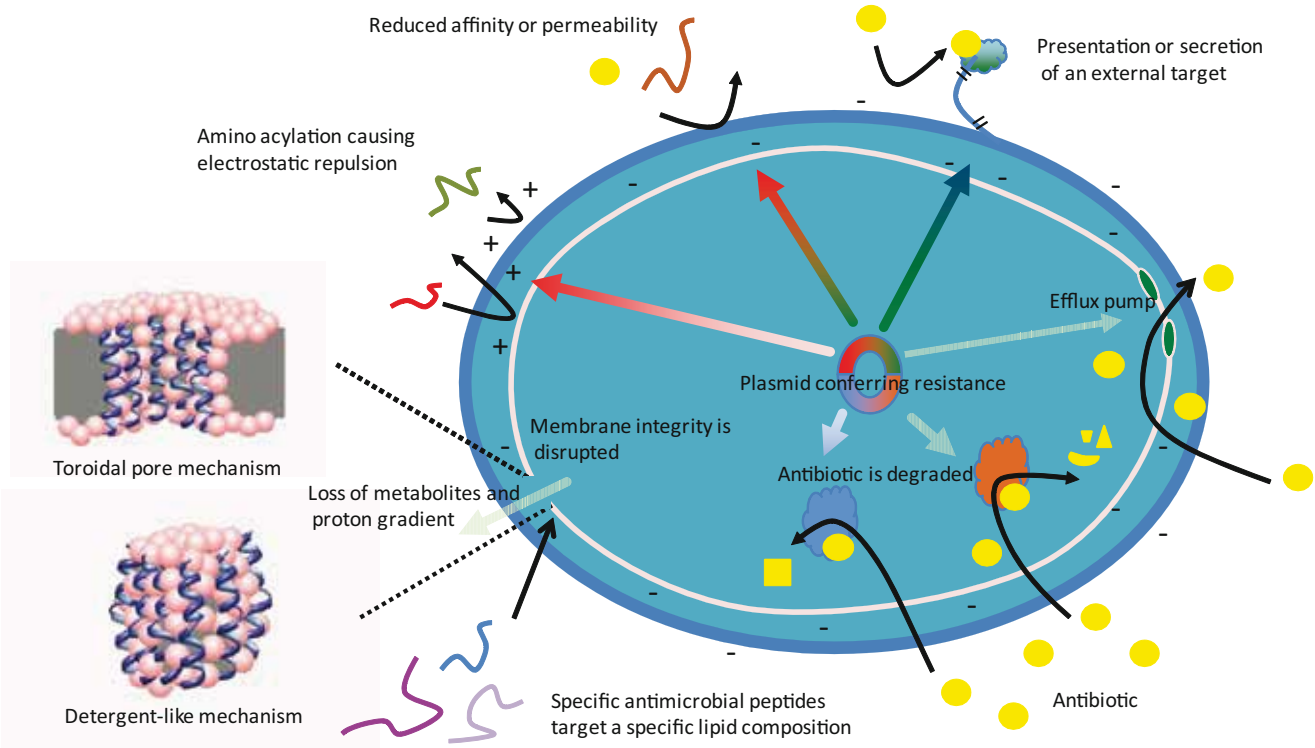

Fig. 1. Common resistance mechanisms to traditional antibiotics (yellow) vs action of an array of antimicrobial peptides (randomly coiled in solution).

configuration is for the peptide to mediate the curvature at the inner or outer surfaces of the toroid. Experimentally, these differences can easily be discriminated by size of the peptide/lipid structure, for example by solid-state NMR. The formation of toroidal pores in liposomes perturb lipid headgroup motion (18,19), but do not fundamentally change the size of the vesicle, whereas solubilised bilayers become much smaller and appear as isotropic peaks (20). Surface plasmon resonance and dual polarisation interferometry can also be used, as detergent mechanisms will irreversibly strip lipid from the supporting chip under flow conditions, while toroidal pores do not (18,21). Recent work suggests that peptides may not even need to form anything so formal as a toroidal pore, as disordered and transient breaches of the membrane may by enough to depolarise the cell (22, 23).

The antimicrobial spectrum of activity of individual peptides is considerably more variable than the usual classification of antibiotics as active against Gram-positive vs Gram-negative (15) bacteria. For example, minimum inhibitory concentration (MIC) values with a 32-fold variation have been reported for maculatin 1.1, isolated from skin glands of the Australian tree frog *Litoria genimaculata* (24). Variation in activities of membrane-acting peptides may be due to the specificity of a peptide sequence for a species-specific membrane headgroup (25) and acyl chain (26) composition, or variable cell-wall characteristics (27).

