Multivalent Antimicrobial Peptides as Therapeutics: Design Principles and Structural Diversities

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Abstract This review highlights the design principles, progress and advantages attributed to the structural diversity associated with both natural and synthetic multivalent antimicrobial peptides (AMPs). Natural homo- or heterodimers of AMPs linked by intermolecular disulfide bonds existed in the animal kingdom, but the multivalency strategy has been adopted to create synthetic branched or polymeric AMPs that do not exist in nature. The multivalent strategy for the design of multivalent AMPs provides advantages to overcome the challenges faced in clinical applications of AMPs, such as: stability, efficiency, toxicity, maintenance of activity in high salt concentrations and under physiological conditions, and importantly overcoming bacterial resistance which is currently a leading health problem in the world. The multivalency strategy is valuable for moving multivalent AMPs toward clinical applications.

Keywords Antimicrobial peptides (AMPs) · Multivalent effect · Multivalent antimicrobial peptides · Structural diversities

Introduction

As the time for the development of resistance to antibiotics by microorganisms continues to decrease, the need to identify and develop new molecules that can overcome the limitations of the present drugs is urgent. Naturally cationic antimicrobial peptides (AMPs) including defensins, which constitute an important component of the innate immune response of most animal species to microbial infections have been considered as a template for the development of new antibiotics due to their unique mechanism of action and that these molecules will have combat antibiotic resistance by microorganisms. (Boman 1995, 2003; Hancock 1997; Zasloff 2002) The world-wide developmental pipeline of new antibiotics has narrowed due to the issues of resistance and the academic community has entered into this void. Some sequence mutation and subsequently structural changes of the natural antimicrobial peptides have been engineered into new molecules which have partially overcome the practical challenges of AMPs for therapeutic use. As a new generation of prospective antibiotics, AMPs offer a broad-spectrum antimicrobial activity with an attractive evolutionary property of action like that of the naturally occurring peptides so that bacteria may find it difficult to develop resistance (Hancock and Chapple 1999; Giuliani et al., 2007; Zasloff 2002). However, in clinical applications, the challenges for AMPs have been (i) toxicity, which is due to their relatively high minimum inhibitory concentration (MIC) or high dosages that are needed, the low selectivity for bacterial cell membrane and their antimicrobial activity being reduced in the presence of salts under physiological conditions, (ii) the short half-life in vivo due to rapid proteolytic cleavage, and (iii) stimulation of an immune response. An approach of targeting mucosal surfaces in topical application may overcome some of these
issues (Hancock 1999). Other strategies that have been used in the face of these challenges include the introduction of D- or other unnatural amino acids into AMPs (Dooley et al. 1994; Welch et al. 2007; Porter et al. 2000) and small organic molecule- or foldamer- or polymer- based antimicrobial peptide mimics (Savage 2002a, b; Scott et al. 2008; Liu et al. 2004; Tew et al. 2002, 2010). The design of multivalent AMPs by conjugation of several or decades of copies of a peptide monomer to scaffold molecules via naturally occurred intermolecular disulfide bridges or unnatural scaffold linkers has overcome some of the problems posed by natural AMPs. The purpose of this review is to highlight the progresses on rational design of multivalent AMPs in addressing these issues.

**Design Principles for Multivalent Antimicrobial Peptides**

Multivalent/polyvalent interactions, as defined by Whitesides, are multiple simultaneous bindings between two biological entities (Mammen et al. 1998). These multivalent interactions prevail in biological system. Multivalency is usually based on these multivalent interactions, which has implications for design and use of multivalent ligands and inhibitors. Through the simultaneous recognition of multiple binding sites multivalent ligands can greatly enhance binding ability by several orders of magnitude in some cases (Rao et al. 1998; Schaschke et al., 2001; Profit et al. 1999) Covalently attaching bioactive compounds to a scaffold or core molecule has proven to be a successful strategy to greatly enhance the affinity of especially weak carbohydrate ligands to bind to bacteria toxins (Zhang et al. 2002; Kitov et al. 2000; Pukin et al. 2007) and bacterial surfaces (Pieters 2007) The development of multivalent antibiotics (vancomycin) as a potential therapeutic agent against infections caused by drug resistant microbes such as methicillin-resistant *staphylococcus aureus* (MRSAs) is well documented, (Li and Xu 2005) and it shows the effectiveness of the multivalent strategy for the design of more effective anti-microbials. Multivalent AMPs can be formed by covalently linking to a scaffold or another core molecule. These multivalent AMPs have been shown to have advantages over their monomeric forms, such as enhanced membrane pore formation (Arnusch et al. 2007), improved antimicrobial activity (Liu et al. 2006), the ability to maintain good antimicrobial activity under physiological conditions (100–150 mM or high-salt conditions) (Tam et al. 2002; Campopiano et al. 2004), improved stability against protease degradation (Tam et al. 2002; Dewan et al. 2009; Young et al. 2009; Falciani et al. 2007; Pini et al. 2008) and low or no cytotoxicity (Kolar et al. 2010; McDermott et al. 2010).

Several AMPs units have been covalently linked together in the same molecule, i.e. dendritic AMPs, in which peptide units are covalently linked to scaffolds or a core molecule. The individual units may act in concert to yield enhanced properties such as improved potency or improved specificity against a target membrane. Such multivalent effects have been achieved by linking several copies of the monomeric form of an AMP to a scaffold that apparently makes AMP units work together in the formation of a pore in a membrane (Arnusch et al. 2007). Thus the topic of multivalent AMPs has many different aspects such as unit length of a sequence, (Pieters et al. 2009) biological applications (Pini et al. 2008), as well as the structural diversities of naturally occurring and synthetically produced multivalent AMPs which will be discussed in this review.

**Structural Diversities of Multivalent AMPs**

The chemical structures of multivalent AMPs vary greatly from naturally occurring or synthetic intermolecular disulfide linked dimers, to synthetic multivalent forms of AMPs, such as the covalently branched/dendrimeric AMPs (Tam et al. 2002; Arnusch et al. 2007) and polymeric AMPs (Liu et al. 2006; Kolar et al. 2010).

