Tackling Antimicrobial Stewardship through Synergy and Antimicrobial Peptides

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REVIEW

Tackling Antimicrobial Stewardship through Synergy and Antimicrobial Peptides

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The unrestricted use of antibiotics has led to rapid development of antibiotic resistance (AR) and renewed calls to address this serious problem. This review summarizes the most common mechanisms of antibiotic action, and in turn antibiotic resistance, as well as strategies to mitigate the harm. Focus is then turned to emerging antibiotic strategies, including antimicrobial peptides (AMPs), with a discussion of their modes of action, biochemical features, as well as potential challenges for their use as antibiotics. The role of synergy in antimicrobials is also examined, with a focus on the synergy of AMPs and other emerging interactions with synergistic potential.

Antibiotic resistance and stewardship

Antibiotic resistance (AR) can be slowed, but not stopped, because it is part of a natural process in which bacteria evolve. New therapeutics will always be necessary in combating pathogenic organisms, even though infection prevention and reduction of antibiotic use are key steps to slowing the current rapid rate of resistance development. In a positive update from the CDC's 2019 Antibiotic Resistance Threats Report, deaths and hospitalizations have decreased since 2013, an important step that was possible because of antibiotic stewardship.[1] However, In the US alone, more than 2.8 million people still contract antibiotic resistant infections annually and an estimated 35,000 people die, leaving room for improvement in our infection prevention and mitigation strategies. Three major venues for antibiotic resistance to occur exist: healthcare facilities, community and the environment, and agriculture, including food production, farming, and animals. Each of these environments creates a space for the mixing of different species of bacteria, antibiotic exposure, and potential for resistance mechanisms to spread.

As shown in Figure 1, there are many types of resistance mechanisms that occur via spontaneous mutation or transfer. These include enzymatic degradation, alteration of the antimicrobial target, efflux of the drug, creating new/redundant cellular processes, and restricting access to intracellular locations through changing membrane lipid composition or membrane proteins and biofilm formation.[2-7] Bacteria inherit these mechanisms through two methods: vertical inheritance where a bacterial cell passes down its resistant gene to its progeny, and horizontal gene transfer (HGT) by which antibiotic resistant genes are integrated into bacteria of potentially different strain and species. HGT is accomplished through conjugation by plasmids, transduction by bacteriophages and natural transformation by eDNA.[8] These inheritance methods speed the development of resistant genes in naïve bacteria.

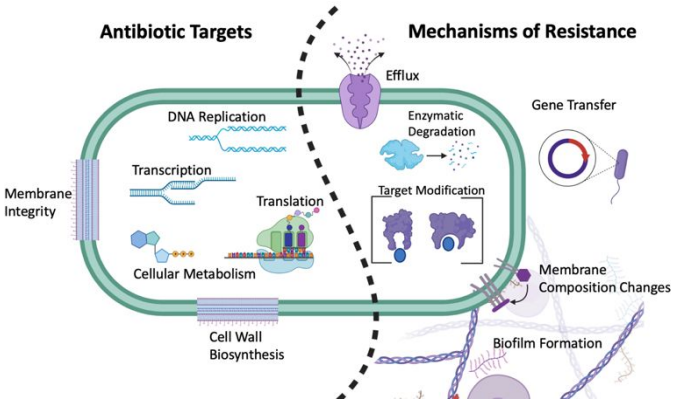

Figure 1. Examples of antibiotic targets and bacterial mechanisms of resistance. Traditional antibiotics and antimicrobial peptides target a variety of cellular processes and can damage DNA as well as binding and preventing DNA replication, transcription and translation. Various antibiotics have also been shown to inhibit mechanisms of cellular metabolism, hinder cell wall biosynthesis and a major mode of action for AMPs is disruption of the membrane activity. Bacteria exhibit an array of mechanisms to prevent these actions including targeted efflux, and degradation of antibiotics. They have also developed mechanisms of increased redundancy of important cellular processes, modification of targets and hindering antibiotic access through biofilm formation and membrane composition changes. Gene transfer is also an important mechanism of acquiring resistance.

