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Review Article

The future for antibiotics: bacterial membrane disintegrators

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Abstract

Bacterial resistance to conventional antibiotics is an ever increasing problem in the treatment of infectious disease. The basic problem is that conventional antibiotics, which generally operate by inhibiting some bacterial enzyme(s), can be rendered ineffective by overuse, natural selection and induced bacterial mutations. A novel class of antibiotics that functions by disrupting the superstructure of bacterial membranes rather than by inhibiting any specific enzyme has presented itself as the future for antibiotics. Most of these bactericidal agents are peptides or peptide-based molecules that have been either isolated from natural sources or synthetically designed. Here we review recent research into the development of peptides as bacterial membrane-disintegrating agents.

Introduction

The widespread use of exogenous antibiotics has increased the antibiotic resistance of pathogenic microorganisms, thus threatening the future medical use of such agents. While antibiotics currently used in the clinic have proven effective against a wide variety of bacterial types, their continued efficacy hinges on avoiding the problem of bacterial resistance. Drugs inhibiting new pathways may offer temporary relief. However, a more appealing, long-term solution is a move toward antibiotic agents that act independently of specific metabolic pathways and thus circumvent resistance issues. The innate antibacterial defense systems found in amphibians, insects and mammals offer a class of molecules fitting such a description: antibacterial peptides. These molecules effectively disintegrate or perforate bacterial membranes, thereby promoting leakage of cell contents and a breakdown of the transmembrane potential. Efforts are currently under way to create more potent, selective bactericidal derivatives and mimics of antibacterial peptides. Designing these novel compounds is greatly facilitated by understanding their mechanism of action and selectivity against microbes. In general, these new antibacterial agents are selective for the negatively charged surface of bacterial membranes relative to the neutral membrane surface of eukaryotic cells. This review explores bacterial membrane- disintegrating peptides and ongoing research into the development of more potent and specific peptide-based antibacterial agents.

The need for new antibiotics

The problem of antibiotic-resistant bacteria is by no means a recent phenomenon. Bacteria have evaded antibiotics from the time that such drugs came into widespread use in the 1930s and 1940s (1). New antimicrobial agents developed in subsequent decades provided a means of controlling bacterial infection. Over time, however, the combination of selective pressures induced by

Table I: Examples of multidrug-resistant bacterial strains.[a]

	S. pneumoniae	Enterococci	*S. aureus*	*E. coli*	*P. aeruginosa*
Penicillin	x		x		
Ampicillin		x	x	x	
Ampicillin-clavulanate		x	x		
Methicillin		x			
Cefotaxine	x				
Erythromycin	x	x			
Vancomycin	none	x	none		
Gentamicin		x	x	x	x
TMP-SMX	x		x	x	x

[a]Data taken from the American Society of Clinical pathologists (104).

Table II: Resistance mechanisms developed by bacterial strains against various classes of antibiotics (2, 4, 105).

Resistace mechanisms	Antibiotic class	Antibiotics	Organisms
Enzymatic Inhibition			
β-Lactamases	β-Lactam	Penicillin, amoxicillin, cephalosporin	*S. aureus, E. coli, P. aeruginosa*
Acetyltransferases, adenyltransferases, phosphotransferases	Aminoglycosides		Enterococci
Esterases, phosphotransferases	Macrolides		Enterobacteriaceae
Permeability Uptake	β-Lactam, aminoglycosides, macrolides		*P. aeruginosa*, enterococci, staphylococci
Porin Channels	β-Lactam, carbapenems		*P. aeruginosa*
	Quinolones, tetracycline, chloramphenicol, β-lactam		*P. aeruginosa, S. aureus*
Target Site Alteration			
Altered penicillin-binding protein	β-Lactam		*S. aureus, S. pneumoniae*
Altered cell wall oligopeptide	Glycopeptide	Vancomycin, teicoplanin	Enterococci
Altered ribosomal target	Tetracycline, macrolides, aminoglycosides	Streptomycin	Enterococci, *E. coli, N. gonorrhoeae*

widespread use of antibiotics and the facile nature of bacterial adaptation resulted in bacterial resistance to a large number of formerly effective drugs (1, 2). Within the last decade or so, the emergence of multidrug-resistant bacteria has been documented, calling attention to the serious nature of the antimicrobial resistance problem (2-6). The situation has progressed to the point that some level of bacterial resistance has been observed against all available antibiotics (1, 2) (Table I).

