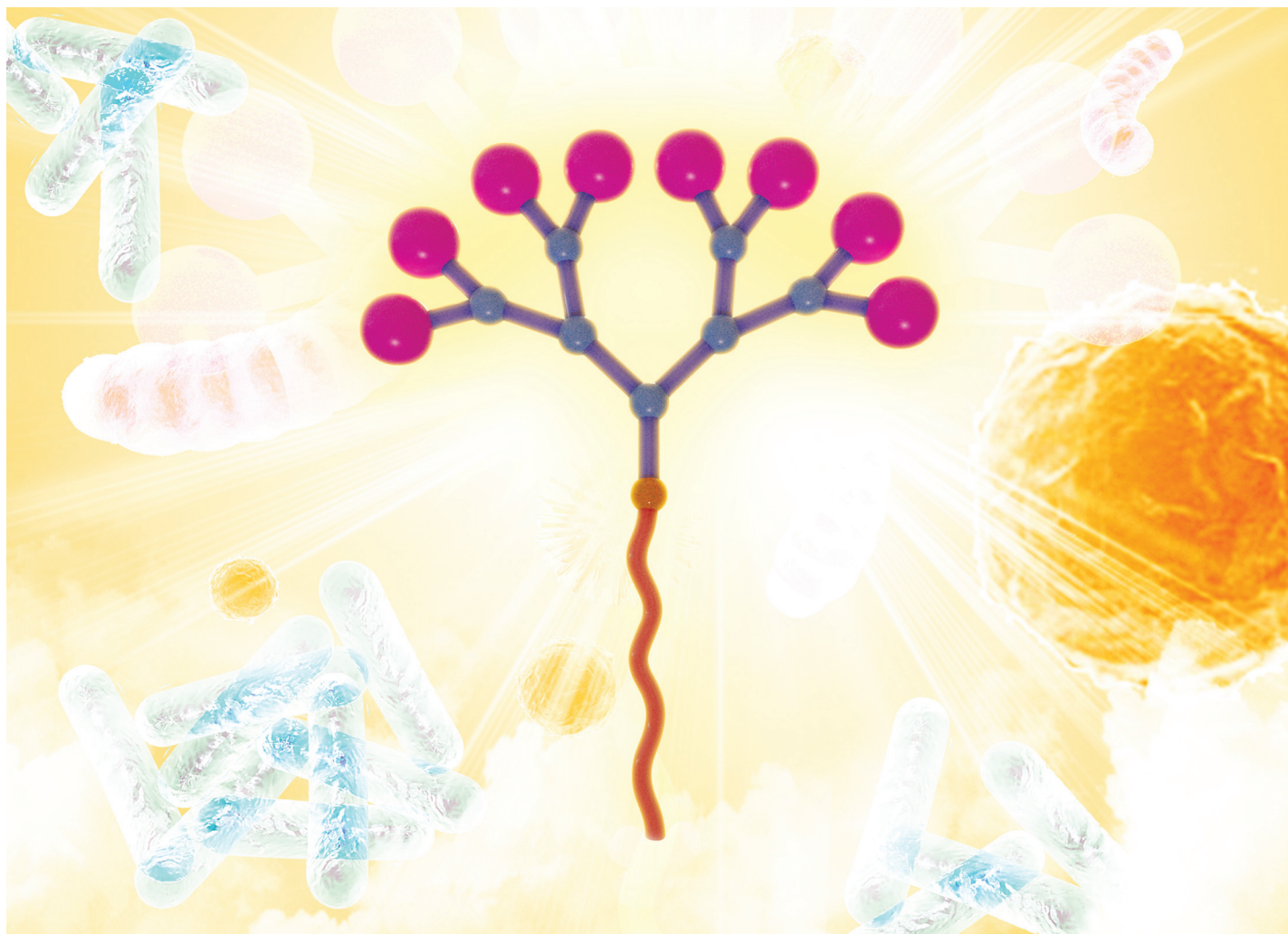


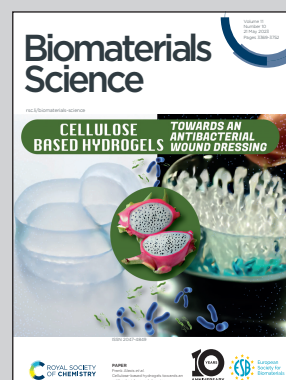


**Showcasing research from Dr Ling PENG's team at Centre Interdisciplinaire de Nanoscience de Marseille, Aix-Marseille University, CNRS, France.**

**Amphiphilic dendrimers against antibiotic resistance: light at the end of the tunnel?**

Amphiphilic dendrimers are becoming a promising solution for antibiotic resistance. These innovative paradigms mimic antimicrobial peptides to achieve strong antibacterial activity while possessing robust stability and a low risk of resistance. The key to their success lies in their exquisite amphiphilicity, which enables them to effectively target bacteria while minimizing harmful effects. By carefully adjusting the dendrimer chemistry, the hydrophobicity-hydrophilicity balance of amphiphilic dendrimers can be precisely fine-tuned for optimal performance in achieving excellent antibacterial activity and selectivity.

**As featured in:**



See Dinesh Dhumal, Ling Peng *et al.*, *Biomater. Sci.*, 2023, 11, 3379.

## MINIREVIEW

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## Amphiphilic dendrimers against antibiotic resistance: light at the end of the tunnel?

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With the alarming and prevailing antimicrobial resistance (AMR) comes an urgent need for novel antimicrobial agents that are not only effective and robust but also do not induce resistance development. Amphiphilic dendrimers are emerging as a promising new paradigm to combat bacterial AMR. They can mimic antimicrobial peptides to produce potent antibacterial activity yet with a low likelihood of generating resistance. In addition, they are stable against enzymatic degradation thanks to their unique dendritic architecture. Importantly, these amphiphilic dendrimers are composed of distinct hydrophobic and hydrophilic entities bearing dendritic structures, which can be precisely designed and synthesized to optimize the hydrophobic–hydrophilic balance yielding potent antibacterial activity while minimizing adverse effects and drug resistance. In this short review, we present the challenges and current state of research in developing amphiphilic dendrimers as new antibiotic substitutes. We start with a brief overview of the advantages and opportunities associated with using amphiphilic dendrimers to combat bacterial AMR. We then outline the specific considerations and the mechanisms underlying the antibacterial activity of amphiphilic dendrimers. We focus on the importance of the amphiphilic nature of a dendrimer that balances hydrophobicity and hydrophilicity via gauging the hydrophobic entity and the dendrimer generation, branching unit, terminal group and charge to allow high antibacterial potency and selectivity while minimizing toxicity. Finally, we present the future challenges and perspectives for amphiphilic dendrimers as antibacterial candidates for combating AMR.