Current clinical antibiotics

Currently used antibiotics target a variety of bacterial cellular mechanisms in an effort to inhibit their growth and reside in five main classes. From most to least common they include: inhibition of cell wall synthesis, inhibition of translation, alteration of cell membranes, inhibition of DNA

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processes and targeting metabolites (Figure 1).[9] Three mechanisms target cell wall synthesis: namely, inhibition of peptidoglycan synthesis (beta-lactams),[10] prevention of cross linkage (vancomycin),[11] and prevention of precursor movement (bacitracin).[12] Inhibition of translation proceeds by binding to various sites of the ribosomal subunits to prevent elongation of the polypeptide. Aminoglycosides irreversibly bind the 30S subunit while tetracyclines block the tRNA 30S binding site.[13, 14] Clindamycin, chloramphenicol and macrolides block elongation by binding the 50S ribosome.[15] Polymyxins and bacitracin (AMPs) both disrupt membranes and are only used topically.[12, 16] Quinolones prevent DNA supercoiling[17] and metronidazole causes DNA damage from the cytotoxic metabolic byproducts it induces.[18] Rifampin inhibits RNA synthesis, while bacitracin, in addition to damaging cellular membranes, also inhibits RNA transcription.[19] Lastly, sulfonamides prevent folic acid synthesis.[20] While these are examples of widely used, on-market antibiotics, the mechanisms of a variety of additional emerging therapies, including antimicrobial peptides, will be reviewed further.

The responsible mitigation of infectious disease relies on a balance of preventing new deadly infections and protecting the microbial ecosystem that is vital to many life processes. By combining antibiotic stewardship and prioritizing the development and approval of new therapies, it is possible to prevent the return of even more widespread disease and death from currently treatable conditions.

Emerging therapeutics

Therapeutic strategies are being explored to combat current and emerging bacterial pathogens, (Figure 2): including antimicrobial peptides, bacteriophage, monoclonal antibodies, combination approaches, quorum sensing inhibitors, and antimicrobial polymers.[9, 21-23] Other considerations for antimicrobial resistance include strategies in antimicrobial delivery, encompassing universal vs targeted delivery and delivery technologies such as liposomes and nanoparticles.[9, 24] Traditional, universally delivered antibiotics have the disadvantage of killing off the healthy natural flora.[25] Targeted delivery approaches would preserve natural flora, keeping the competition present to disallow overgrowth of resistant pathogenic organisms.

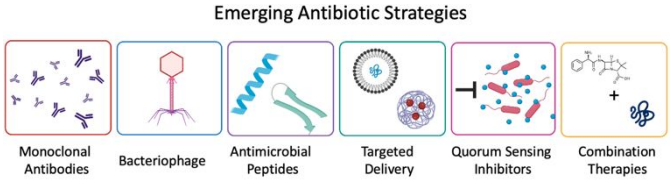

Figure 2. Emerging Antibiotic Strategies that represent potential novel treatment options for microbial infection.

Monoclonal antibodies

In light of their potential, monoclonal antibodies may be underleveraged.[21] Low cross reactivity with human cells is due to the drastic difference between bacterial and human antigens and their specificity ensures low damage to normal flora and low toxicity to human cells.[26, 27] They are easy to produce, but expensive and have low shelf stability. While they may limit virulence and keep selective pressure low, thus preventing resistance, they may not work as a monotherapy.[28] There are currently four FDA-approved antibody treatments that all target exotoxins, which is a limited application of this strategy.[29]

Bacteriophage

Phage are bacteria-specific viruses that anchor to bacterial cell membrane, inject their genetic material, and take over the hosts DNA replication and ribosomal machinery. The phage are then built within the host cell, which eventually leads to cell lysis and continuation of the phage life cycle. The opportunities are varied for the application of phage as a biopharmaceutical, including service as a CRISPR-Cas9 delivery system, as a bacterial killing machine in their own right, and for intracellular delivery of other antimicrobial instructions.[30-33] Engineered phage have induced expression of non-lytic antimicrobial peptide DNA, which showed rapid killing of target organisms.[34] Work has also been reported where phagemid was used to deliver CRISPR-Cas9, where the CRISPR nuclease activity was used to cleave antibiotic resistance genes.[35] While these applications are promising, several hurdles exist. Resistance to phage has developed and this should be carefully monitored to not destroy natural biological balances.[36] Without biotechnological development, the immune system will develop a response and clear phage upon subsequent infections (i.e., one time use only).[37] This technology also has the potential to induce pathogenicity and virulence in commensal and non-target pathogenic bacteria by moving genetic elements between bacteria, although much work needs to be done on this front to further elucidate the danger.[31, 37] While there are hurdles, the technology presents an elegant way to utilize naturally occurring phenomena.