Clinically available antibiotics generally act through disruption of various metabolic pathways by targeting specific enzymes and/or membrane proteins. Bacterial genetic mutations and natural selection can lead to enzymatic inhibition of antibiotic drugs, altered porin channels, antibiotic efflux and alteration of target proteins, which can greatly attenuate antibiotic potential. The dependence of current drugs on inhibition of specific pathways is thus a major weakness and the reason why new agents in this class, while effective in the short term, are inevitably usurped by bacterial mutations (Table II).

Bacterial membrane-disintegrating peptides, which have their origin in naturally occurring cationic peptides, are promising alternatives to conventional antibiotics. These novel molecules operate by interacting with cellular components distinct from those targeted by conventional antibiotics. Moreover, because they act by disrupting the integrity of the bacterial cell membrane, the risk of developing drug resistance is greatly minimized. These agents demonstrate antibacterial activity against a broad spectrum of microorganisms and offer a platform for the development of simpler molecules operating via the same mechanism. With an interest in designing novel antibiotics, understanding structure-activity relationships in membrane-disintegrating peptides has become an area of active research in recent years.

Antibiotics in nature: membrane-disintegrating peptides

Cationic, membrane-disintegrating antibacterial peptides are found in the innate defense systems of a range of organisms including plants (7), insects (8, 9),

Table III: Classes and origins of natural peptide membrane disintegrators.

Peptide	Organisms	Ref.
α-Helical		
Temporin	Amphibians	106
Melitin	Insects	20
Peptaibols	Fungi	23
Cecropins	Insects, mammals	69
Dermaseptins	Amphibians	107
Maginins	Amphibians	108
β-Sheet		
Defensins	Insects, plants, mammals	7, 9, 27
BPI	Mammals	109
Lactoferricin	Mammals	30, 110
Protegrin	—	111
Extended		
Indolicidin	Mammals	33
PR39	Mammals	34
Looped		
Gramicidins	Bacteria	112
Polymyxins	Bacteria	36, 37

amphibians (10) and mammals (11-13). In insects and amphibians, injury induces production of antimicrobial peptides as a type of immune response to prevent local infection. In mammals, antimicrobial peptides are present at high levels in neutrophils, the most important cells involved in the immediate response against microbes. A comprehensive database maintained at the University of Trieste, Italy, currently contains over 800 peptides (14) and a database of a subclass of antibacterial peptides includes hundreds of other sequences (15).

Antibacterial peptides display varied lengths, sequences and secondary structures, but have two distinguishing common features: a net positive charge typically +2 to +6 and an overall amphipathic fold imparting polar and hydrophobic faces to the molecule (16). The cationic nature of antibacterial peptides promotes selective interaction with the negatively charged surface of bacterial membranes over the neutral surface of eukaryotic membranes (17), whereas the amphipathic conformation promotes bacterial cell lysis (18). Antibacterial peptides are typically classified into 4 structural categories: β-sheet, α-helix, extended and covalent or disulfide-looped (Table III, Fig. 1).

Conformational analyses via circular dichroism, nuclear magnetic resonance and infrared spectroscopies reveal differences in how the various structures of antibacterial peptides are stabilized. Peptides in the β-sheet category, for example, typically contain 1-3 disulfide bridges that act to stabilize the β-sheet structure in solution and when in contact with bacterial membranes. The looped class demonstrates similar structural behavior due to the presence of its constrained ring. In contrast, α-helical and extended antibacterial peptides are typically disordered in aqueous solution but form amphipathic conformations when in contact with model membranes or dissolved in hydrophobic, membrane-mimicking solvents or detergent systems (19).

α-Helical peptides

Peptides with α-helical structure are perhaps the most well-studied class of antibacterial peptides. These peptides fold as amphipathic helices with a polar face composed primarily of cationic lysine and/or arginine residues. While a number of α-helical peptides exhibit high levels of antibacterial activity and selectivity, some, such as melittin found in bee venom (20), are potently hemolytic. Magainin, a 23-residue peptide, is a member of the α-helical class and is found in frog skin secretions (21). This peptide has received much attention in the literature, including reports of broadened bactericidal efficacy through various sequence modifications, derivatizations and development of magainin-based model peptides, as detailed below.