**The Naturally Occurring or Synthetic Intermolecular Disulfide Linked Homo- and Hetero-Dimers of AMPs**

Bacteria produce proteases and peptidases that hydrolyze and inactivate the antibiotics which is also a common mechanism for bacterial inactivation of AMPs (Peschel and Sahl 2006). A hallmark of several naturally occurring cationic antimicrobial peptides is the presence of pairs of disulfide bonds. It has been shown that the disulfide bonds convey peptide resistance against proteolytic degradation. However, the intramolecular disulfide bonds are not essential for antimicrobial properties of defensins (Maemoto et al. 2004; Rozek et al. 2003; Wu et al. 2003; Liu et al. 2008). While several naturally occurring antimicrobial peptides contain 3 or 2 pairs of rigid intra-molecular disulfide bonds, peptides that are linked by inter-molecular disulfide bond are uncommon. Recently, a number of molecules with inter-molecular disulfide bonds creating homo- and hetero-dimers have been reported (Yomogida et al. 1996; Batista et al. 2001; Hornef et al. 2004). In addition, since the intermolecular disulfide bond could reinforce the activity of two individual chains, the antimicrobial activities of several synthetic intermolecular disulfide linked dimers have been examined. In this section, we will highlight the antimicrobial
properties of homo- and hetero-dimers with the natural as well as synthetic intermolecular disulfide bonds.

Yomogida et al. (1996) reported the first cationic antimicrobial peptide (CAP11) with an intermolecular disulfide bond isolated from Guinea pig neutrophils granules. CAP11 contains two identical chains that are rich in basic amino acid residues (8 arginine and 9 lysine per chain) and linked through Cys41 (Table 1). CAP11 has potent antimicrobial activity against both Gram-positive and Gram-negative bacteria and the activity was not altered by the presence of 150 mM NaCl. CAP11 was also active against retroviruses. The S-pyridyl ethylated monomer also showed comparable antimicrobial activity compared to its dimer (Okuda et al. 2006). However, the dimer had a two fold increase in LPS-binding affinity compared to the monomer. Lee et al. (2001) reported a homodimer (dicynthaurin) isolated from hemocytes of the tunicate, 

Halocynthia aurantium (dicynthaurin) isolated from hemocytes of the tunicate, 

Distictin, from skin extracts of the tree-frog Phyllomedusa distincta. (Batista et al. 2001) Distictin contains 22-(chain A) and 25-(chain B) amino acid residues and it showed marked antimicrobial activity against Gram-positive and Gram-negative bacteria, comparable to conventional antibiotics (Table 1). The individual chains showed low antimicrobial activity (Dalla Serra et al. 2008). Synthetic homodimers of two chains (i.e., A–A and B–B), however, had comparable antimicrobial activity with that of the heterodimer. These results clearly reveal the improvement in antimicrobial activity of moderately active peptides through intermolecular disulfide bonding. Interestingly, it has been shown that distintin forms a non-covalent dimer at pH 6.8 that exists as a compact bundle of four helices. This full parallel four-helix structure confers excellent stability (>6 h) against proteolytic enzymes such as trypsin, chymotrypsin, and subtilisin (Raimondo et al. 2005). However, both the monomeric chains A and B existed as a monomer and were completely degraded by these enzymes. Thus it was suggested that the covalent association of the two chains improves the antimicrobial activity and the non-covalent association protects against proteolytic degradation (Raimondo et al. 2005). Solid state NMR structural studies of distintin in model lipids indicated that distintin refolds from a compact four helix structure into a more open two helix structure (Resende et al. 2009). The enhanced antimicrobial activity was attributed to this open structure wherein the helix formed by chain B anchors the dimer in the membrane interface whereas the chain A interacts weakly with the bilayer.

Wang et al., reported the heterodimer from hemocytes of tunicate, Halocynthia aurantium (Jang et al. 2002). Halocidin contains two chains, a 18 residue leucine rich peptide connected intermolecularly through cys18 to a 15 residue peptide which lacks the first three N-terminus residues of the 18 residue chain. The antimicrobial activity of halocidin, the 18 and 15 residue chain monomers, and their corresponding homodimers were investigated. The 15 residue monomer and its homodimer showed poor antimicrobial activity. However halocidin, 18 residue chain monomer, and its homodimer (di18Hc) exhibited excellent antimicrobial activity against multi-drug resistant 
P. aeruginosa
and methicillin-resistant S. aureus. The antimicrobial activity of di18Hc against a number of clinical isolates was investigated in the presence of monovalent and divalent cations (Jang et al. 2003). The homodimer retained its activity in the presence of up to 300 mM NaCl. However, addition of MgCl₂ decreased the activity substantially: about 4 fold decrease in activity was observed at 5 mM MgCl₂ against MRSA while the activity was lost against multi-drug resistant 
P. aeruginosa.

The reported presence of a number of covalent homo- and hetero-dimers in mouse intestinal tissue, known as cryptidin-related sequence (CRS) peptides, further extend the repertoire of naturally occurring disulfide-bonded covalent dimers (Hornef et al. 2004). The antimicrobial activities of CRS4C-1a peptide sequence in its monomeric and dimeric forms were investigated (Hornef et al. 2004). It has been suggested that the homodimer not only exhibited superior broad spectrum antimicrobial activity but also killed the bacteria almost instantaneously compared to the monomer (Table 1). The diversity of covalently linked homo or hetero dimeric CRS family of antimicrobial peptides, might potentiate an efficient protection of the gastrointestinal mucosa from infection with enteric pathogenic microorganisms. It is likely that covalent dimerization in vivo is a mechanism for expanding the diversity of this innate defense system.

Morrison et al. (2002) identified a new β-defensin related (Defr1) gene from mouse that is highly expressed in heart and testis. The matured form contains 34 amino acid residues and unlike other β-defensins, Defr1 contains odd number (five instead of six) cysteine residues. A synthetic Defr1 peptide, which existed as an intermolecular disulfide-linked dimeric form of 34-residue monomer, exhibited antimicrobial activity against Gram positive bacteria (S. aureus), and Gram negative bacteria (P. aeruginosa,
Table 1  Antimicrobial properties of intermolecular disulfide linked homo-/hetero dimers