QSIs

Quorum sensing is microbial information exchange that increases bacterial resistance through formation of biofilms, induction of efflux pumps and antibiotic production.[38] It allows bacteria to alter gene expression based on cell densities, available nutrients, and the presence of other species. Autoinducers are the extracellular signaling molecule that communicates between cells, creating a feed-forward mechanism. Autoinducers induce the production of more autoinducers.[39]

The ability to interfere with this process, and thus weaken enmeshed bacterial communities, has proceeded through a variety of mechanisms. Other bacteria have inhibited the synthesis of the signal molecules, have enzymatically degraded signal molecules, acted as binding inhibitors for receptor sites, and interfered with signaling molecule's ability to bind gene promoters, thereby inhibiting gene expression.[38-41] All these methods are viable and investigated strategies for therapeutic purposes. Several qualifications for QSIs have been described in the literature, including the use of a small molecule that

inhibits QS gene expression, being highly specific with no adverse effects on the bacteria or the host, and chemical stability. The criteria are thought to lower the harm to commensal bacteria, and reduce selective pressure, disfavoring the development of drug resistance. [42, 43]

These methods provide a few examples of the unique and varied strategies to be explored to combat infection. It is imperative to invest in different methodologies to be successful. While these methods utilize a variety of microbial, cell biology and biochemical techniques, the strategies employed in developing antimicrobial peptides is rooted in protein biochemistry.

Antimicrobial peptides

Antimicrobial peptides (AMPs), also known as Host Defense Peptides (HDPs), are one avenue of current interest for the design of novel therapeutics. Found naturally in all forms of cellular life, including bacteria, they are short, gene-encoded polypeptides that generally exhibit a high degree of positive charge and hydrophobicity.[44] These peptides can be categorized into a variety of types, including but not limited to defensins,[45] magainins,[46] cathelicidins,[47] and cecropins,[48] all of which display varying structures and functions.[44] The structural classes of cationic AMPs include disulfide bonded β-sheet peptides (defensins), amphipathic α-helical peptides (magainins/cecropins), extended peptides with a single predominant amino acid (indolicidin), and loop-structured peptides (bactenecin).[49-51] Examples of the various structural classes are illustrated in Figure 3.

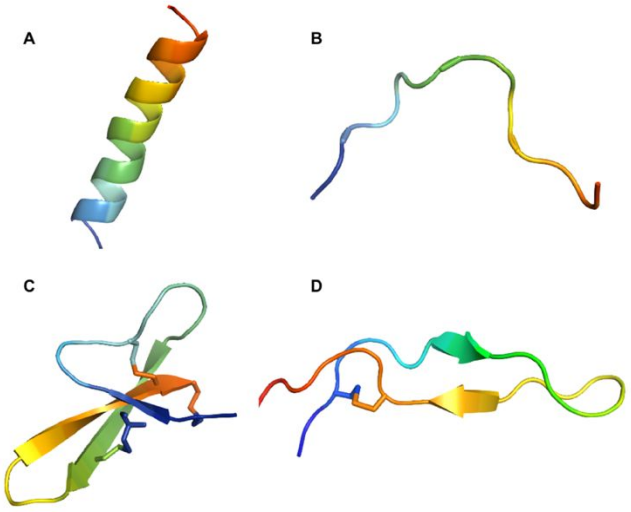

Figure 3. Four representative structures of AMP structural classes including A) alpha-helical magainin-2 (PDB 2MAG); B) extended indolicidin (1G89); C) beta sheet defensin (1DFN); and D) the loop peptide lactoferricin (1LFC).

The different methods of categorization are not all inclusive but provide a framework for interpreting the antimicrobial activity. While some AMPs have potent broad-spectrum activity, the more modest biocidal activity, especially found in physiologically relevant conditions such as high salt and cation presence, has led to the understanding that direct antimicrobial activity is only one aspect of the role of these peptides role in physiological systems. The ability of these peptides to modulate the immune system[52] has led to the differential use of the terms AMP and HDP, with AMP utilized when referencing their ability to directly inhibit or kill bacteria, and HDP referring to their immunomodulatory effect. While many mammalian AMPs are thought to exert modest activity via direct antimicrobial activity in physiological conditions, and also function by modulating immune regulation,[50, 53, 54] several amphibian skin AMPs display comparable antimicrobial efficacy in a variety of physiological conditions. This suggests that they may primarily serve as direct antimicrobial agents rather than immunomodulators.[55] Peptides derived from non-mammalian systems may serve as more effective direct antimicrobials than their mammalian derived counterparts, like cathelicidins. While cytotoxicity against mammalian cells can be evident for certain AMP's, or at higher dose, selectivity normally targets bacteria as a result of the higher negative charge associated with bacterial cell membranes, favorable transmembrane potentials for bacteria, and the higher stability normally associated with mammalian cell membranes through their molecular components.