Peptaibols, a unique group of membrane-active peptides produced by fungi to fend off bacteria (22-24), are α-helical peptides characterized by a sequence of 10-20

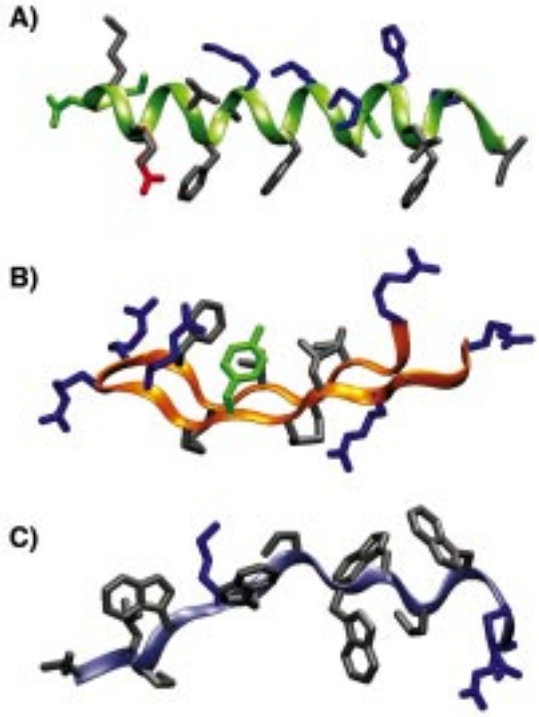

Fig. 1. NMR structures of peptides belonging to different structural classes. A) α-Helical magainin (122), B) disulfide-stabilized β-sheet protegrin (123, C) extended indolicidin (124). Side chains in each of the structures are colored according to chemical character: basic residues are blue, acidic are red, polar are green and hydrophobic are gray. Note the segregation of cationic side chains to one face of the magainin helix and the ends of the protegrin sheet. Produced from Protein Data Bank files A) 2MAG, B) 1PG1 and C) 1G89 with VMD software (125).

amino acids with a high proportion of the $C_{\alpha\alpha}$-disubstituted α-aminoisobutyric acids as their positively charged groups, an N-terminal acetyl group and a C-terminal alcohol. A subclass of this group, the lipopeptaibols, run 6-10 residues in length and have a short, N-terminal linked fatty acid (25).

β-Sheet peptides

Other antimicrobial peptides achieve amphipathicity by folding into antiparallel β-sheets that are often stabilized by one or multiple disulfide bonds. Two examples in this category are the defensins and the protegrins. Human defensins are cationic, arginine-rich peptides linked by 3 disulfide bonds into a 3-stranded β-sheet (26). They show activity at micromolar concentrations against bacteria, fungi and enveloped viruses, although the precise mechanism of their activity is unclear (27). Protegrins have been isolated from porcine leukocytes (28) and demonstrate membrane permeation and destabilization in model membrane systems (29). They are active against bacteria in the micromolar range (30).

Extended peptides

The extended class of antibacterial peptides is made up of primarily members containing an unusual population of amino acid residues in their sequences. Examples include the histidine-rich histatin found in saliva (31), proline- and arginine-rich PR-39 found in pig intestine (32) and two peptides with unusually high concentration of tryptophan, an amino acid with a normally low frequency in proteins. Indolicidin (33), derived from bovine neutrophils, is a 13-residue peptide with 5 tryptophan residues. It has been shown to be effective against both Gram-negative and Gram-positive bacteria (34). Tritrpticin is also 13 residues in length, contains 3 tryptophan residues, and shows bactericidal and fungicidal activity (35). The high tryptophan content of indolicidin and tritrpticin may be important for membrane activity, as tryptophan side chains display a tendency toward membrane interaction and integration near the membrane-water surface (34).

Looped peptides

Looped peptides were the first agents to be widely used as antibiotics. Polymyxin B, for example, has been used as a topical antibiotic for decades (36). The polymyxins are a family of looped peptides that contain several positively charged diaminobutyric acid residues. Recently, polymyxin M (also called mattacin), a peptide rich in diaminobutyric acid and containing an 8-carbon acyl chain at one end, was isolated from the Gram-positive bacterium *Paenibacillus kobensis M* (37). Polymyxin M generally demonstrates greater activity than polymyxin B against Gram-negative and Gram-positive bacteria. Gramicidin S, a 10-residue cyclic peptide synthesized by Gram-negative bacteria, exhibits bactericidal activity against primarily Gram-positive strains, although the peptide has been shown to be active against Gram-negative bacteria and fungi as well (38, 39). Gramicidin S forms an amphipathic β-sheet structure in membranes leading to membrane leakage and depolarization (40). It should be noted that gramicidins are a structurally diverse group of peptides; gramicidin A, for example, is helical while gramicidin S is looped.

Nonpeptidic membrane disintegrators

In addition to the naturally occurring peptides, several small-molecule membrane disruptors have been identified. The most prominent of these is squalamine, which is amphipathic not by the nature of its folded structure but by the presence of polyamine chains about a steroid core (41). The bactericidal mechanism of squalamine, despite its small size, is similar to membrane-disintegrating peptides. Both bacterial membrane leakage measurements and transmission electron microscopy demonstrate that squalamine perforates bacterial membranes and promotes leakage of cell contents (42, 43).