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Antimicrobial resistance (AMR) is an urgent global health crisis. In 2019, an estimated 4.95 million deaths were associated with bacterial AMR, a figure likely to exceed 10 million

per year by 2050, surpassing cancer-related deaths.<sup>1,2</sup> In addition, AMR not only affects humans but also animals and the environment.<sup>3,4</sup> The emergence of AMR is a combined product of bacterial evolution alongside immense selective pressure caused by the inappropriate use of antibiotics.<sup>5</sup> Importantly, multidrug-resistant bacteria frequently accumulate genes that contribute to the development of resistance

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against multiple antibiotics, leaving clinicians with limited or no therapeutic choices. Pathogens of particular importance include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species that are collectively described by the acronym “ESKAPE”.<sup>6,7</sup> These pathogens are related to high mortality risk infections and can evade the biocidal activity of both conventional and new-generation antibiotics. Therefore, novel antibacterial agents are urgently required to fight against bacterial AMR.

The development of new antibiotics has, however, halted in recent decades, largely because of difficulties related to scientific, regulatory, and economic concerns.<sup>8–10</sup> Most antimicrobial agents used today were discovered in the 1950s, and even the latest “novel” antibacterial drug class, the lipopeptides such as vancomycin, has been available for routine use since the end of the 20th century.<sup>11</sup> Alternative strategies to combat AMR include the use of antibiotics in combination with other antibiotics or adjuvants and the use of antibacterial therapies that utilize bacteriophages, photodynamic treatments, antibodies, antimicrobial peptides (AMPs), nucleic acid therapeutics, and nanoparticles.<sup>12–19</sup>

Among these different approaches, AMPs are particularly appealing because they possess unique properties that enable a broad spectrum of antibacterial activity while not generating resistance.<sup>14,19</sup> AMPs are amphiphilic, combining cationic surface charges and hydrophobic components. Their primary mechanism of action relies on the electrostatic interactions with the negatively charged bacterial surface through their positively charged amino acid residues. This is followed by subsequent penetration of their hydrophobic entity into the bacteria cell membrane to cause membrane disruption, and hence cell killing and bacterial death.<sup>12,20</sup> As the primary interaction of AMPs with bacterial membrane is not mediated through a specific target, the development of antimicrobial resistance is highly unlikely and inconsistent. In addition to their primary mechanism of action, AMPs may also exert anti-

bacterial activity *via* immunomodulatory mechanisms that neither harm host cells nor induce drug resistance.

Despite these promising features, AMPs have not yet progressed towards their clinical use. This is mainly due to their peptide composition leaving them open to inherent proteolytic instability and short half-lives with elusive antimicrobial potencies *in vivo*.<sup>19</sup> Efforts made to overcome these disadvantages and impart higher antibacterial potency, have led to AMP mimics offering improved efficacy and stability as well as less adverse effects as potential antibiotic substitutes. AMP mimics<sup>21</sup> include peptides bearing D-enantiomeric or  $\beta$ -amino acids, peptoids, and polymeric antimicrobials, and a unique family of compounds called dendrimers which are of particular current interest.<sup>22</sup> Dendrimers are composed of a core, repetitive branching units and terminal groups (Fig. 1A), and have a precise chemical structure, radial dendritic architecture, and multivalent cooperativity confined within a small volume.<sup>23</sup> Notably, the dendritic structural feature creates steric hindrance and impedes access to enzyme active sites, hence enhancing proteolytic stability.

Multiple attempts have been made to develop peptide dendrimers as potential mimics of natural and synthetic AMPs based on their close chemical and structural relationship.<sup>21,24</sup> However, while peptide dendrimers do demonstrate a greater stability than linear peptides, they still suffer from enzymatic degradation. Poly(amidoamine) (PAMAM) dendrimers, conceptualized in the later 70s as peptide mimics<sup>23,25</sup> have gained increasingly more interest as antibacterial candidates thanks to their high biocompatibility and resilience to proteolytic degradation.<sup>26</sup> High-generation PAMAM dendrimers are highly effective against various bacteria but also exhibit high cytotoxicity even at low concentrations.<sup>27–29</sup>

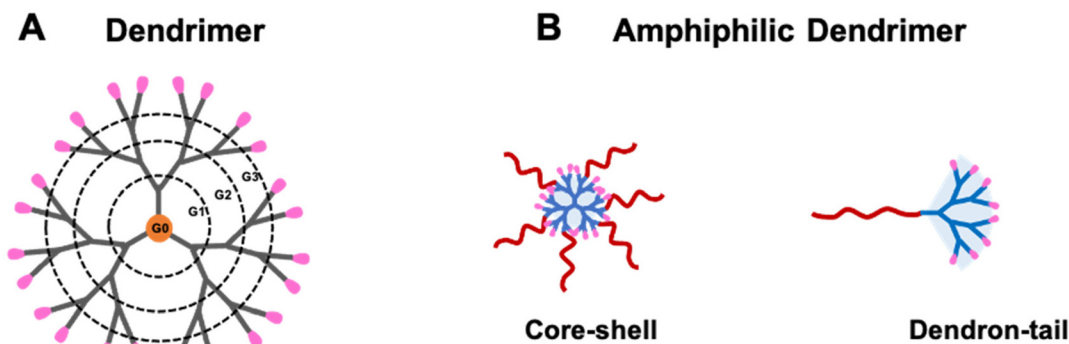
To overcome these limitations, small amphiphilic dendrimers have been conceived and synthesized as novel antibacterial candidates with the aim of further enhancing chemical and enzymatic stability and thereby improving antibacterial potency while reducing eventual toxicity. Amphiphilic dendrimers are composed of distinct hydrophobic and hydrophilic entities, and can be precisely designed and synthesized with core-shell or dendron-tail structure (Fig. 1B).<sup>30,31</sup> Specifically, amphiphilic dendrimers offer the inherent antimicrobial activity of detergent-like amphiphiles as well as the chemical and structural stability of dendrimers. In addition, their amphipathic nature enables these dendrimers to self-assemble into nanostructures, thus further improving their proteolytic stability and simultaneously allowing their accumulation at the infection site *via* the enhanced permeability and retention (EPR) effect.<sup>32</sup> The EPR effect refers to the specific enrichment of macromolecules or nanosized particles within tumor or inflammation lesions owing to the leaky vasculature and dysfunctional lymphatic drainage found specifically at these locations.<sup>33,34</sup> Although the EPR effect has been widely exploited for nanotechnology-based cancer treatment,<sup>35,36</sup> it was first discovered during studies of inflammation involving a bacterial infection where vascular permeability was found to be increased.<sup>37</sup> Similarly, infected tissue displays dysfunctional