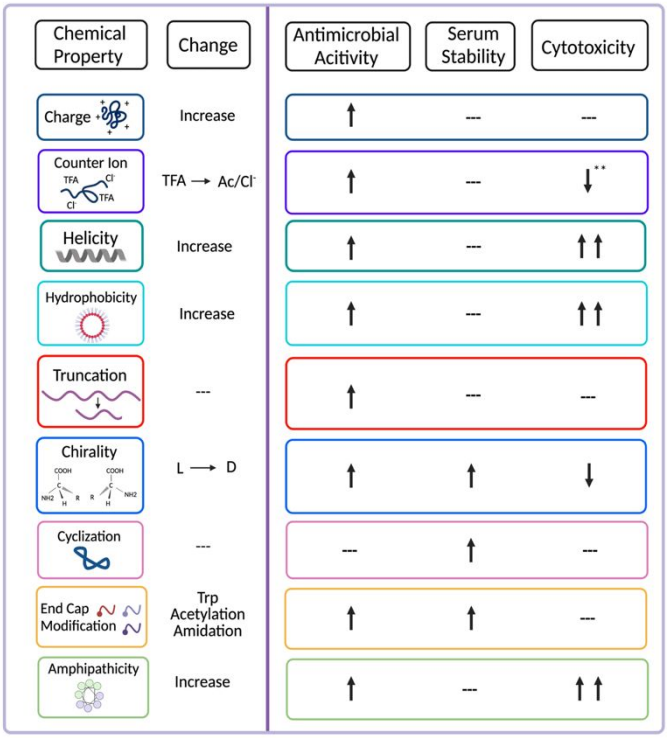

Figure 4. Biochemical strategies utilized for AMPs, showing their impact on antimicrobial activity, serum stability and cytotoxicity. Upward arrows indicate an increase in the property and downward arrows indicate a decrease in the property. The application of these strategies is highly dependent on the individual systems. **Acetate has increased cytotoxicity in some peptides.

AMP structure-function relationship

Much effort has gone into the strategic design of peptides with preferential activity (Figure 4). While the structural diversity of peptides and the membrane diversity of bacteria make this a difficult challenge, several general trends have

been elucidated. An increase of positive charge (cationicity) increases activity, with the net charge being more important than the size of the peptide. Replacing lysine residues with arginine residues as sources of positive charge was also shown to improve the therapeutic index of a series of peptides.[56] For alpha-helical peptides, helicity improves not only their antimicrobial activity, but also cytotoxicity toward human erythrocytes. The effect on cytotoxicity is more prominent than the improvement of AMP activity, making increased helicity a poor choice for improving the therapeutic index. Increased hydrophobicity has shown to improve activity against gram-positive bacteria but also increases cytotoxicity.[57] The amphipathicity of the molecule also negatively contributes to the therapeutic index with imperfect separation being shown to improve activity but also increase cytotoxicity.[51] These trends demonstrate a trade-off between activity and potential deleterious effects on human tissues.

Another method of improving activity in peptides is by utilizing diastereomers. Conversion of L → D amino acids has been shown to retain antimicrobial activity but eradicate the hemolytic activity of a variety of peptides.[58, 59] However, the broad applicability of that strategy remains to be rigorously tested as studies show an alteration in the secondary structure that can create preferential binding to the negative phospholipids rather than zwitterionic lipids found in mammalian cells.[59] Utilizing chirality to disrupt resistance mechanisms also depends on the antimicrobial mode of action. This strategy is well suited to membrane disruption but may hinder the action of peptides that require chiral recognition, such as membrane channels, or inhibition by enzyme disruption. Other modifications that improve peptide stability include terminal amidation or acetylation, and cyclization.[60, 61] Salt resistance is another important factor in improving AMP activity as many peptides lose potent activity in high salt concentrations. Positioning tryptophan and β-naphthylalanine at the N- and C-terminus have also been shown to improve serum stability as well as increase salt tolerance.[62] Truncating peptides while retaining their activity is another strategy for increasing the likelihood of use as it reduces the complexity of the manufacturing process.[63]

Another point for consideration is the choice of counter ion. Trifluoracetic acid (TFA) is often used in the final cleavage and deprotection step of synthesized peptides. Peptides bound with TFA, acetate, and chloride all exhibited differential cytotoxicity to bacterial and mammalian cells with TFA being undesirable for its propensity to increase cytotoxicity in mammalian cells.[64] Counter ion salt cytotoxicity was shown to be peptide dependent, adding another consideration to the development of AMPs.