Bactericidal activities

The bactericidal activity of any of these antimicrobials is lower than that of conventional antibiotics. While pharmaceutical drugs such as ampicillin and vancomycin are effective at the nanomolar level, naturally occurring antimicrobial peptides typically show minimal inhibitory concentrations in the low micromolar range against Gram-negative bacteria and in the high micromolar range against Gram-positive bacteria (Table IV).

Mechanism of action

Despite the diversity of sequences and folded structures of peptide antibiotics, their bactericidal mechanism is strikingly similar throughout all classes. Antimicrobial peptides exert their effects through a 2-step mechanism: electrostatic interactions with the cell surface and membrane permeabilization via pore formation, membrane dissolution or disruption due to membrane curvature strain. Secondary effects include membrane depolarization and the influx of antibacterial peptides through the perforated membrane whereby they may induce additional effects within the bacterium (44).

Cell selectivity

The interaction between an antibacterial peptide and the bacterial membrane imparts specificity to the peptide

Table IV: Bactericidal activities of natural peptides against Gram-positive and Gram-negative bacterial strains.[a]

			Gram-positive				Gram-negative		
	Class	Units	*B. subtilis*	*M. luteus*	*E. faecalis*	*S. aureus*	*P. aeruginosa*	*E. coli*	Ref.
Cecropin	α-Helical	μM		1.4			2.6	0.4	113
Magainin	α-Helical	μM				>80	76	38	71, 76
Defensin A	β-Sheet	μM		0.6		>50		>50	114
Protegrin	β-Sheet	μg/ml			2.7	1.7	0.5	0.75	111
Indolicin	Extended	μg/ml				2-4		20-30	34, 115
Helioferins	Lipopeptaibol	μg/ml	3	1.5		1.5	>100	>100	23
Polymyxin M	Looped	μM					100	3-12.5	37
Polymyxin B	Looped	μg/ml			40	26	0.2	1.8	100, 116, 117
Gramicidin S	Looped	μg/ml	3.1		3.1	1.5	6.2-25	3.1	77
Squalamine	Small, steroid	μg/ml			1-2	1-2	4-8	1-2	100, 116, 117

[a]Values are minimal inhibitory concentrations (MIC).

and is the first step in the bactericidal mechanism. The external leaflet of bacterial cell membranes is composed of zwitterionic phosphatidylethanolamine and anionic phosphatidylglycerol lipids, lipopolysaccharides for Gram-negative bacteria and teichoic acid groups for Gram-positive bacteria, giving all bacterial membranes a net negative charge. On the other hand, the exterior leaflet of eukaryotic cells is electrically neutral, typically containing zwitterionic phospholipids and cholesterol. Antimicrobial peptides achieve selectivity for bacterial cells almost exclusively by exploiting this difference in membrane surface charge, although the presence of cholesterol in eukaryotic membranes and ethanolamine lipids in bacterial membranes may also play a role (45-47). Cationic charges on the antibacterial peptide promote interaction with the negatively charged bacterial polysaccharides and membranes. Because eukaryotic membranes lack anionic components, interactions with bactericidal peptides are attenuated.

Bactericidal mechanism

Evidence suggests that the steps following electrostatic interaction between the peptide and membrane are: first, interfacial partitioning of the peptide and accumulation on the target membrane; second, peptide structural changes (conformation, aggregation and orientation) induced by interactions with the lipid bilayer; third, membrane permeabilization and depolarization; and finally, rapid bacterial cell death as a result of leakage and depolarization.

Three models have been presented to help explain the membrane-disintegrating behavior of lytic peptides: the barrel-stave, carpet and toroidal pore models (Fig. 2). For additional information on these models, a number of excellent reviews on this subject are available (48-50).

In the barrel-stave model, the membrane is permeabilized by the formation of transmembrane pores composed of a bundle of amphipathic helices, similar to ion channel-forming membrane proteins (48, 51-53). Following electrostatic interaction with the cell surface, bactericidal peptides insert directly into the membrane with their hydrophobic face aligned toward the lipid tail groups and their hydrophilic face lining the pore “channel”. The strong interactions between peptide side chains and lipid hydrocarbon chains is an integral part of this mechanism, leading to a dominance of hydrophobic interactions and making the presence of hydrophobic residues key to the bactericidal activity of these peptides. The pore-forming process can occur at low surface concentrations, since only two peptides are required to dimerize and form a nascent channel. Subsequent recruitment of additional peptides may then enlarge the pore.