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**Fig. 1** Cartoon illustrations of (A) a dendrimer and (B) amphiphilic dendrimers. (A) The dendrimer structure is composed of a central core, repeat branching units forming consecutive levels or generations (G1, G2 and G3) and terminal groups on the surface (shown in pink). (B) Amphiphilic dendrimers of core-shell and dendron-tail structures composed of distinct hydrophobic (red) and hydrophilic (blue) entities. Reproduced from ref. 30 with permission from ACS Publications, copyright 2023.

lymphatic drainage resulting from increased interstitial pressure and tissue destruction. Consequently, nanosystems are expected to exploit the EPR effect to preferentially deliver antibacterial agents to sites of infection, thereby improving therapeutic efficacy and reducing adverse effects<sup>32,38</sup> such as that achieved with nanotechnology-based cancer therapy.

In this short review, we first highlight the advantages and opportunities associated with amphiphilic dendrimers as antibacterial candidates to treat AMR using representative examples. We then outline the specific considerations and the mechanisms underlying the antibacterial activity of amphiphilic dendrimers. Importantly, the amphiphilicity and the hydrophobic-hydrophilic balance play critical roles in yielding amphiphilic dendrimers with high potency and selectivity towards resistant bacteria while minimizing cytotoxicity to host cells. We conclude by presenting our view of the future challenges and perspectives for amphiphilic dendrimers as antibacterial candidates.

## Amphiphilicity imparts and promotes antibacterial activity

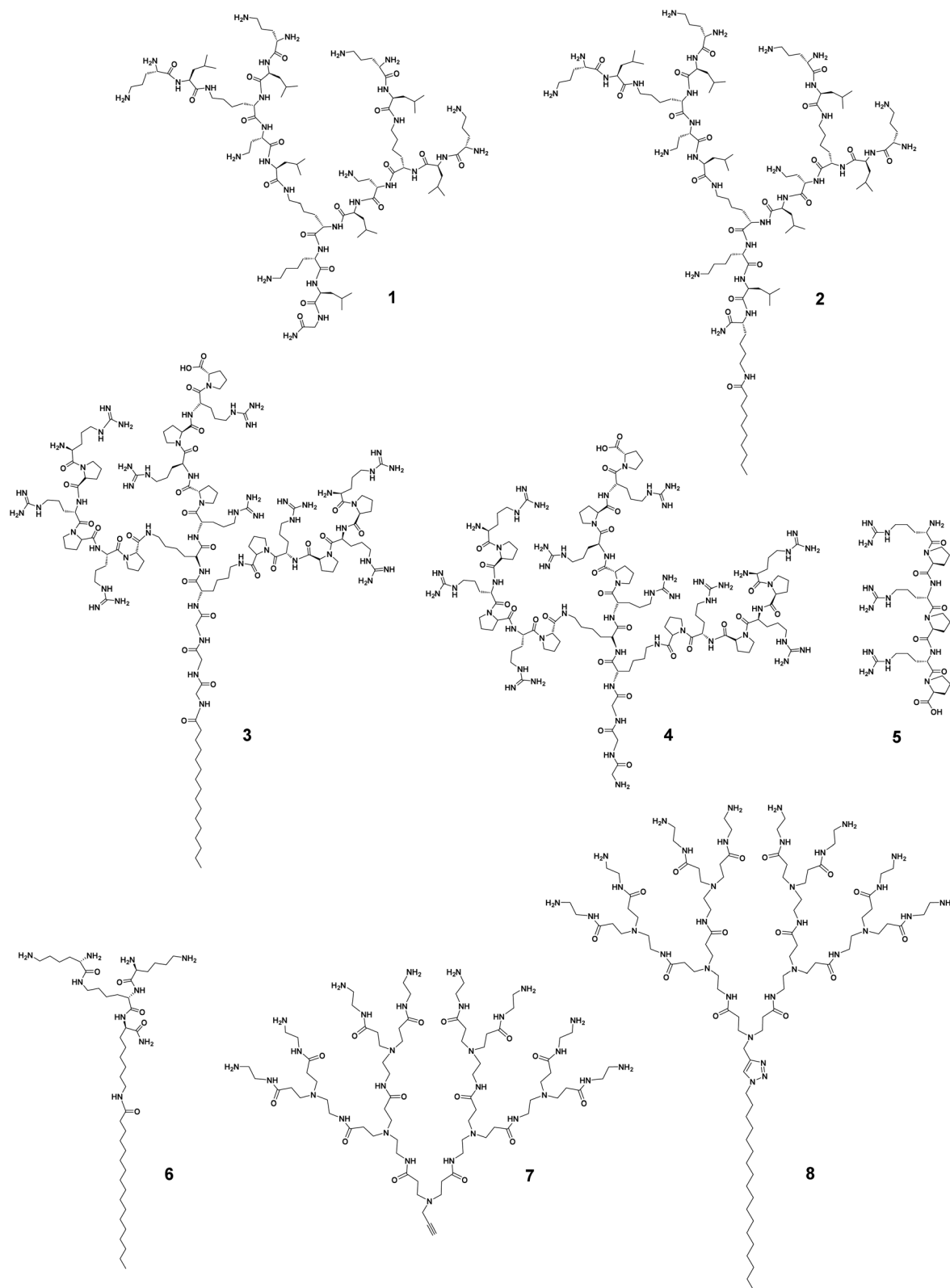
The antibacterial activity has been studied in many peptide dendrimers with a view to creating AMP mimics.<sup>22</sup> Prof. Jean-Louis Reymond and colleagues have worked intensively in this regard. Recently, they reported that imparting amphiphilicity to peptide dendrimers *via* the conjugation of a lipidic chain considerably increased the antibacterial activity against various drug-resistant bacteria.<sup>39</sup> Specifically, the peptide dendrimer of the second-generation composed of the repeating unit lysine-leucine, **1** (Fig. 2), was active against only *P. aeruginosa*. After conjugating a small C<sub>10</sub> hydrophobic fatty acid chain to **1**, the resulting amphiphilic dendrimer **2** (Fig. 2) was highly potent against both Gram-negative and -positive bacteria as well as multidrug-resistant bacteria (Table 1).<sup>39</sup> This study highlights the importance of imparting amphiphilicity to the dendrimer in order to improve antibacterial activity,

permitted in this example by introducing a hydrophobic chain.