| Peptides                  | Amino acid sequence                      | Average Antimicrobial activity (μM) against | Reference               |
|--------------------------|------------------------------------------|---------------------------------------------|-------------------------|
|                          |                                          | G<sup>+</sup> pathogens | G<sup>-</sup> pathogens |                          |
| CAP 11 (Monomer)         | GLRKKFRKTRKRLKKGTQGRKVRKAWKREYQQPVPPCRI | -                                           | 0.1                     | Okuda et al. 2006       |
| CAP 11                   | GLRKKFRKTRKRLKKGTQGRKVRKAWKREYQQPVPPCRI | 0.09                                       | 0.09                    | Yonogida et al. 1996    |
| Cynthaurin               | ILQGRKVLGLKAGGSLHRKKAAATGYKTKKLGRKIGKTGRKVWKAWREYGQIPYPCR | N/A                                         | N/A                     | Lee et al. 2001         |
| Cynthaurin Distinctin A-A homodimer | NLVSGLRKKFRKTRKRLKKGTQGRKVRKAWREYGQIPYPCR | 23.4                                       | 35                      | Batista et al. 2001     |
|                         | Chain A                                   |                                             |                         | Dalla Serra et al. 2008 |
|                         | Chain B                                   |                                             |                         |                         |
|                         | Halocidin                                 |                                             |                         |                         |
|                         | di-18Hc                                   |                                             |                         |                         |
|                         | di-15Hc                                   |                                             |                         |                         |
|                         | Defr1                                     |                                             |                         |                         |
|                         | CRS4C-1a dimer                            |                                             |                         |                         |
|                         | CRS4C-1a monomer                          |                                             |                         |                         |
|                         | bis-LLP1                                  |                                             |                         |                         |
|                         | LLD1                                     |                                             |                         |                         |
|                         | Analogue 5                               |                                             |                         |                         |
|                         | bis-magG3C                                |                                             |                         |                         |
|                         | magG3C                                   |                                             |                         |                         |
|                         | bis-TL1                                  |                                             |                         |                         |
|                         | TL1                                      |                                             |                         |                         |
|                         | Magamin (F12W, N22C) dimer                |                                             |                         |                         |
|                         | Sushi (S3)                               |                                             |                         |                         |
|                         | S3-C27S                                  |                                             |                         |                         |
|                         | Bactenecin                               |                                             |                         |                         |
|                         | Parallel dimer                           |                                             |                         |                         |
|                         | Antiparallel dimer                        |                                             |                         |                         |
|                         | PST 13-RK                                |                                             |                         |                         |
|                         | Tat(W)                                   |                                             |                         |                         |
|                         | di-Tat(W)-C                              |                                             |                         |                         |

The position and disulphide bond connectivity is shown at the intermolecular disulphide bond (C–C) between two monomers sequences. The homo-/hetero-dimers with intermolecular disulfide bond are shown in rows below with linkage of the intermolecular disulfide bond (C–C) (are shaded in grey) for the corresponding sequences. The naturally occurring homo/heterodimers are highlighted in bold. (Color figure online) N/A<sup>a</sup>: MIC data not available.
E. Coli and B. cepacia) at a concentration of 50 μg/ml. The antimicrobial activity of Defr1 against S. aureus, E. Coli, and B. cepacia was observed to be reduced in NaCl concentrations of 30–150 mM, but the activity against P. aeruginosa was independent of salt concentration. Defr1 was found to have potent activity against P. aeruginosa, killing 90% of bacteria (LD$_{90}$) at 10 μg/ml (Morrison et al. 2002). Subsequently, the same group reported structure–activity relationships in defensin dimers of ß-defensin related peptides (Defr1). The first description of a covalently inter-molecular disulfide cross-linked dimer of the defensin family provided further evidence that the potent antimicrobial activity of a defensin is linked to its ability to form stable higher order structures, which are multivalent AMPS (Campopiano et al. 2004). A six cysteine variant peptide Defr1 Y5C of Defr1 was synthesized and its antimicrobial activity was significantly lower than Defr1 (Tables 1 and 2). Mass spectrometry analysis indicated that 34-residue Defr1 formed a covalent dimer as a mixture of intermolecular disulfide-linked dimer isoforms whereas Defr1 Y5C existed as its monomer with three pairs of intra-molecular disulfide bonds due to the lack of an odd cysteine residue. In contrast, Defr1 dimers were covalently stable and were only dissociated into monomers by the addition of reducing agent. The striking difference in the antimicrobial activity was attributed to the formation of a stable covalent dimer of Defr1 through intermolecular disulfide bonds. The oxidized Defr1 Y5C, its reduced form and the reduced form of Defr1 have similar/comparable antimicrobial activity due to their very close similarity in sequence of monomers and being in their monomeric forms; however, the oxidized Defr1 is a mixture of dimeric isoforms with two pairs of intra-molecular disulfide bonds and one pair of intermolecular disulfide bond and had significantly higher activity than its reduced form (monomeric form), Defr1 Y5C (in its the oxidized form) and the reduced form of Defr1 5YC (Tables 1 and 2). Furthermore, Defr1 (in its covalently dimeric form) almost maintained activity against Pseudomonasa aeruginosa in the presence of 25, 50 and 150 mM NaCl and it displayed 100% killing at 25, 50 mM NaCl, and 84% killing at 150 mM NaCl, and 25% killing even at 300 mM NaCl whereas Defr1 5YC (in its monomeric form) was almost inactive. The activity of Defr1 Y5C was extremely salt-sensitive, killing only 10% of 1 × 10$^5$ bacteria in the presence of 25 mM NaCl and being completely inactive at 50 mM NaCl. The striking difference in antimicrobial activity and salt-insensitivity of the covalent dimer Defr1 compared to Defr1 5YC (the monomeric form) highlighted the unique contributions of the covalent dimerization.

Inspired by the synergism when two putative peptides are combined through a disulfide bond, a number of homodimers have been synthesized. Tencza et al. (1999) synthesized three homodimers with intermolecular disulfide bonds derived from Lentivirus Lytic peptides (LLP1, Table 1). In addition, these authors also synthesized a magainin analogue (mag G3C) as a monomer and the disulfide bonded homodimer (Tencza et al., 1999). The monomers and their corresponding homodimers have comparable antimicrobial activity against P. aeruginosa. However, the dimers are more potent against S. aureus than their corresponding monomers. In addition, the disulphide-bonded dimers have potent bactericidal activity against methicillin-resistant S. aureus and vancomycin-resistant E. faecalis.

Dempsey et al. (2003), reported the synthesis of another disulfide-dimerized magainin analogue (mag N22C) by air oxidation. The antimicrobial activity of the monomer and the dimer were tested against Gram-negative pathogens. The homodimer displayed >4 fold increase in antimicrobial activity compared to the monomer. However, the dimer induced more haemolysis compared to the monomer. The increased antimicrobial activity of the dimer was attributed to the stronger affinity to the negatively charged membrane.