Future Generation Antimicrobial Agents

Just as the chemical weapons used within the microbial world were harnessed for the earliest antibiotics, so antimicrobial peptides appear to be promising leads as a next-generation class of antimicrobial agent (17, 28). Part of the attraction lies in the novelty of the membrane bilayer target, but also, so the argument goes, in the lower likelihood of microbial resistance.

The matter of resistance is, however, the topic of some debate. In principle, several of the recognised mechanisms of resistance would be of less utility to microbes as countermeasures to antimicrobial peptides (**Fig. 1**): (**a**) One of the most significant and general mechanisms is the active pumping of chemicals back out of the cell by drug transporters, whereas membrane-disrupting peptides act extracellularly. (**b**) Another major mechanism of resistance is enzymatic inactivation of antimicrobial agents, such as β-lactamase activity. In the case of unmodified antimicrobial peptides, this would most logically come in the form of a protease. While such proteases (or more general binding factors) have been identified against individual peptides in isolated settings (e.g. (29)), the general sequence variability of antimicrobial peptides is so significant that a successful resistance factor would have to be fairly nonspecific, at which point it would reduce microbe fitness possibly to the point of lethality. (**c**) Microbes can adapt the antibiotic's targeted structure. In the case of antimicrobial peptides, bacteria may modify their cell membrane or cell wall structure by amino-acylation and other modifications, and/or increasing crosslinking to reduce electrostatic attraction and penetration of the cationic peptides. These phenomena have already been observed (27), and both may be an important part of the explanation of variable peptide activities over different species. It is possible that these factors are sufficiently genetically complex, or confer enough of a survival disadvantage on resistant variants, that the likelihood of resistance might be minimised through careful public health management (30).

Indeed, carefully administered public policy regarding the next generation of antibiotic agents – whatever they will be – would seem to be an unquestionable imperative. As the aphorism goes, those who do not study history are destined to repeat it. This suggests that we should aim to maintain as much of the balance between man and microbe as possible, and not use the antibiotics unnecessarily. A fairly obvious policy would be to make the antibiotic agents unavailable for routine administration to livestock. An optimistic step on this front was recently taken in a US federal court judgement (31), which ruled that the FDA must end over three decades of circumlocution and act on 1977 proceedings to withdraw approval for the subtherapeutic agricultural use of penicillin and tetracyclines. A less obvious idea would be to reserve the next new class of antibiotic agents as a last-resort measure, to minimise exposure and selection of resistant microbes, at least until additional new classes are discovered. If antimicrobial peptides do evolve into becoming the next class of antibiotic agents, we should also make every effort to maintain their diversity. Antibiotic research typically aims to achieve broad-spectrum activity, no doubt due more to the understandable practicalities of cost-efficient production and distribution. In fact, administration aside, orthogonal narrow-spectrum, targeted antibiotic agents might be more sensible from a public health perspective. We can not doubt that resistance factors to any single compound already exist in the microbial population; the only control we really have is to limit the extent to which we select for the bacteria with these factors. Broad-spectrum antibiotics select for all bacteria that harbour these resistance factors, and increase the likelihood of lateral transmission of these factors across multiple infectious strains. Resistance to orthogonal strain-specific antibiotic agents, on the other hand, is less likely to transfer from unrelated strains which experience no selective pressure from the antibiotic agent.

All of these measures would limit the economic return on research and development, and therefore represent a considerable impediment to industry, which is already discouraged from the search for new antibiotics (6,32). Consequently, a considerable fraction of this research has come from academia. The expertise of industry through all stages of pharmaceutical development is nevertheless crucial in the development of new antibiotics. The situation calls for an integration of global public health and therapeutic goods regulators, cooperating with teams from industry and academia. Although this is difficult to imagine with the inertia inherent in modern bureaucracies, it is not without precedent: the World Health Organization coordinated just such an enormous campaign, and across several diametrically opposed political systems of the day, to eradicate smallpox 35 years ago (33).

Coda

Magainin, an antimicrobial peptide from the African clawed frog *Xenopus laevis*, nearly did make it to market about ten years ago. Its rejection after Phase 3 by the FDA was arguably more a maladroit act of the bureaucracy than anything to do with the peptide's safety or efficacy (32), and was regarded by many as a worrisome sign. Ironically, it may have been the right decision for the wrong reasons; with the alarming rate of emergence of multiple-antibiotic resistant 'superbugs', the time for magainin and other antimicrobial peptides may yet come. If and when it does, it will be better that bacteria have not had a chance to develop resistance to these agents through easy availability before then. It may also be fitting that it was an administrative complication that kept the peptide out of the market, as indications suggest that current regulatory administrations are not integrated with public health closely enough to properly execute policy for a new class of antibiotic agents. In the meantime, university-based scientists will continue to do everything they can to provide the basic research necessary to understand antimicrobial peptide specificity and activity, in preparation for the day when this understanding will be needed for clinical application.

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