Recently, Lai *et al.* reported similar findings.<sup>40</sup> They synthesized amphiphilic peptide dendrimers composed of a C<sub>16</sub>-alkyl chain attached to the peptide dendron with arginine-proline repeating motifs. Remarkably, the amphiphilic dendrimer **3** (Fig. 2) had a minimum inhibitory concentration (MIC) over 60-fold lower (geometric mean MIC of 2.2  $\mu\text{g mL}^{-1}$ ) than that of the corresponding peptide dendrimer **4** (138  $\mu\text{g mL}^{-1}$ ), whereas the linear peptide homolog **5** showed no activity at all (Table 1). These data in comparison to linear peptides support the importance of the dendritic structure and its unique multivalency for higher antibacterial activity, which can be further increased upon imparting amphiphilicity to the peptide dendrimer. The amphiphilic feature of **3** also enabled the dendrimers to self-assemble into nanostructures for more potent antibacterial activity.<sup>40</sup> A similar observation was reported by Gide *et al.*, where nanostructures formed by the amphiphilic lipidated polylysine dendrimer **6** showed broad spectrum activity against both Gram-negative and -positive bacteria, including multidrug-resistant strains as well as biofilms.<sup>41</sup> Collectively, these studies demonstrate that imparting amphiphilicity can effectively promote and enhance the antibacterial and antibiofilm activities of conventional peptide dendrimers, allowing greater potency and a broader spectrum.

Potent antibacterial activity shown by high-generation cationic PAMAM dendrimers demonstrated the interest of positively charged dendrimer terminals able to interact with negatively charged bacterial surface.<sup>45</sup> Unfortunately, however, high-generation PAMAM dendrimers are not only toxic but also difficult to synthesize in a defect-free form, whereas low-generation PAMAM dendrimers, though not toxic, lack antibacterial activity.<sup>46</sup> The addition of a hydrophobic alkyl chain (C<sub>18</sub>) to the small PAMAM dendron **7** generated the amphiphilic dendrimer **8** (Fig. 2) that was highly effective against Gram-negative and -positive bacteria as well as drug-resistant strains with MIC values of 6.0  $\mu\text{g mL}^{-1}$  (Table 1), whereas **7** without amphiphilicity showed no notable antibacterial activity at all.<sup>44</sup>





**Fig. 2** Imparting amphiphilicity to promote antibacterial activity of dendrimers. Chemical structures of the amphiphilic dendrimers **2**, **3**, **6** and **8** in comparison with those of the dendrimers **1**, **4**, and **7** and the linear peptide **5**.



**Table 1** Impact of the structural features of the dendron-tail amphiphilic dendrimers on the antibacterial activity

| Dendrimer structure variation | Dendrimer number | Hydrophobic chain | Generation | Surface functionality | MIC [ $\mu\text{g mL}^{-1}$ ] |                |                |                      | Hemolysis ( $\text{HC}_{50}$ ) [ $\mu\text{g mL}^{-1}$ ] | Ref. |
|-------------------------------|------------------|-------------------|------------|-----------------------|-------------------------------|----------------|----------------|----------------------|----------------------------------------------------------|------|
|                               |                  |                   |            |                       | <i>S. aureus</i>              | MRSA           | <i>E. coli</i> | <i>P. aeruginosa</i> |                                                          |      |
| Hydrophobic tail              | 1                | No chain          | 2          | Primary amine         | — <sup>a</sup>                | — <sup>a</sup> | >85            | >64                  | >1700                                                    | 39   |
|                               | 2                | C <sub>10</sub>   | 2          | Primary amine         | 64                            | — <sup>a</sup> | 1              | 3                    | 650                                                      |      |
|                               | 3                | C <sub>16</sub>   | 2          | Guanidine             | 11.85                         | 11.85          | 2.96           | 11.85                | 265                                                      | 40   |
|                               | 4                | No chain          | 2          | Guanidine             | >384.86                       | >384.86        | 21.8           | 87.21                | — <sup>a</sup>                                           |      |
| Hydrophobic tail length       | 13               | C <sub>6</sub>    | 2          | Primary amine         | >500                          | >500           | >500           | — <sup>a</sup>       | >500                                                     | 42   |
|                               | 14               | C <sub>12</sub>   | 2          | Primary amine         | 15                            | 15             | 60             | — <sup>a</sup>       | >500                                                     |      |
|                               | 15               | C <sub>18</sub>   | 2          | Primary amine         | 5                             | 8              | 10             | — <sup>a</sup>       | 23                                                       |      |
| Generation dependence         | 9                | C <sub>14</sub>   | 3          | Primary amine         | 3.9                           | — <sup>a</sup> | 7.8            | — <sup>a</sup>       | 10                                                       | 43   |
|                               | 10               | C <sub>14</sub>   | 2          | Primary amine         | 1.95                          | — <sup>a</sup> | 3.9            | — <sup>a</sup>       | 63                                                       |      |
|                               | 11               | C <sub>14</sub>   | 1          | Primary amine         | 3.9                           | — <sup>a</sup> | 3.9            | — <sup>a</sup>       | >5000                                                    |      |
| Terminal functionality        | 8                | C <sub>18</sub>   | 3          | Primary amine         | 6                             | 6              | 6              | 6                    | 194                                                      | 44   |
|                               | 18               | C <sub>18</sub>   | 3          | Tertiary amine        | 63                            | 42             | 6              | 65                   | 214                                                      |      |
|                               | 19               | C <sub>18</sub>   | 3          | Guanidine             | 200                           | >200           | 200            | 100                  | 159                                                      |      |
|                               | 20               | C <sub>18</sub>   | 3          | Carboxylic acid       | 100                           | 75             | 100            | >200                 | >400                                                     |      |

<sup>a</sup> “—”: not available.