Metal binding domains

Metal binding domains are another biochemical feature that is found in natural AMPs and utilized as a strategic addition in synthetic AMP development to improve or change the activity of peptides. A variety of metal ions, including copper, zinc, manganese, and sodium confer important activity to AMPs.[65] One mode of action is by metal sequestration.[66] Metals are physiologically important to bacterial cells and the release of peptides like calprotectin, psoriasin and microplusin have been shown to inhibit bacterial growth by binding to essential metal cations.[67]

Over 250 natural AMPs possess an amino-terminal copper and nickel (ATCUN) motif, though the structural diversity of these peptides make it impossible to provide a one size fits all approach to the role of the ATCUN in their sequence.[68] Several examples of naturally occurring ATCUN-containing peptides include the fish piscidins,[69] tick ixosin,[70] tunicate clavanins,[71] human hepcidins and histatins,[72, 73] and amphibian tachykinins,[74] limnonectins,[75] and nigroains.[76] The phylogenetic and structural diversity of these peptides shows that the application of the ATCUN motif as a potent coordinator of antimicrobial activity cannot be overlooked. While the role of the ATCUN motif across these peptides has not been fully elucidated, some work has been done, including recent work on the piscidin family and ixosin that indicate that the ATCUN motif and its metal binding properties play a distinct and important physiological role in preventing microbial infection.[77-79] The domain has been utilized in our lab to confer alternative activity on antimicrobial peptides that did not naturally contain an ATCUN motif.[80-83]

Zinc-binding peptides have also shown important antimicrobial activity. For example, thanatin, a zinc binding peptide, was shown to have clinical activity against the highly problematic and resistant New Delhi metallo-β-lactamase-1 (NDM-1) by stripping the lactamase of its required zinc cofactor.[84] Dermcidin, another zinc binding AMP, was shown to be more active when oligomerized, which was stabilized by zinc binding.[85] Clavanin A is another zinc-binding peptide that has been shown to cleave genomic DNA, showing another mode of action of metal binding peptides.[71] These examples illustrate the diversity of activity conferred by metal ions to AMPs and the potential of utilizing metal binding domains to improve or create alternative antimicrobial activity.

The biochemical features highlighted are highly dependent on a variety of factors and the rational design of peptides is more complicated than simply improving on them. The contribution to tertiary structure, outsized effects of single amino acid residues, and complex interplay with different bacterial strains make this approach a complicated endeavor. One common feature of every AMP is that they must first interact with the bacterial membrane.

Mechanisms of membrane interactions

Membrane composition is vitally important to how they interact with peptides. A variety of models have been developed that describe the disruption of bacterial membranes by AMPs. Either 22 residues as an alpha helix, or 8 residues as a beta sheet, are required to span the membrane.[69] However, many instances in the literature show that 22 residues are not necessary to disrupt the membrane,

leading to different hypotheses on the biophysical mechanism of disruption. The percentage of different membrane species is also important. Protein to lipid ratio, membrane surface charge, cell type and curvature strain all impact the ability of an AMP to interact with a membrane, and by which method it will do so.[86-90] The membrane disruption models include toroidal pore formation, a carpet model and the barrel stave model, lipid oxidation, and membrane thinning or thickening (Figure 5). The toroidal pore and barrel stave model both propose membrane spanning mechanisms. In the toroidal pore the peptide inserts itself perpendicularly, disrupting the membrane curvature and causing separation of the polar head groups and lipid tails.[91] The barrel stave model begins with accumulation on the surface of the membrane and then integration to form a pore.[90] These methods differ in that the toroidal pore proceeds via electrostatic interactions while the barrel stave mode of action also utilizes and interacts with the hydrophobic lipid tails.[92] The carpet model of pore formation relies on the accumulation of peptide at the surface, but rather than inserting into the membrane creates a critical threshold of peptides that destroys the membrane integrity allowing the leakage of the cellular contents or depolarization.[90]