Yau et al. (2001), examined the antimicrobial properties of a 34-residue peptide derived from the 3rd domain of Factor C (S3). The peptide had potent antimicrobial activity (16 nM) against 30 clinical strains of P. aeruginosa. In addition, S3 maintained its antibacterial activity even at 300 mM NaCl. The work by Li et al. suggested the importance of an intermolecular disulfide bond in the antimicrobial S3 dimer for binding, disruption, and neutralization of lipopolysaccharide (LPS) (Li et al. 2004).

Recently, improved antimicrobial activity as well as salt-resistance has been demonstrated for parallel and anti-parallel homodimers derived from bactenecin (Lee et al. 2008). The parallel dimer exerted a two fold increase in

| Table 2 | Antimicrobial activity of Defr1 and Defr1 Y5C in the oxidized (dimeric form) and reduced forms (monomeric forms) (red) |
|---------|---------------------------------------------------------------|
| Organism | Strain | MBC$^a$ |
| Gram-negative | | |
| P. aeruginosa | PAO1 | 6 | 50 | 50 | 100 |
| E. coli | ATCC 25922 | 8 | 100 | >100 | 50 |
| Gram-positive | | |
| S. aureus | ATCC 25923 | 10 | >100 | >100 | >100 |
| E. faecalis | ATCC 29212 | 6 | 100 | >100 | ND$^b$ |
| Fungi | | |
| C. albicans | J2922 | 3 | 25 | 25 | 50 |

Data taken from Campopiano et al. (2004)
antimicrobial activity against Gram-positive bacteria whereas its activity was unaltered against Gram-negative bacteria compared to the monomeric bactenecin. However, the antiparallel dimer exerted even stronger activity than the monomer against Gram-positive and the Gram-negative pathogens. The activity of the monomer against S. aureus was completely lost and it retained only <40% activity against E. coli in 150 mM NaCl. The two dimers, on the other hand, retained their antimicrobial activity in the presence of 150 mM NaCl.

In an effort to understand the specificity, Yang et al. (2009), investigated the effect of dimerization of a tritrypticin analogue (PST 13-RK, KKKFPWWWPFKKK-NH₂) formed by intermolecular disulphide bond at the C-terminus position. Interestingly, the dimer had similar antimicrobial activity but increased toxicity to eukaryotic cells. More recently, Zhu and Shin (2009), have investigated the potency and stability of a disulfide-linked dimeric analogue of cell penetrating peptides Tat(W). In addition to the stronger antimicrobial activity against Gram-positive bacteria, the disulfide-linked dimer showed rapid bactericidal activity compared to the monomer. However, both the monomer and dimer were rapidly cleaved by trypsin.

It can be concluded that intermolecular disulfide bonds confer the following properties that may enhance the therapeutic potential of antimicrobial peptides: (i) high potency i.e., ability to kill bacterial at lower concentrations than the monomer; (ii) high selectivity i.e., ability to kill bacteria with no or low eukaryotic toxicity; (iii) ability to kill bacteria more rapidly, thereby, controlling bacterial expansion; (iv) ability to retain the antimicrobial activity under high salt concentrations, and (v) enhanced resistance to proteolytic enzymes. Based on the studies mentioned above, dimerization of a less potent antimicrobial peptide increased the antimicrobial potency significantly whereas a marginal increase in the activity for an active sequence. The choice of sequence and position of disulphide bond plays an important role in determining the therapeutic potential of these peptides. However, the design and synthesis of homo- or hetero-dimers, tetravalent or other oligomeric multivalent forms of an AMP based on intermolecular disulfide linked multimers is limited by the complexity of the oxidation products (e.g., a mixture of dimer isomers with different disulfide linked patterns), separation of oxidation products (Campopiano et al. 2004; Hornef et al. 2004; Wu et al. 2003) and one or two types of side products due to high oxidation levels involved in the synthesis (Liu et al. 2009). The concept of multivalent AMPs based on inter-molecular disulfide cross linked dimer for the increased antimicrobial activity and its anti-salt property was further supported and proved by Lys-based branched or polymeric form multivalent AMPs, which are discussed in the following sections.

### Synthetic Unnatural Multivalent AMPs

The unnatural-linked multivalent AMPs have different chemically structural considerations, depending on the choice of the scaffold or core molecule. The availability of a wide variety of scaffold or core molecules provide constructs that allows several copies of the peptides to be appended simultaneously (Fig. 1). Core molecules can be structurally diverse, ranging from amino acids (e.g., lysine or glutamic acid dendrimers) (Tam 1988; Sadler and Tam 2002), simple amino, carboxylic and hydroxyllic cores (Sadler and Tam 2002) or small organic molecule-based dendrimers (Arnusch et al. 2007), and even to polymeric chains. (Arimoto et al. 1999; Liu et al. 2006; Kolar et al. 2010; McDermott et al. 2010). In this section, we discuss three types of multivalent AMPs and detail their advantages over disulfide-linked dimers.

#### Design of Dendrimeric or Branched Multivalent AMPs based on α-Amino Acid Scaffolds

Sadler et al., published a comprehensive review of peptide dendrimers (Sadler and Tam 2002), which focused on the types of peptide dendrimers. Applications of peptide

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Schematic representation of dendrimeric/branched and polymeric multivalent peptides (a). The dendrimeric/branched core or scaffold molecules can be Lys or other molecules. One lys for divalent AMPs, three Lys for tetravalent AMPs and seven Lys for octavalent AMPs; (a–c) The core or scaffold may contain 2, 3, 4 or 8 connecting units that can be used to create divalent, trivalent, tetravalent and octavalent AMPs (a–c). d The peptide can be covalently linked to a polymerizable monomer or polymeric unit to produce peptide-polymer conjugates.
Dendrimers include use as biomedical diagnostic reagents, protein mimetics, anticancer and antiviral agents, vaccines and drug and gene delivery vehicles. The ε-amino group of the lysine and the carboxyl group of the glutamic acid have been used as a core for the synthesis of multivalent AMPs. Tam (1988) pioneered the synthesis and study of bioactive peptides containing a lysine core. ε- and ε-amino groups of lysine are used for the generation of two, four, and eight copies of short peptide sequences (Tam et al. 2002).