Importantly, the amphiphilic dendrimer **8** retained activity against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm. Altogether, these exemplary studies strongly support the importance and impact of amphiphilicity to transform dendrimers from non-active or weakly active to highly active candidates against antibiotic-resistant bacteria.

## Fine-tuning amphiphilicity to balance antibacterial activity and toxicity

The potent antimicrobial efficacy shown by some amphiphilic dendrimers can unfortunately be associated with high toxicity. This is not surprising since many AMP-mimicking amphiphilic dendrimers exert their activity by first interacting with and then disrupting the bacterial membrane. A similar mechanism can occur towards eukaryotic cell membranes, thereby causing cytotoxicity. The trade-off between the desired antibacterial activity and undesired cytotoxicity is primarily and critically modulated by the hydrophilic–hydrophobic balance of the amphiphilic dendrimers and needs careful gauging to maximize the activity while minimizing the toxicity. This hydrophilic–hydrophobic balance is influenced by several structural characteristics of the amphiphilic dendrimers, such as the nature of the hydrophobic entity, dendrimer scaffold, surface functionality, charge density, as well as the number and size of the terminal groups. Minor changes to these factors can significantly tilt this balance. Consequently, substantial efforts have been dedicated to finding an optimum hydrophilic–hydrophobic balance and charge density that avoids cytotoxicity without compromising the antibacterial activity.

The cytotoxic and hemolytic potential of amphiphilic dendrimers can be critically influenced by dendrimer generation and the nature of the exponentially increasing terminal units.

Chen *et al.* studied amphiphilic polyester dendrimers as antibacterial candidates.<sup>43</sup> These dendrimers are composed of alkyl chains and polyester dendrons based on 2,2-bis(methylol)propionic acid (bis-MPA). Notably, the prime candidate **9** carrying a C<sub>14</sub> alkyl chain (Fig. 3) exhibited highly potent antibacterial activity (MIC of 4.0–8.0  $\mu\text{g mL}^{-1}$ ) without any notable cytotoxicity or hemolytic activity, even at concentrations >5000  $\mu\text{g mL}^{-1}$ . However, the first-generation dendrimer **11** (Fig. 3) was highly hemolytic ( $\text{HC}_{50}$  10–63  $\mu\text{g mL}^{-1}$ ), while the second-generation dendrimer **10** (Fig. 3) was moderately hemolytic ( $\text{HC}_{50}$  2500  $\mu\text{g mL}^{-1}$ ), although they both showed antibacterial activity (Table 1). This could be explained by the overall hydrophobicity of the antimicrobial candidate, the higher generation dendrimer having more dendrimer terminals bearing positive charges hence lowering its hydrophobicity and thereby also its toxicity.<sup>43</sup> This study demonstrated the critical nature of an optimum amphiphilic balance in the molecular structure of the dendrimer in order to achieve negligible toxicity while retaining activity against a broad spectrum of bacteria.

Another exemplary study was made with the core–shell amphiphilic dendrimers synthesized by Worley *et al.*<sup>47</sup> These dendrimers are PAMAM dendrimers functionalized with a nitric oxide donor and a hexyl aliphatic chain as the dendrimer terminal. Notably, the toxicity of these dendrimers increased with the generation number: the generation 4 dendrimer exhibited greater toxicity than the generation 1 dendrimer ( $\text{IC}_{50}$  = 75 and 1150  $\mu\text{g mL}^{-1}$ , respectively). This is understandable considering the exponentially increased number of hydrophobic terminals with increasing dendrimer generation, translating to high toxicity. Interestingly, the generation 3 dendrimer **12** (Fig. 3) was the only generation capable of eradicating both Gram-negative and -positive biofilms at nontoxic concentrations (10–50  $\mu\text{g mL}^{-1}$ ) well below the  $\text{IC}_{50}$  values (450  $\mu\text{g mL}^{-1}$ ).