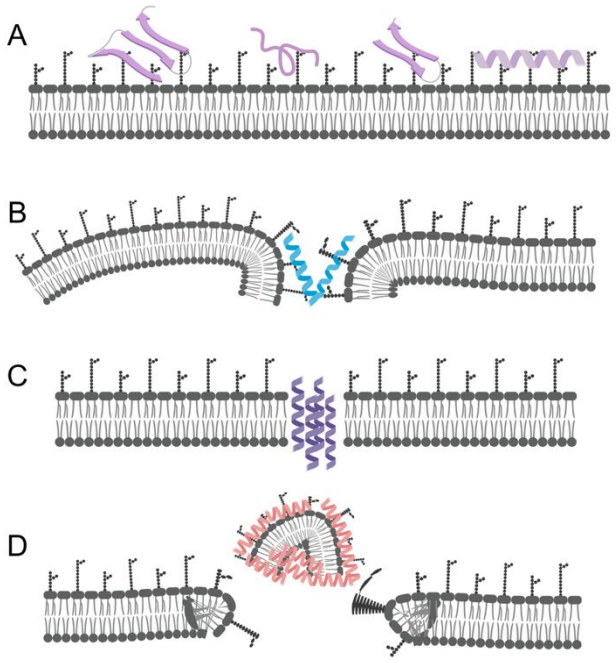

Figure 5. Selected mechanisms of membrane disruption with A) membrane disruption begins with accumulation of peptide on the surface; B) toroidal pore formation; C) barrel stave pore formation; and D) the carpet model.

All these models rely on the release of intracellular contents or membrane depolarization for disrupting bacterial activity and are considered biocidal modes of action. While many peptides are membrane disruptors, some operate more like cell penetrating peptides, slipping through the membrane to act on intracellular targets.[93] Others may form transient pores, allowing the intracellular accumulation of peptides while the bacterial membrane recovers. All of these membrane interactions are key to the activity and viability of these peptides and play a pivotal role in the use of antimicrobial peptides in combination.

AMPs targeting other biological molecules

Other strategies have included testing AMPs with different classes of biological molecules, such as enzymes and histones. The human AMP LL37 with RNase1 (an enzyme), which is also transported to the extracellular space, acted synergistically to kill *E. coli*, where the membrane pore formation by LL37 allowed entry of RNase1.[94] This shows a case of potential natural immune responses determined by in vitro assays. Another example is the observation of synergy with histones and AMPs, where H2A was shown to aid pore formation by LL37 and magainin-2, which then enabled it to enter cells, reorganize bacterial DNA, and prevent any bacterial recovery.[95]

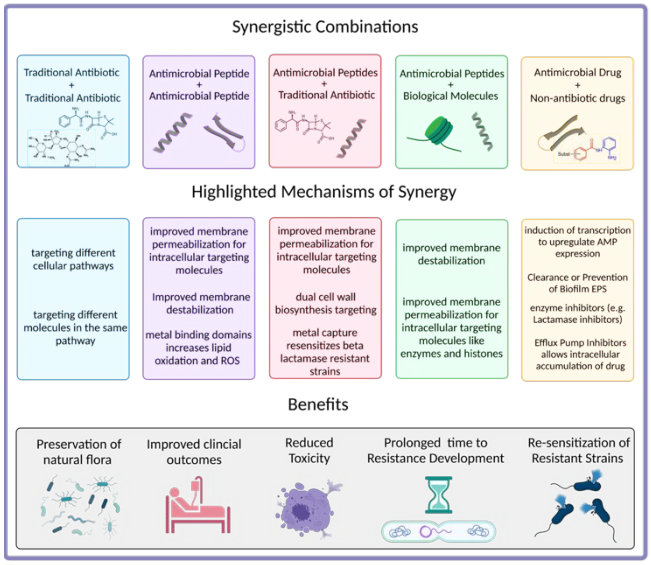

Figure 6. Synergistic interactions can be described for a variety of combinations of synthetic, biological and organic molecules. These interactions proceed through a variety of mechanisms, of which a few examples are given. While not all of these mechanisms confer the benefits shown, they all provide at least one, and generally more, of the beneficial outcomes as described.