Dendrimeric/branched multivalent AMPs can be directly synthesized by solid phase peptide synthesis (SPPS) via the use of several double Fmoc Lys. (e.g., 1 Lys for dimer, 3 Lys for tetramer and 7 Lys for octamer) (Fig. 1a). The synthesis does not need any additional steps for synthesis of a scaffold or core molecule, which is used to link several copies of an AMP. This strategy has many advantages over the corresponding linear analogues such as stability against proteases (Tam et al. 2002; Bracci et al. 2002; Lozzi et al. 2003; Falciani et al. 2007; Young et al. 2009; Dewan et al. 2009) and lack of salt sensitivity, (Tam et al. 2002; Dewan et al. 2009) and the two important criteria that limit the therapeutic potential of AMPs. Pini et al. (2008) and Pieter et al. (2009) have partially reviewed some examples developed by this method, which focused on different biological functions and membrane permeabilization, respectively. Here we would like to summarize progress with this type of Lys-based dendrimeric or branched multivalent AMPs.

In their first report, Tam et al. (2002) examined the effects of increasing the number of branches and peptide sequences on antimicrobial and haemolytic activity. A four residue RLYR and an octapeptide RLYRKVYG sequences that represent the topological motifs of the tachyplesin-1 were appended to monomer, trimer, and heptamers to produce divalent, tetravalent, or octavalent peptides, respectively. (Figs. 1a, 2 compares the effect of increasing the repeating units of linear sequence/branch numbers on therapeutic index (defined as the effective concentration required to cause 50% haemolysis divided by geometric mean minimum inhibitory concentration). For linear peptides, a plateau is observed beyond a trimer for the linear series as well. However, for branched peptides the value increases exponentially with increasing branches.

The use of AMPs in vivo has been largely limited by their short half-lives, since peptides are susceptible to endogenous proteases and peptidases. In an attempt to compare the stability of linear and branched peptides, Tam et al. examined the antimicrobial activities of these peptides in the presence of trypsin (Tam et al. 2002). The linear peptide lost their antimicrobial activity within the first few minutes after digestion whereas the tetravalent RLYR retained ~80% antimicrobial activity even after 24 h. Similar results were obtained for the tetravalent and octavalent forms of RLYRKVYG (~65% activity after 24 h). Thus, branching improves the proteolytic resistance, the shorter sequence being more resistant compared to the longer one.

Kallenbach’s group has conducted an extensive study with a branched tetramer (RW) 4D [(RW)4K2K] in which four copies of sequence (RW) were linked to a Lys scaffold (Fig. 3) (Liu et al. 2007; Hou et al. 2009; Young et al. 2009). In a demonstration, Liu et al. (2007), examined the effects of increasing the repeat units for a dipeptide (RW) on antimicrobial and haemolytic activity. Similar to the results obtained by Tam et al. a plateau was observed beyond a trimer for the linear series as well. However,
(RW)_{4D} had about 10 fold increase in therapeutic index compared to the linear tetramer. These studies suggested that increasing the number of repeats through branching amplifies the antimicrobial properties without affecting the toxicity. In addition, the tetramer (RW)_{4D} inhibits bacterial growth in both planktonic and biofilm states. Live/Dead cell assays revealed that most bacteria in a preformed biofilm lose viability after treatment with this peptide and this result is in marked contrast to the existing report that antimicrobial peptides are ineffective against mature bacterial biofilms (Hou et al. 2009).

Tetramer (RW)_{4D} displays enhanced activity against multi-drug resistant (MRD) bacteria and shows protease stability. Young et al. used similar assay and compared the stability of tetramer (RW)_{4D} with indolicidin (Young et al. 2009). After indolicidin was treated with 100 nM trypsin for 1 h or 1 mM trypsin for 2–5 min, about 25–30% reduction in the antimicrobial activity was observed for indolicidin peptide whereas the tetramer did not lose the activity even after 24 h. By contrast, the antibacterial activity of (RW)_{4D} was unaltered even at high trypsin concentration (up to 1 mM) over various time intervals (up to 24 h) (Young et al. 2009).

Our group has recently reported the structural parameters for antimicrobial activity, human epithelial cell cytotoxicity and killing mechanism of synthetic monomers and branched dimer derived from the C-terminus region of hBD3 (e.g., V2-monomer: RGRKVVRRKK) and V2-dimer derived from the C-terminus region to toxic and killing mechanism of synthetic monomers and branched dimer derived from the C-terminus region (Zhou et al. 2010). The branched V2-dimer showed potent broad-spectrum antimicrobial activity whereas the V2-monomer was only active against P. aeruginosa. V2-dimer showed higher antimicrobial activity against P. aeruginosa ATCC 9027 (Log Reduction: 2.82; almost equals to 99.9% bacteria being killed at 2.74 μM) compared to V2-monomer (Log Reduction: 2.02, equals to 99% bacteria being killed at 4.8 μM). Using fluorescence correlation spectroscopy (FCS), significant aggregation and some leakage of rhodamine dye were observed with anionic lipids POPG with V2-monomer at the concentration of 10 μM and no significant aggregation or disruption of vesicles was observed for V2-monomer and V2-dimer tested against POPC. V2-dimer induced more leakage and aggregation than the monomeric form. V2-dimer is the most effective antimicrobial peptide, with aggregation of POPG vesicles observed at concentrations as low as 1 μM.

In a systematic study on the effect of covalent dimerization on antimicrobial activity, Dewan et al. reported antimicrobial action of prototypic amphiphilic cationic decapeptides (Ac-G-X-R-K-X-H-K-X-W-A-NH2, X was didehydrophenylalanine for monomer ΔFm, γ-aminobutyric acid for Um, or phenylalanine for Fm) and their branched dimers (Dewan et al. 2009) (Table 3). Among these monomers, only ΔFm was modestly anti microbial (MICs of 110 μM (E.coli) and 450 μM (S. aureus). Interestingly, a linear dimer of ΔFm, viz. (ΔFm)2 turned out to be highly potent against E. coli (MICs of 2 μM and minimum bactericidal concentration (MBC) of 2 μM) and modestly potent against S. aureus (MICs of 20 μM and MBC of 20 μM). In contrast, the branched dimer of phenylalanine (Fd) and didehydrophenylalanine (ΔFd) showed a strong increase in potency, the latter was found to be a potent antimicrobial against both E. coli (MIC of 2.5 μM) and S. aureus (MIC of 5 μM) and had about 9 fold increase in anti S. aureus activity compared to the Fd substitution. In addition, ΔFd exhibited highest affinity for LPS, rapid bacterial killing, no haemolytic activity and excellent proteolytic resistance against trypsin. Thus studies with branched dimers of Fm and Um have indicated that covalent dimerization represents a scaffold for potentiation of antimicrobial peptides. Furthermore, these authors also examined the proteolytic stability of the peptides in the monomeric form (i.e., ΔF, F, and U) in the presence of cytosolic extracts, trypsin, and chymotrypsin. The phenylalanine and γ-aminobutyric acid analogues were degraded readily whereas the didehydrophenylalanine remained intact. Thus, resistance against proteolysis in branched peptides could be enhanced by appropriate substitution of unnatural amino acids.