In the carpet model, peptides first interact with the membrane surface and then cover it at a comparatively high surface concentration, which reaches a threshold beyond which the membrane becomes permeable (19, 54-56). The peptides act in a detergent-like manner to disrupt the membrane via the formation of transitory pores. At no time during this process do peptides insert into the membrane. Rather, they interact electrostatically with phospholipid head groups and then rotate in such a way that their hydrophobic face is positioned in the hydrophobic part of the membrane with the peptide axis parallel to the membrane plane. Disruption of the membrane occurs either by dissolution of large portions of the membrane (*i.e.*, membrane solubilization) or by creation of unstable curvature strain in the membrane.

The toroidal pore model combines aspects of both the barrel-stave and carpet mechanisms. Membrane interactions are the same as in the carpet model, but membrane permeability develops at a somewhat lower peptide concentration. As in the barrel-stave model, pores are lined with peptides. However, toroidal pores have lipids inserted between helices to form a mixed transmembrane pore (57).

Although most of the information on the mechanism of action of antibacterial peptides has been obtained using peptides from the α-helix class, mechanisms appear to be common among all peptides in any class (47). In addition, even though each of these models emphasizes membrane permeabilization, there is evidence that membrane permeabilization may not necessarily be linked to

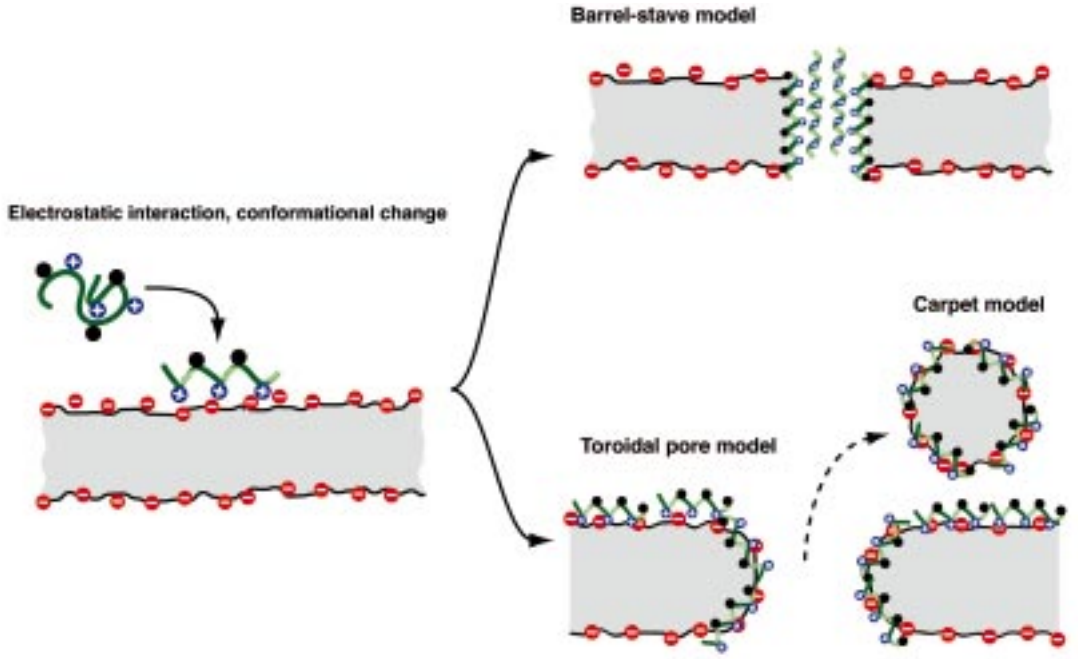

Fig. 2. Models of antibacterial peptide membrane disruption. The first step in all models is electrostatic binding of the peptide to the membrane surface (left). The peptides bind in low numbers in the barrel-stave model (top right) and subsequently form transmembrane pores as bundles of a few peptides. Peptides orient perpendicular to the membrane, and the hydrophobic face of the peptide interacts directly with the lipid hydrocarbon chains. The carpet and toroidal pore models (bottom right) share common initial features, in which peptides bind in large numbers to the membrane surface and when a threshold concentration is reached, either solubilize large portions of the membrane (carpet model) or induce toroidal pores in the membrane (toroidal pore model). In either of the latter models, peptides are parallel to the membrane surface and interact with both the lipid headgroups and, due to penetration into the bilayer, with portions of the lipid tails close to the membrane surface.

bactericidal activity in all cases. It is possible that, in some instances, membrane disruption is but a means to transport molecules into the cell, wherein peptides attack some as yet unknown secondary target(s) (44).