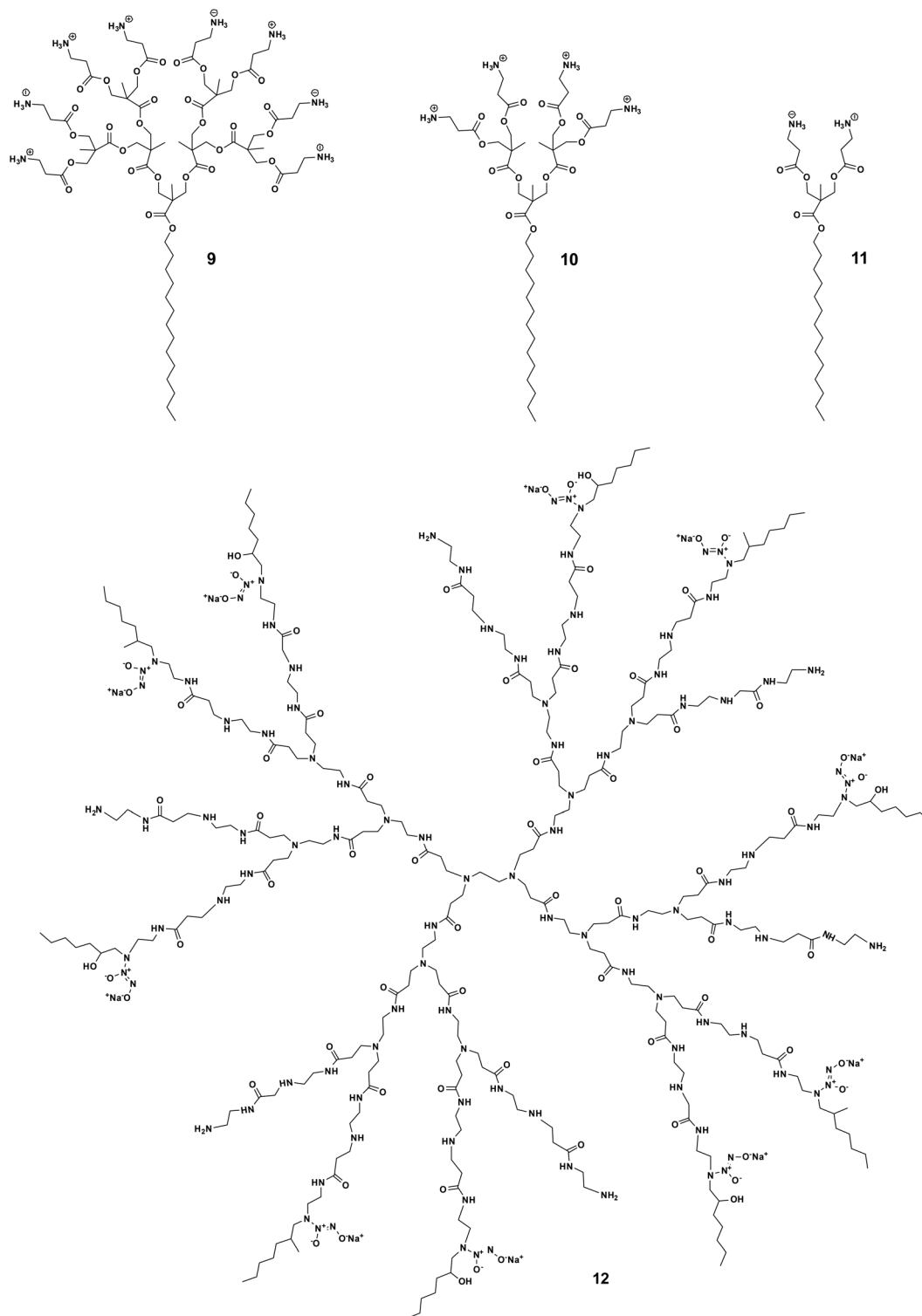
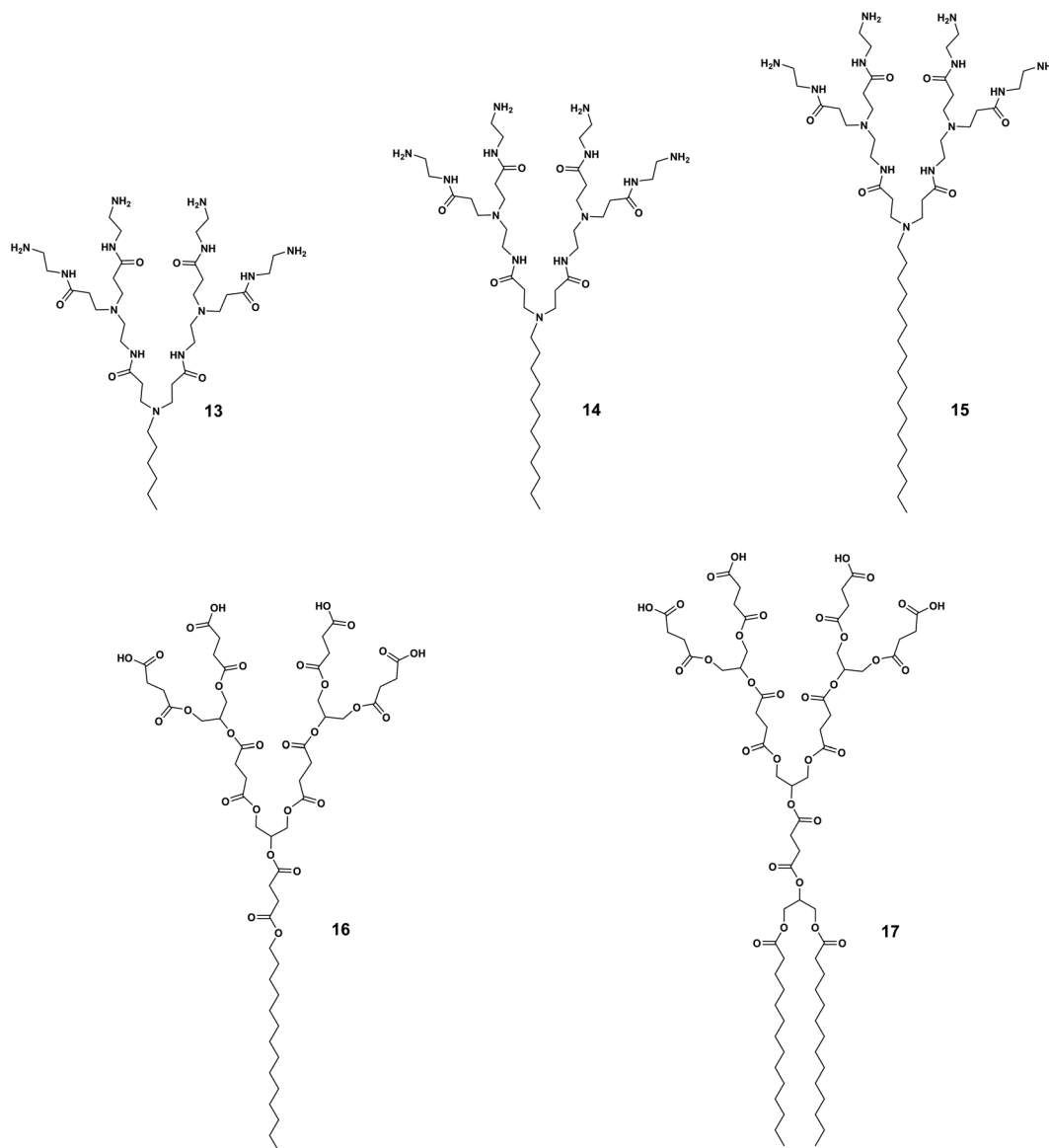


Fig. 3 Impact of dendron generation on the trade-off between activity and toxicity shown by the amphiphilic dendrimers 9, 10, 11 and 12.

$\text{mL}^{-1}$ ). These results again indicate that fine tuning of the hydrophobic-hydrophilic balance between dendrimer generation and functional group density with amphiphilic characteristics can effectively maximize antibacterial action while minimizing toxicity to mammalian cells.

For a given dendrimer generation number, the increase in the hydrophobic tail length can increase the antibacterial activity as well as the toxicity of amphiphilic dendrimers. Guo *et al.* synthesized amphiphilic dendrimers 13–15 (Fig. 4) based on a PAMAM dendron with varying lengths of alkyl chains.<sup>42</sup>





**Fig. 4** Impact of hydrophobic chain length and number on the balance between antibacterial activity and toxicity as demonstrated by the amphiphilic dendrimers **13**, **14**, **15**, **16** and **17**.