An elegant demonstration of the structure–activity relationship of branched peptides has been reported by Pini et al. (2005). These authors assembled a 10 residue peptide QKKIRVRLSA on a tetrabranched lysine scaffold (M6) and examined the antimicrobial activity. M6 displayed potent antimicrobial activity against several Gram-negative pathogens but showed poor activity against Gram-positive pathogens. To assess the activity spectrum, alanine scanning was performed wherein each residue in the QKKIRVRLSA sequence was replaced with an alanine residue and analyzed for antimicrobial activity (Fig. 4). The results indicated that K2A, I4A, V6A, and L8A substitutions lead to a profound decrease (>15 fold reduction) in the antimicrobial activity whereas other residues affected the activity marginally suggesting the importance of bulky hydrophobic side chains in the branched peptides. To investigate the lysine-arginine disparity, these authors replaced each of the lysine 2 and 3 with arginine. While K2R replacement increases the activity slightly K3R and the double replacement (i.e. K2R, K3R) lead to 8 or 9 fold decrease in antimicrobial activity (Table 2). Furthermore, shortening the sequence by removing the Q1 resulted in 3 fold decrease in the antimicrobial activity. These results clearly showed that M6 was optimized for antimicrobial activity against Gram-negative bacteria.

Recently, two independent studies compared the antimicrobial properties of a branched dimer and an intermolecular
Table 3 Antimicrobial properties of branched peptides containing lysine and glutamic acid core

| Peptide | Sequence | Average Antimicrobial activity (μM) | Reference |
|---------|----------|-------------------------------------|-----------|
|         |          | G+       | G-       |          |           |
| R4      | RLYR<sup>a</sup> | >500     | >500     | Tam et al. 2002 |
| (R4)<sub>2</sub> | RLYRR<sup>a</sup>LYR<sup>a</sup> | 3.5      | 16.9     |          |
| (R4)<sub>4</sub> | (RLYR)<sub>2</sub>R <sup>a</sup> | 1.1      | 14.4     |          |
| D<sub>2</sub>R4 | (RLYR)<sub>4</sub>K<sup>a</sup> | 4.4      | 4.2      |          |
| D<sub>4</sub>R4 | (RLYR)<sub>4</sub>[K<sub>2</sub>K]<sup>a</sup> | 0.7      | 0.83     |          |
| R8      | RLYR<sup>a</sup>VYGR<sup>a</sup> | 37.3     | 51.6     |          |
| D<sub>2</sub>R8 | (RLYR)<sub>2</sub>VYGR<sup>a</sup> | 3.5      | 16.9     |          |
| D<sub>4</sub>R8 | (RLYR)<sub>4</sub>[K<sub>2</sub>K]<sup>a</sup> | 0.7      | 0.83     |          |
| D<sub>8</sub>R8 | (RLYR)<sub>8</sub>[K<sub>2</sub>K]<sup>a</sup> | 0.6      | 0.5      |          |
| M5      | [KIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | not active | 10.1 | Liu et al. 2007 |
| M6      | [QKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | not active | 0.8   | Pini et al. 2005 |
| M31     | [AKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 3.3      |          |          |
| M32/M4  | [QAKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 16.3     |          |          |
| M33     | [QKATIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 1.6      |          |          |
| M34     | [QKKARVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 12.9     |          |          |
| M35     | [QKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 4.2      |          |          |
| M36     | [QKKIRALRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 12.8     |          |          |
| M37     | [QKKIRVALSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 3.3      |          |          |
| M38     | [QKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | >12.9    |          |          |
| M39     | [QKKIRVRLA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 3.6      |          |          |
| M28     | [QKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 0.6      |          |          |
| M29     | [QKKIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 6.1      |          |          |
| M30     | [QKRIRVRLSA]<sub>4</sub>K<sub>2</sub>K<sup>a</sup> | 7.5      |          |          |
| PST13-RK | KKKFPFWWPFFKK<sub>4</sub>-NH<sub>2</sub> | 3.5      | 2.2      | Yang et al. 2009 |
| di-PST13-RK-K | KKKFPFWPFK<sub>4</sub>-K<sub>2</sub>K<sup>a</sup> | 2.7      | 2.1      |          |
| Tat(W)  | GRKKRRQRRRPQ<sub>4</sub>-NH<sub>2</sub> | 2.0      | 2.0      | Zhu and Shin 2009 |
| di-Tat(W)-K | GRKKRRQRRRPWQ<sub>4</sub>-K<sub>2</sub>K<sup>a</sup> | 1.0      | 1.0      |          |
| Fm      | AcGFRKFKFWA<sub>4</sub>-NH<sub>2</sub> | >500     | >500     | Dewan et al. 2009 |
| Um      | AcGFRKHKUWA<sub>4</sub>-NH<sub>2</sub> | >500     | >500     |          |
| ΔFm     | AcΔFRKΔFHΔFWA<sub>4</sub>-NH<sub>2</sub> | 450      | 110      |          |
| (ΔF<sub>m</sub>)<sub>4</sub> | AcΔFRKΔFHΔFWAAPQΔFRKΔFHΔFWA<sub>4</sub>-NH<sub>2</sub> | 20      | 2        |          |
| Fd      | Ac (GFRKFKFWA) <sub>4</sub>K<sup>a</sup> | 45      | 3        | Khrushcev et al. 2007 |
| ΔFd     | Ac (ΔFRKΔFHΔFWA) <sub>4</sub>K<sup>a</sup> | 5       | 2.5      |          |
| Ud      | Ac (GFRKHUKUWA) <sub>4</sub>K<sup>a</sup> | 30      | >100     |          |
| RlAR    | RLYR<sup>a</sup> | not active | 250     | Khrushcev et al. 2007 |
| ΔE(RlAR)<sub>4</sub> | not active | 12      |          |          |
| Klar    | not active | 15      |          |          |
| ΔE(Klar)<sub>4</sub> | not active |          |          |          |

The branched K/E is highlighted in represented in italics. The branched peptides are highlighted in rows below (in grey) whereas the linear peptides are highlighted in rows above for the corresponding sequences

<sup>a</sup> The antimicrobial activity was at low salt conditions
disulfide linked homomodimer. Yang et al. (2009) dimerized a 13 residue peptide, KKKFPWWWPFKKK by cysteine oxidation and incorporation of branched lysine at the C-terminus and compared their antimicrobial properties with the monomer. While neither of the dimers affected the antimicrobial activity significantly, the therapeutic index dropped considerably from 47 for the monomer to 2 for the dimers suggesting a loss in selectivity when the potent monomer was dimerized. Similar to these studies, Zhu and Shin (2009) compared the effects of dimerization of a cell penetrating Tat sequence GRKKRRQRRRPWQ, on antimicrobial activity. Membrane depolarization assays indicated that both the dimers killed S. aureus rapidly compared to the monomer. However, monomer, homodimer and branched dimer were rapidly cleaved by trypsin within a few minutes. The two examples showed the antimicrobial activity and stability of dimers was dependant on monomer sequences and the locations of dimerization in comparison with those of dimers mentioned above.