The membrane-disintegrating nature of peptide antibiotics makes them much less susceptible to bacterial resistance than conventional antibiotics (Table II). Nevertheless, the development of resistance is possible (58), and, in some cases, has been observed. Resistance may develop, for example, through modification of molecules in the cell envelope (59). In Gram-positive organisms teichoic acids may be modified with D-alanine, resulting in a free amine, or phosphatidylglycerol lipids may be lysylated (60), thereby reducing the net negative charge of the membrane and attenuating interaction with cationic peptides. In Gram-negative bacteria, modifications of the lipopolysaccharide (LPS) membrane component could reduce the net negative charge, or an additional fatty acid could be added to the lipid A portion of LPS to increase the stability of the bilayer, thereby reducing its susceptibility to disruption by antibiotic peptides (61). In addition, it is possible that outer membrane proteases could cleave antimicrobial peptides (62) or efflux pumps could expel them from the cell (63). While these mechanisms of resistance have been observed in isolated cases, the complexity of such modifications minimizes the likelihood of general resistance to membrane-disintegrating peptides.

Recent developments in peptide antibiotics

The common mechanism of action and structural diversity of antimicrobial peptides provide a range of opportunities for further development. A database of designed or modified synthetic peptides currently lists hundreds of entries, demonstrating the scope of the development effort (64). Many strategies have been used to develop these active, specific antimicrobial peptides, including sequence analysis of natural peptides with high activity and specificity leading to the synthesis of simpler analogs (65-67), combinatorial libraries of peptides to screen for the most active variant (68) and systematic studies of structural properties and membrane interactions (69, 70). Many of the resultant peptides have the desirable properties of broad bactericidal activity and specificity for bacterial rather than eukaryotic cells. Given the relatively large number of sequence modifications, derivatizations and hybridizations performed with naturally occurring and synthetic membrane-disrupting peptides, it is impossible to cover all of the promising work that has been done towards the development of peptide

Table V: In vitro bactericidal activities of designed or modified membrane-disrupting peptide sequences and synthetic peptide mimics.[a]

Molecule[b]	Units	Gram-positive				Gram-negative				Ref.
		B. subtilis	*E. faecalis*	*E. faecium*	*S. aureus*	*S. epidermis*	*P. aeruginosa*	*E. coli*	Hemolysis	
β-Peptides										
β-17 (APC40)	μg/ml	0.8		12.5	3.2			6.3	64	101
AP40	μg/ml	1.6		50	12.5			25	178	101
#4	μg/ml	0.8		3.1-6.3	6.3			6.3	280	102
#7	μg/ml	0.8		6.3	12.5				22	103
Dendrimeric Peptides										
Linear RLYR	μM		0.9		1.8		0.5	1	338	90
8-Demdrimer RLYR	μM		0.8		0.5		0.3	0.5	1514	90
Designed Peptides										
KIGAKI	μg/ml				8		32	8	>500	85
Leucine-lysine peptide	μM				0.5			3.5	>50	118
SC4[c]	μM				0.35		0.01	0.003-0.25	>100	83
Designed tachyplesin 3 (cTP)	μM		0.3		0.4		0.7	0.1		119
Peptide Mimics										
P5 (cecropin-magainin hybrid)	μM	0.78				1.56	0.78-1.56		>100	120
GS14K4 (gramicidin S)	μg/ml	25	1.5		50	2	3.1-25	3.1	200	77
Pexiganan (magainin analog)	μg/ml		128		8	4	16	16		121
Non-Cys, D-Pro protegrin	μg/ml	25		12.5-25	50			6.3		86
Non-Cys, amino-Pro protegrin	μg/ml	12.5		12.5	25			3.1		86
D-isomer protegrin	μg/ml	6.3		6.3	12.5			3.1		86
Small Molecules										
SM-25 (squalamine derivative)	μg/ml		3.13		0.78	0.39	0.78	1.56	50	100
Squalamine derivative #3	μg/ml		3.1		0.4		2	3	29	100, 117, 118
Synthetic Polymers										
Arylamide polymer 8-mer	μg/ml	16		12-25			31-62	12-50		103

[a]Values are minimal inhibitory concentrations (MIC). [b]Molecule names are those reported in the respective references. [c]Values for SC4 are LD_{50}

antibiotics. We have instead chosen specific works that exemplify key properties of peptide antibiotics and represent the variety of approaches used to develop novel antibacterial peptides and related molecules (Table V).

Structural and compositional characteristics imparting bactericidal activity

A wealth of information on the structural and compositional requirements of antibacterial peptides exists in the literature, although the majority of it focuses on peptides in the α-helix class. These systematic studies have investigated effects from changes in net charge, helicity, hydrophobicity and hydrophobic moment on lipid interaction, permeabilization, bactericidal activity and hemolysis. Important factors for bactericidal activity established through structure-activity relationships are amphipathicity, hydrophobicity and the presence of basic amino acid residues and defined secondary structures (71, 72).