Synergy as a therapeutic strategy

Synergistic drug interactions are utilized in a broad variety of contexts that include treatment of cancers, viral and bacterial infections, and others.[96-98] Synergistic interactions are combinations of drugs that have an effect that is larger than the additive independent contributions of the individual drugs (Figure 6). While there are a variety of methods and mathematical interpretations, each debated in regard to the interpretation of the data from vitro experiments,[99] the method of combining drugs to create more effective treatments is in broad use. It is also a promising therapeutic strategy for bacterial infection. Synergy can occur through two primary mechanisms. First, in vivo, promiscuous synergy can produce off target interactions through non-specific mechanisms that result in an enhancement of efficacy.[97] The second mechanism, which is amenable to in vitro study, is specific synergy, whereby the two drugs either act in concert

to inhibit complimentary pathways or aid the action of the other to inhibit essential cellular processes.[99]

There are a variety of benefits to utilizing synergy as a therapeutic strategy. Synergistic interactions may decrease production costs due to a lower need for each drug for efficacy. There is also an increased chance for tolerable toxicity (commonly problematic in the case of cancer treatments) or reduced toxicity and off-target effects in the case of antimicrobials. [100] Resistant strains may be re-sensitized to previously efficacious antibiotics by introducing a synergistic interaction.[101-103] This has been shown with multidrug resistant tuberculosis. The most widely used treatment, rifampicin, was able to regain activity against MDR TB when treated in combination with beta-lactams.[104, 105] The use of antibiotics in combination also has the potential to prolong the development of antimicrobial resistance because of the multiple modes of action and increased efficacy.[106]

Traditional antibiotic synergy

The use of traditional antibiotic drugs in combination has been a widely utilized therapeutic strategy for treating pernicious bacterial infections.[107] Another example of current clinical antibiotics that are used in combination is that of beta-lactams with aminoglycosides for infective endocarditis caused by *Enterococcus* and *Streptococcus* species.[108] These traditional drug combinations have either re-sensitized a previously effective drug, or are intrinsically more effective when used together. Clinical outcomes for patients become increasingly difficult as resistance genes are spread. Rigorous work has not been done to fine tune the optimal combinations of these drugs as they are combining drugs already in clinical use, decreasing the necessity of clinical trials even as the benefit of that may be important.[100]

Synergy of antimicrobial peptides

A strategy that is currently being widely explored is the synergy between antimicrobial peptides. Naturally occurring, these peptides are thought to act synergistically in their native environment. Some believe that AMPs are highly specific to the host and targeted bacterial species, and that the minor variations and redundancy/co-evolution of AMPs is evidence of this.[109] It can be difficult to truly ascribe all the synergistic interactions of these peptides in vitro because they may interact with other immune components and physiological conditions that an in vitro assay cannot adequately account for.[109] They may also experience synergy with more than one other species, which is also difficult to capture with currently utilized FICI assays. CRISPR gene editing has allowed the testing of AMPs in in vivo settings. Drosophila knockouts of ten different AMPs showed a high specificity, and often additive or synergistic activity of AMPs in vivo, and provide a useful tool for studying naturally synergistic combinations and how these peptides interact within the innate immune system.[110]

Biochemical mechanisms of AMP synergy

While in vivo studies are unique, they are limited in scope. A variety of synergistic pairs have also been elucidated in vitro, which allows for determining biochemical mechanisms. For example, magainin-2 and PGLa, from *Xenopus laevis*, are synergistic by creating a functionally more stable pore in the bacterial membrane: magainin-2 resides on the surface and stabilizes the PGLa homodimers spanning the membrane.[111] This pair highlights the common membrane disrupting mechanism of many AMPs. A variety of studies have also shown how pore-forming peptides allowing entry of a peptide with an intracellular target, creating synergy.[112-114] The pore-forming abilities of AMPs have also been studied to determine synergy with traditional drugs.[115-117] The intracellular targets of AMPs, and the role of synergy therein, is an area of study poised for additional attention.

Synergy with metal binding peptides

A variety of peptides have exhibited activity by utilizing a metal binding domain to potentiate their synergy. For example, ixosin and ixosin B, from the tick *Ixodes sinensis,* displayed one mechanism where the ATCUN binding domain naturally displayed synergy. The ATCUN-containing ixosin mediated oxidation of lipids and led to the accumulation of ixosin at the membrane, rather than accumulating intracellularly, thereby enhancing activity.[118] Another notable example is the ability of zinc binding peptides to re-sensitize bacteria with the infamous New Delhi metallo-β-lactamase-1 (NDM-1) to beta-lactams.[84] The synergy between antimicrobial peptides with metal binding domains has also been studied with other AMPs. While the mechanisms are not fully elucidated, the ATCUN motif has been shown to confer enhanced synergistic activity with natural and synthetic peptides beyond that shown by their non-ATCUN containing counterpart.[83] The enhanced formation of reactive oxygen species (ROS), conferment of lipid oxidation, and alternative mechanisms of action may contribute to this activity.