Khrushchev et al. (2007) reported the first example of glutamic acid based dendrimeric peptides and examined the antimicrobial properties. Divalent and tetravalent copies of four residue peptides KLAR or RLAR have been synthesized and their antimicrobial activity was examined against E. coli. A sharp increase in the anti E. coli activity was observed for both tetravalent peptides compared to the divalent analogues.

Based on these observations, lysine scaffold based multivalent AMPs confers several advantages over monomers, linear peptides or intermolecular disulphide-linked dimers. These include (i) ease of synthesis i.e., requires fewer steps than linear analogues and disulfide-linked dimer, (ii) multiple copies can be prepared which is difficult for the disulphide linked peptides, (iii) high potency under physiological salt concentration against broad spectrum of pathogens, (iv) significant retention of activity in the presence of proteolytic enzymes and cytosolic extracts.

Multivalent AMPs Formed with a Small Organic Molecule Scaffold or Core via “Click Chemistry”

Arnuschi et al. (2007) designed divalent, tetravalent and octavalent forms of magainin 2 by the use of alkyne substituted dendrimers based on 3,5-di-(2-aminoethoxy) benzoic acid repeating units as a core molecule via a copper-mediated 1,3 dipolar cycloaddition reaction named “click” chemistry. Figure 5 shows structures of dendritic core molecules (compounds 1–3) and the linkage spacer (structure 4) between peptides and dendritic core molecules. The dendritic core molecules are covalently linked to AMPs by a chemo- and regio-selective coupling of an alkyne moiety of dendritic core molecule to an azide-functionalized magainin 2 peptide, yielding a 1,3-disubstituted 1,2,3-triazole-linking spacer (structure 4) via click chemistry to prepare multivalent magainin 2 including divalent, tetravalent and octavalent compounds (Fig. 5). The authors synthesized multimers with 2, 4, and 8 copies of magainin 2 and examined the efficacy of these analogues in releasing carboxy fluorescein dye from model lipids. Predictably, the dye release potency greatly increased with number of copies. An enhancement of around 100 fold was obtained for the tetravalent compound, an enhanced membrane pore formation action of almost 600-fold was observed with octavalent magainin 2 (Tables 4 and 5). The antimicrobial activity of magainin 2 and the multivalent peptides were evaluated against B. subtilis. The divalent form of magainin 2 was about 5-times more potent than magainin 2 and the tetravalent analogue had the same activity as magainin 2 (Pieters et al. 2009). (When we calculated their relative potency, unit of MIC was converted from μg/ml into μM.) Thus, contrary to lysine core, the 3,5-di-(2-aminoethoxy) benzoic acid-based dendritic core molecules seemed to be less efficient in amplifying the antimicrobial properties.

Multivalent AMPs Formed by Attaching Peptides to Reactive Polymer Scaffolds

The principles of multivalency not only can be applied by attaching several copies of AMPs (less than ten) to a small molecule scaffold or core (already shown above), but also can be applied to attaching several copies or even many copies (several tens or even hundreds) of AMPs to an active biopolymer and synthetic polymer with several or numerous repeating units. Hjørringgaard et al. (2009)
reported synthesis of templated multimers of AMPs, in which six copies of antimicrobial peptide alamethicin were covalently bound to cyclodextrin scaffolds via “click chemistry”). Cyclodextrins (CDs) are a family of compounds made up of sugar molecules bound together in a ring (cyclic oligosaccharides, including α-, β- and γ-CD with six, seven and eight sugar units, respectively), a family of naturally occurring biopolymer, which are

Table 4 Leakage results for 5(6)-carboxyfluorescein from mixed DOPC/DOPG vesicles (25 µM, 1/1)

| Compound      | L₅₀ (nM) | Relative potency (per peptide) | Molecular mass (kDa) | Real potency (µg/ml) |
|---------------|----------|--------------------------------|----------------------|---------------------|
| Octavalent    | 14       | 107 (13.4)                     | 22.5                 | 0.31                |
| Tetravalent   | 130      | 11.5 (2.9)                     | 11.2                 | 1.49                |
| Divalent      | 230      | 6.5 (3.25)                     | 5.48                 | 1.26                |
| Divalent      | 250      | 6 (3)                          | 5.48                 | 1.37                |
| Magainin      | 1500     | 1 (1)                          | 2.47                 | 3.7                 |

Data taken from Arnusch et al. (2007)

Table 5 Leakage results for 5(6)-carboxyfluorescein from DOPC vesicles (25 µM)

| Compound      | L₅₀ (nM) | Relative potency (per peptide) | Molecular mass (kDa) | Real potency (µg/ml) |
|---------------|----------|--------------------------------|----------------------|---------------------|
| Octavalent    | 17       | 255 (73)                       | 22.5                 | 0.38                |
| Tetravalent   | 22       | 454 (113)                      | 11.2                 | 0.25                |
| Divalent      | 250      | 40 (20)                        | 5.48                 | 1.37                |
| Divalent      | 370      | 27 (13.5)                      | 5.48                 | 2.03                |
| Magainin      | 10000    | 1                              | 2.47                 | 24.7                |

Data taken from Arnusch et al. (2007)
produced from starch by means of enzymatic conversion. Alamethicin represented one of the antimicrobial peptides, constructed of 20 residues of which eight were α-amino-isobutyric acids, Aib. Alamethicin inserts into lipid bilayers via the N-terminus, aggregates and forms voltage-gate channels, which accounts for its antibiotic activity. CD has been functionalized with azide groups. Alamethicin has been synthesized using automated solid phase peptide synthesis. A linker containing the alkyne group has been incorporated into the peptide. Then the multimers of antimicrobial peptide alamethicin were synthesized by coupling of an alkyne group of peptide to the azide groups of CD scaffolds to form multivalent form of alamethicin. These multivalent AMPs can be used as model artificial channels and will be compared to the monomeric species in electrophysiological experiments as well as labeling experiments using NMR spectroscopy, to probe the structure and function of these channels. Furthermore, the structures of the artificial channels can be examined using NMR in membrane environments.