As the hydrophobic chain length increased from  $C_6$  to  $C_{18}$ , the hemolytic activity increased along with antibacterial activity. For example, the amphiphilic dendrimer **15** was more active and also more hemolytic than **13** and **14** (Table 1). A similar trend in hemolytic activity was observed for amphiphilic peptide dendrimers.<sup>39</sup> The elongation of the fatty acid chain from  $C_6$  to  $C_{24}$  in the peptide dendrimer **2** led to a gradual increase in hemolysis, while the antibacterial activity increased until a plateau was reached.<sup>39</sup>

In a rare and surprising example, Meyers *et al.* reported that the carboxylic acid-terminated amphiphilic dendrimer **16** connected to a single aliphatic chain (Fig. 4) was more toxic to HUVEC cells than the amphiphilic dendrimer **17** carrying double aliphatic chains (Fig. 4).<sup>48</sup> The selectivity index of the double chain dendrimer **17** was improved as this had a lower

MIC against tested Gram-positive bacteria in comparison with that of the single chain dendrimer **16**.<sup>48</sup> Currently, only saturated alkyl chains and their effect on activity and toxicity have been studied; knowledge on the effect of unsaturated fatty acids and other types of lipid chains as the hydrophobic part of amphiphilic dendrimers is lacking.

The hydrophobic–hydrophilic balance of the amphiphilic dendrimers can be additionally altered by the terminal functionality, charge, and charge density, as well as the steric size, hence impacting the trade-off between activity and toxicity. We have recently studied the antibacterial activity of amphiphilic PAMAM dendrimers composed of a long  $C_{18}$  alkyl chain and a PAMAM dendron bearing one of the following terminals: primary amine (**8** in Fig. 2), tertiary amine (**18**, Fig. 5), guanidine (**19**, Fig. 5), or carboxylic acid (**20**, Fig. 5).<sup>44</sup> The primary



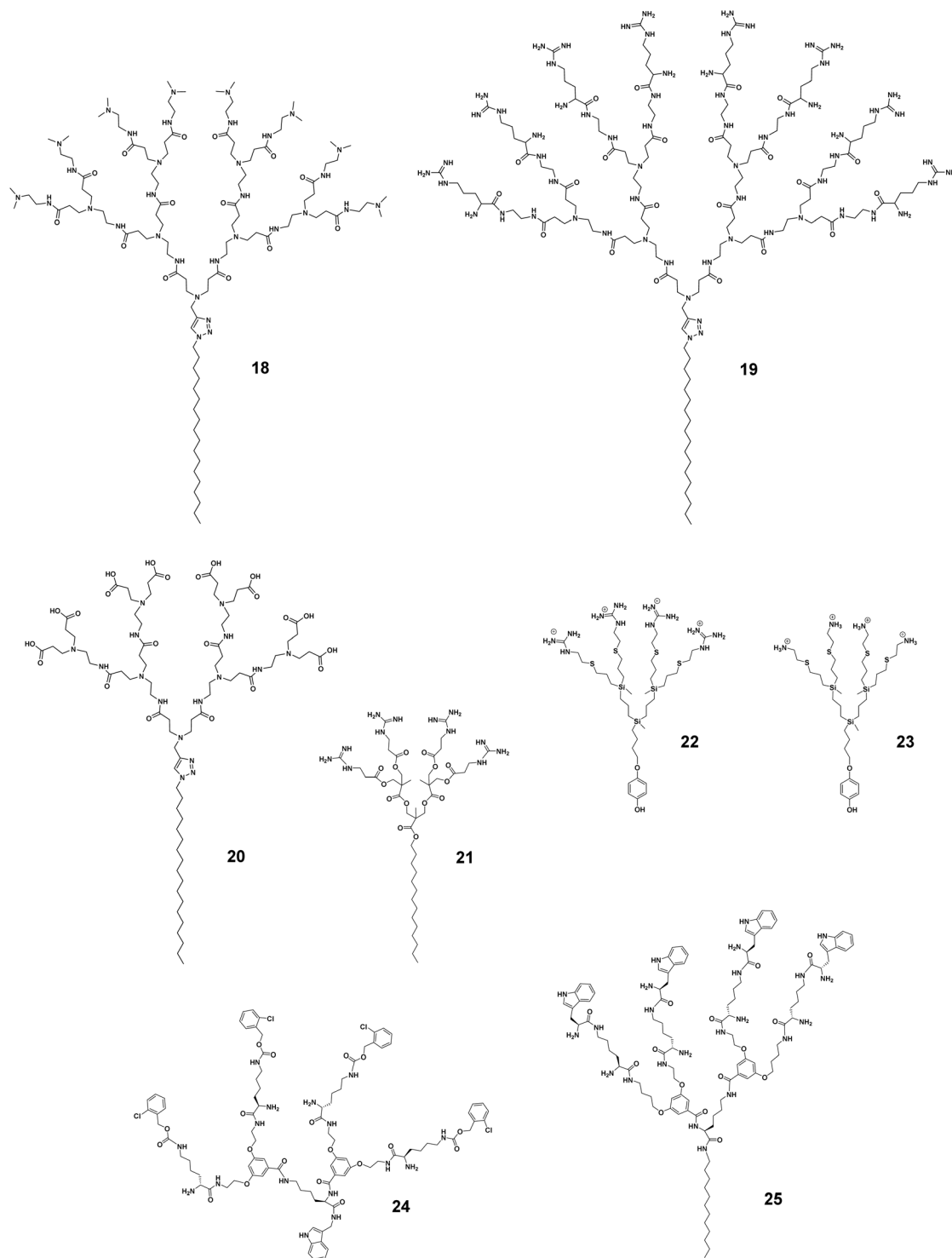


Fig. 5 Impact of terminal functionalities on the activity and toxicity of the amphilic dendrimers 18, 19, 20, 21, 22, 23, 24 and 25.