Synergy with non-antibiotic compounds

Synergy has also been exhibited where non-antibiotic compounds have enhanced the activity of regular antibiotics.[119, 120] One example is the induction of natural AMPs by aroylated phenylenediamines, which induce expression of cathelicidins in vivo.[121] A more classic example is that of clavulanic acid acting as a suicide inhibitor of beta-lactamases to restore the activity of amoxicillin.[122] Efflux pump inhibitors prevent the antibiotic resistance mechanism commonly employed by bacteria to export intracellularly accumulated drugs, re-establishing their efficacy.[123] These inhibitors have also been shown to prevent the formation of biofilm, another important player in antibiotic resistance.[124-127] Eradication of biofilms, while not itself an antibiotic treatment, is an important strategy for increasing microbial susceptibility.

Other strategies for biofilm inhibition, beyond efflux pump inhibitors, are also being widely explored in the literature.[128-130]

These examples highlight several strategies for combination and synergistic treatment of bacterial infections. Combinations of traditional clinically-approved drugs are already in use, but there is vast potential for use of other synergistic combinations to improve clinical outcomes for resistant infections.

Challenges to development

There are several aspects of these synergistic interactions that may impede in vivo applicability and need to be considered. Do both the drugs reach the target at the same time? Ensuring the co-application of synergistic pairs can be accomplished by targeted delivery systems but may be a difficult prospect for orally-delivered antibiotics. Are there implications for the in vivo system where the drugs cannot be co-administered because of an off-target effect? This is less likely with AMPs but a consideration for organic antibacterial drugs. Many HDPs are currently in clinical trials and may stimulate the immune system in unanticipated ways, especially in combination with other potential antimicrobial candidates. In vitro testing is not able to decipher these nuanced questions. While most of the peptides in clinical trials are of human origin, the use of non-human AMPs may be a better strategy for reducing immunogenic overreaction and limiting resistance. One benefit, however, is that commensal bacteria may already have resistance, which is significant for maintaining the natural microbiome.

The utility of the fractional inhibitory concentration (FIC) assay, widely used in the literature for determining lead hits for synergy, has been questioned in the literature.[99, 100] In an editorial in the *Journal of Antimicrobial Chemotherapy*, the author encourages a conservative interpretation of FICI data and argues that additive FIC values are meaningless because of the high degree of potential experimental error in FIC indices, and thus, only synergistic, antagonistic or "no interaction" should be interpreted.[131] While some take issue with the in vitro FICI, the fact remains that nuance is often lacking in in vitro studies that are pursuing applications in vivo, especially for human drug treatments. The practice of claiming synergy when only shown in non-pathogenic *E. coli* should be eliminated, and broad-spectrum synergy or efficacy in clinically relevant strains should be established in vitro before any literature data is given serious consideration. The in vitro tool still provides a valuable building block for discovery, and while more resource intensive in the lab, relative to the MIC measurement; it remains the best method for synergy determination in vitro.

Conclusions

The impact of pathogenic organisms on human health and industry necessitates a multi-pronged approach to mitigate their negative effects. Understanding the role of microbes in the environment, the evolution of antimicrobial resistant strains, and the impact of biochemical features of these pathogenic organisms will lead to improved therapeutic strategies. While antibiotic stewardship and environmental regulation is important for maintaining a healthy microbial community and is protective of human health, there is also a need for new therapeutic approaches. Traditional antibiotics were often discovered by screening mixtures with antibiotic properties and isolating naturally occurring compounds. Combining that tradition with the rational design of new molecules, as well as diverse new strategies, is key to mitigation of bacterial infections. These new strategies, such as utilizing bacteriophage, monoclonal antibodies and QSI's are inventive ways forward. Other strategies include the use of natural or synthetic AMPs in combination, combining traditional drugs with AMPs, and more imaginative strategies, such as stimulating the immune response and employing combinations of non-antibiotic drugs and enzymes, are all potential applications that utilize synergy as a therapeutic strategy. The future of antimicrobial therapy resides in combination therapeutics and biotechnology. These strategies will improve our ability to combat resistance and manage clinical outcomes for critically ill patients.

Author Contributions

JAC elaborated the theme of the review. JMG drafted and then finalized the review with JAC.

Conflicts of interest

There are no conflicts to declare.

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