Using analogs of magainin-2 and model membrane systems, Wieprecht *et al*. demonstrated that a high cationic angle on the peptide increases interaction with negatively charged membranes and that membrane permeabilization is increased by having a small charged angle and low membrane charge (73). Similar experiments varying the hydrophobic moment and overall hydrophobicity of magainin-2 analogs showed a decrease in membrane specificity but an increase in both bactericidal and hemolytic activity with increased hydrophobic moment or hydrophobicity (74, 75). These results suggest that the selectivity of a peptide (*i.e.*, one binding bacterial rather than eukaryotic cells) is optimized by a high net positive charge, a large cationic face and low hydrophobicity, thus increasing binding to negatively charged bacterial membranes and decreasing permeabilization of uncharged eukaryotic cells. These conclusions are supported by work with the model helical peptide KLA1 (69, 76).

Modifications of the looped/β-sheet gramicidin S peptide using diastereomers at different positions along the chain to modify amphipathicity of the β-sheet showed that amphipathicity (and a large hydrophobic surface) decreases the therapeutic ratio (bactericidal activity/hemolytic activity) by increasing hemolysis and decreasing bactericidal activity, and that a threshold for this behavior exists (77). It appears that peptides having different secondary structures demonstrate the same relationship between hydrophobicity and the ability to indiscriminately permeabilize membranes.

Modified synthetic peptides

One approach that has been used in the design of antibacterial peptides is to modify the sequences of naturally occurring peptides in order to mimic their behavior in a smaller molecule, increase their bactericidal activity and/or reduce their hemolytic activity.

Mayo *et al*. (78, 79) designed a series of β-sheet-forming peptide 33mers (βpep peptides) by substituting functionally key amino acid residues from the β-sheet domain of 55 kDa human neutrophil bactericidal/permeability increasing protein (BPI) (80-82) into the antiparallel β-sheet scaffold of α-chemokines and using basic principles of protein folding to enhance peptide water solubility and β-sheet conformational stability. At least two βpep peptides (βpep-19 and βpep-25) were more active than BPI, showing bactericidal activities in the 10 nM range (83). βpep peptides fold as amphipathic β-sheets (84), with their polar face composed of multiple lysine and arginine residues and their hydrophobic face composed primarily of aliphatic side chains. Dissection of βpep-25 led to the identification of a dodecapeptide (SC4) that formed a helical structure and was even more potent than the parent βpep-25 against a broad spectrum of bacteria (83). SC4 demonstrated activities in the low single digit nanomolar range against Gram-negative bacteria and submicromolar range against Gram-positive bacteria. In addition, SC4 showed relatively low hemolysis of human red blood cells even at millimolar concentrations and no apparent lytic effects up to the millimolar level against endothelial cells, fibroblasts or leukocytes.

An 18-residue peptide based on the repeating sequence KIGAKI was modeled after α-helical sequences, but was designed to shift preferred amphipathicity from an α-helical to a β-sheet conformation (85). In model membrane systems, the KIGAKI peptide demonstrated increased selectivity for mimics of bacterial membranes relative to its α-helical relatives. Bactericidal activities for the KIGAKI peptide were similar to those observed for α-helical peptides, but its β-sheet conformation yielded somewhat reduced hemolysis.

In an effort to simplify the synthesis of protegrin, a cysteine-linked β-sheet peptide, Lai *et al*. replaced cysteine residues in the sequence with threonine, a statistically known β-sheet former, and used D-proline in the beta-hairpin region to promote turn formation (86). These substitutions eliminated the peptide's disulfide bridges, but maintained much of the β-sheet conformation. The authors concluded that while structure was important for activity, the distribution of charge in the molecule also played an important role, as did the level of hydrophobicity. For example, substituting alanine for threonine produced a much less effective peptide.

Muhle *et al*. designed a family of cyclic β-sheet peptides composed of putative LPS-binding sequences. Several members of the family demonstrate high selectivity for Gram-negative bacteria, which display LPS moieties on their surface, and have bactericidal activity in the tens of nanomolar range (87).

A number of hybrid peptides have been made in an attempt to capture qualities of either parent peptide. Boman *et al*. (88) produced a cecropin-melittin hybrid that exhibited reduced hemolytic activity as compared to melittin, but increased bactericidal activity as compared to cecropin. A cecropin-magainin hybrid called P18 produced by covalently linking residues 1-8 of cecropin A and residues 1-12 of magainin-2 (89, 90), shows similar effects, with the hybrid improving biological activities of the parent sequences.