The emergence of vancomycin-resistant bacteria (e.g., vancomycin-resistant *Enterococcus* (VRE); vancomycin-resistant *S. aureus* (VRSA)) is of concern clinically and in hospitals, because vancomycin is the last resort for the treatment nosocomial infections due to multidrug-resistant gram-positive bacteria. The hypothesis is that noncovalent associated dimer of vancomycin plays an important role in the recognition events of the lipid intermediates. This hypothesis promoted synthetic chemists to design covalently-linked dimer or multivalent or polymeric forms of vancomycin (Arimoto et al. 1999; Lu et al. 2007). Arimoto et al., reported that the first multivalent polymer of vancomycin greatly enhanced its antimicrobial activity against vancomycin-resistant *Enterococci* (VRE, VanA and VanB) (Arimoto et al. 1999; Li and Xu 2005). Polymeric forms of vancomycin (methanol) show the enhancement 8-63 fold in potency against VRE with retention of practical MIC values against *S. aureus* and *Enterococci* (Fig. 6; Table 6). Based on the design principle of poly/multivalent interactions between receptors and ligands, this was a pioneering method to produce multivalent forms of antibiotics (e.g., glycopeptide vancomycin) to overcome the drug resistance against VRE. It has been shown that the choice of the linker structure significantly affects the antimicrobial profiles of multivalent vancomycin. Subsequently, the same group designed rigidly-linked vancomycin dimers by the use of actinocin, a natural previleged scaffolds as linkers of vancomycin dimers, the in vitro and in vivo data displayed that the dimers exhibited excellent antimicrobial activities in the murine infection model and improved their in vivo efficiency against resistant bacteria (Lu et al. 2007).

In another example, Kallenbach’s group designed polymeric forms of cationic antimicrobial peptide. The design of polymer-AMPs conjugates were based on the action mechanism of AMPs (e.g., the formation of transmembrane pores) by the application of the design principle of multivalency. The authors reported that 4-residue peptides (RWRW and RRWW) were linked to the biocompatible reactive poly(maleic anhydride) (PMA) to yield polymeric peptides containing 39–40 monomeric units (Liu et al. 2006) (Figure 7). Relative to the free peptides, the polymer-peptide conjugates show a 15- or 10-fold improvement in IC₅₀ against *E. coli* and *B. subtilis*

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**Table 6** Antibacterial activities (MIC, µg/ml) of the monomeric vancomycin and polymeric vancomycin

| Compound            | *S. aureus* | *Enterococci* | VRE (VanA) | VRE (VanB) |
|---------------------|-------------|---------------|------------|------------|
| Vancomycin          | 0.2         | <0.5          | >250       | 125        |
| Polymeric Van       | NA          | 31            | >250       | 31         |
| (emulsion condition)|             |               |            |            |
| Polymeric Van       | 2.3         | 2             | 31         | 2          |

Data taken from Arimoto et al. 1999 and Li and Xu 2005

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**Fig. 6** The structures of polymeric form of vancomycin. Redrawn structure from Li and Xu (2005)

**Fig. 7** Structure of polymeric AMPs based on 4-residue peptides (RWRW and RRWW) and a polymaleic anhydride (PMA) chain. Redrawn structure from Liu et al. (2006)
(Table 7) without provoking more severe hemolysis of red blood cells. This clearly highlights multivalency or polyvalency as a useful route to enhance the activity of antimicrobial peptides with high therapeutic potential for treating microbial or viral infection (Malamud et al. 2007).

In a conference report, the method of click chemistry was applied by Cai and McDermott et al. to covalently conjugate cathelicidin derivatives (LL-37 or LL-25) to polymerized-liposomes (polydiacetylene, PDA) or fluororous polymer surfaces, a 100 fold antimicrobial activity was achieved with low cytotoxicity (Kolar et al. 2010; McDermott et al. 2010).

**Concluding Remarks**

Multivalent forms of antimicrobial peptides or other type of antibiotics (e.g., glycopeptide vancomycin), have been discussed, in which several or decades of copies of peptides were attached to a series of structurally diverse scaffold or core molecules. One of the advantages for application of design principle of multivalency lies in the structural diversity of many types of scaffolds molecules, the control and the tuning the multivalency. It has been clearly shown that the choice of scaffold molecules or the linker structures and the multivalency significantly affects the antimicrobial profiles of multivalent AMPs. There is considerable evidence that covalent dimerization or multimerization/polymerization of antimicrobial peptides provides advantages as a strategy to overcome the challenges of AMPs, such as drug resistance, killing ability, cytotoxicity and stability) to move them toward potentially clinical applications. However, the quantitative structure–activity relationship (QSAR) of multivalent AMPs and the physical parameters of these multivalent AMPs for the improvement of their biological functions are still poorly understood on a molecular level. The design strategy of multivalency was proved as a useful and practical route to greatly enhance the activity of antimicrobial peptides with high therapeutic potential for treating microbial or viral infection.

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**Table 7** Summary of bioassay results with polymeric peptides and the monomeric peptides

| Compound       | Monomer content | IC50, μg/ml E. Coli | HD50, ng/ml red blood cell E. Coli | Hemolytic index (HI) | HD50, ng/ml red blood cell B. Subtilis | Hemolytic index (HI) B. Subtilis |
|----------------|-----------------|---------------------|-----------------------------------|----------------------|----------------------------------------|---------------------------------|
| Ac-RWRW-NH2    | 1               | 510                 | 142                               | 1850                 | 3.6                                    | 13.0                            |
| Ac-RRWW-NH2    | 1               | 586                 | 131                               | 2280                 | 3.9                                    | 17.4                            |
| PMA-RWRW       | 39              | 33                  | 10                                | 128                  | 3.9                                    | 12.8                            |
| PMA-RRWW       | 40              | 39                  | 12                                | 135                  | 3.5                                    | 11.2                            |
| PMA Control    | 40              | NAa                 | NAa                               | >1000                | NA                                     | NA                              |

*No killing was detected. Data taken from Liu et al. (2006)
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