Tam *et al.* have synthesized dendrimeric peptides, molecules in which 2, 4 or 8 peptides are linked to a multifunctional core (91). Dendrimers with short, repeating arginine- and leucine-containing peptides yielded molecules with potent antimicrobial activity and decreased hemolytic activity relative to monomeric peptides of the same sequence.

Borrowing the idea from naturally occurring polymyxins and lipopeptaibols, several investigators have recently produced fatty acid conjugates of antibacterial peptides in order to improve bactericidal activity. In these investigations, addition of fatty acid moieties increased bactericidal activity of cathepsin G (92, 93) and lactoferrin peptides (94, 95). Antibiotic activity was similarly increased in magainin (96) and a cecropin-melittin hybrid (97) by fatty acid conjugation. Fatty acids with dodecyl or octadecyl tails conjugated to SC4 greatly enhanced the activity of this peptide, particularly against Gram-positive bacteria including even anthrax and other strains resistant to conventional antibiotics (98). It appears that conjugation of a hydrophobic fatty acid moiety increases peptide bactericidal potency by enhancing membrane interactions and stabilizing the structure of the peptide in the membrane-bound state. The down side to the peptide acylation strategy, however, is the generally increased lysis of human erythrocytes, which may limit the useful of such agents as antibiotics in humans. Nevertheless, this strategy presents an interesting approach to design novel antibiotics that function as membrane disintegrators.

Other novel membrane-disintegrating agents

Several groups are working to transfer some of the membrane-disintegrating properties of antibacterial peptides to simpler synthetic molecules. Some small-molecule mimics of polymyxin B (99) and squalamine (100) have also been reported. These molecules mimic their parents by creating small amphiphilic agents that generally appear to function by disrupting membranes. However, they also tend to allow other hydrophobic drugs into cells and lack specificity for bacterial cell membranes. Overall, they appear to be less effective than their parent compounds.

Porter *et al*. synthesized β-amino acid-containing peptides that adopt an amphipathic helical conformation (101, 102). In this system, optimal activity was achieved with a smaller proportion of charged amino acid residues

Table VI: Cationic-peptide derived molecules in commercial development.

Company	Peptide/Type	Clinical indication	Development stage
Topical Agents			
Genaera	Locilex/pexignan (magainin derivative)	Diabetic ulcer infections	Completed phase III
Micrologic Biotech	MBI-226	Catheter infections	Phase III
	MBI-594AN (α-helix)	Acne	Phase III
Intrabiotics	Iseganan (protegrin analog)	Ventilator-associated pneumonia	Phase III
Xoma	Mycoprex (BPI-derived)	Acne	
Demegen	Histatin analogue (P-113)	Gingivitis	Phase II
Systemic Agents			
Xoma	BPI	Meningococcal meningitis	Phase III
Entomed	Heliomycin (ETD-151)	Antifungal	Preclinical
AM Pharma	Lactoferricin	Antibacterial, antifungal	Preclinical

compared to other membrane-disintegrating helical peptides. Adjusting the positions of β-amino acid residues was found to decrease hemolytic activity, but also antibacterial efficacy. The nonnatural backbone of such molecules should provide increased resistance to proteolysis *in vivo*, imparting a unique property to these molecules.

Producing biomimetic antimicrobial polymers is an approach that employs a simple and inexpensive synthesis using a single monomer unit. Tew *et al*. (103) have produced synthetic arylamide polymers that effectively mimic the structural requirements of antibacterial peptides through variation in the positions of primary amine and *tert*-butyl groups along the polymer backbone. The resulting molecules, which are amphipathic, can be made in different lengths from long (n=60) to short (n=8) polymers. Shorter variants tend to be more bactericidal and are effective at concentrations of 10-50 μg/ml against Gram-positive and Gram-negative strains.

Conclusions

Although this review has covered only a small part of the field of antibiotic peptides, the general consensus as to what makes an effective antibacterial agent is a high incidence of positively charged and hydrophobic groups in the context of an amphipathic conformation. Several investigations into modified antimicrobial peptides have led to effective bactericidal agents in the commercial realm. However, the hemolytic activity of most of these molecules has generally limited their use to topical applications, such as treatments for acne, diabetic foot ulcers and catheter infections (Table VI). Nevertheless, it should be apparent from this review that there are a few identified peptides that have low effective therapeutic concentrations and minimal hemolytic effects. Because of the increasing problem of resistance of bacteria to conventional antibacterial agents, the future of antibiotics lies in the production of agents that function by selectively disintegrating bacterial membranes. Efforts are currently focusing on the design of new chemical entities that mimic the amphipathic nature of membrane-disintegrating peptides and increase their therapeutic index by enhancing selectivity for bacterial cell membranes.

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