amine dendrimer **8** was the most active dendrimer against both Gram-negative and -positive bacteria as well as drug-resistant bacteria (Table 1). The tertiary amine dendrimer **18** showed similar activity against *E. coli* as **8** but had a better safety profile, probably due to the more dispersed cationic charge over the larger tertiary amine terminal when compared with that of the

primary amine terminal. It should be mentioned that the carboxylate-terminated dendrimer **20** failed to show any antibacterial activity but was also neither toxic nor hemolytic, as supported and confirmed by results from other groups.<sup>42,49</sup>

Surprisingly, the guanidine-terminated dendrimer **19** was inactive against all bacteria tested.<sup>44</sup> This finding correlate



with the results previously reported with guanidine molecular umbrella **21** (Fig. 5).<sup>43</sup> In addition, **19** was found to be toxic, which can be attributed to the guanidine terminals that can strongly interact with the eukaryotic cell membrane. These results contradict those previously published supporting arginine-rich moieties as a useful means to generate antibacterial candidates.<sup>50</sup> For example, the amphiphilic carbosilane dendrimer **22** bearing guanidine terminals was very active against *E. coli* and *S. aureus* with an MIC value of 2.0  $\mu\text{M}$ , which was similar to that of the amine-terminating dendrimer analogue **23**.<sup>51</sup> The antibacterial activity of the arginine-terminated dendrimer **22** is reminiscent of some highly potent AMPs, such as indolicidin and tritrpticin, which are rich in arginine. Nevertheless, this is just one consideration as these peptides are also rich in tryptophan, which imparts essential and balancing hydrophobicity to these peptides.<sup>52</sup> We therefore suggest that the carbosilane dendritic scaffold may provide adequate hydrophobicity to these dendrimers balancing the effects of their highly cationic terminals.<sup>51</sup>

Another aspect to be considered while designing more selective antimicrobial peptide dendrimers is the incorporation of hydrophobic amino acids at the peptide dendrimer terminals. Sowińska *et al.* designed two groups of polylysine peptide dendrimers using orthogonally substituted lysine as a core, and the dendrimer terminals were either substituted with lysine or tryptophan with 4 or 8 positive charges, respectively.<sup>53</sup> The dendrimers carrying four positive charges such as **24** (Fig. 5) had a broad spectrum of activity with MIC values of 15–31  $\mu\text{M}$ , whereas those with tryptophan terminals were 4–8 times more active. As an example, the dendrimer **25** (Fig. 5) bearing a  $\text{C}_{12}$  alkyl chain as a core and tryptophan terminals, showed marked antibacterial activity against multidrug-resistant clinical isolates of different bacteria strains with a mean MIC value of 7.0  $\mu\text{M}$ . Unfortunately, dendrimer **25** (Fig. 5) was also highly cytotoxic at 20  $\mu\text{M}$ . Apparently, the dodecyl chain in combination with the tryptophan amino acid residues conferred significant hydrophobicity to the dendrimer and rendered it cytotoxic. Nonetheless, this study also highlighted the importance of the terminal group hydrophobicity and its impact on amphiphilicity, and thereby on the complex activity–toxicity relationship.<sup>53</sup>

## Amphiphilic dendrimers have increased stability

As linear peptides, many AMPs can be easily degraded by various enzymes and therefore show poor stability and low bioavailability as drug candidates. Thanks to their dendritic structural feature, dendrimeric peptides or peptide dendrimers are different, showing considerable resistance to proteolytic degradation. The dendritic architecture forms a steric hurdle for enzymes, hindering their access to active sites for hydrolyzation of these peptides. Amphiphilic dendrimers show even greater resilience towards enzymatic degradation by virtue of their self-assembly into nanostructures, thus prolonging their

circulation time. As such, amphiphilic dendrimers often stably retain their antibacterial activity in the presence of sera, salts, and enzymes.

For example, the peptide dendrimer **26** had good antibacterial activity but was not stable in the presence of serum (Fig. 6).<sup>39</sup> Addition of a  $\text{C}_{10}$ -alkyl chain to **26** yielded the amphiphilic dendrimer **27** that allowed an increase in serum half-life from 6 to 20 h (for **26** and **27**, respectively). This is in line with literature reports that lipidation can be used to prolong the circulation of peptides or nucleic acids *via* binding to serum proteins.<sup>54</sup> Further studies on **27** showed extremely promising antibacterial efficacy in animal models in which the peptide dendrimers lacking a hydrophobic chain had failed. These results indicate that imparting amphiphilicity to peptide dendrimers is a valuable strategy to enhance both the serum stability and antibacterial activity, and may determine the clinical translation of these dendrimer candidates.

In 2019, Wang J. *et al.* identified that the arginine–proline (RP) sequence prevented the enzymatic degradation of AMPs in the presence of trypsin and chymotrypsin, but that the activity of these peptides was hampered in the presence of salts.<sup>55</sup> They further integrated the RP sequence into amphiphilic peptide dendrimers to capitalize on the dendritic structure and improve the stability.<sup>40</sup> The authors identified the two most potent amphiphilic peptide dendrimers, **3** and **28** (Fig. 6), bearing a hexadecanoid acid tail ( $\text{C}_{16}$ ) with a peptide dendron generation of 3 and 2, respectively. Both dendrimers were active with respective mean geometric MIC values of 2.2 and 2.9  $\mu\text{M}$  against nine different bacterial strains. Importantly, they both remained bactericidal in the presence of either monovalent or divalent cations, even after 48 h of serum incubation. When exposed to high salt concentrations (150 mM NaCl or 2.0 mM  $\text{CaCl}_2$ ), the MIC value of **3** against *E. coli* was only slightly increased to 3.0 or 2.0  $\mu\text{M}$ , respectively. A similar trend was observed for **28**, the MIC of which was increased by 3.5- and 2.5-fold under the same conditions, respectively. Furthermore, the MIC values against *E. coli* were slightly increased 0.5-fold for **3** and 1-fold for **28** in the presence of 8.0  $\mu\text{g mL}^{-1}$  of trypsin and chymotrypsin. Collectively, these results demonstrate that amphiphilic dendrimers exhibit considerable resilience and stability against enzyme degradation, serum sensitivity, and high salt concentration destabilization.<sup>40</sup>

## Amphiphilic dendrimers mainly act on bacterial membrane

Encouraged by the antibacterial performance of amphiphilic dendrimers, studies on their mechanisms have been actively pursued. Current results suggest that many amphiphilic dendrimers mediate membrane depolarization and disruption, representing the primary mechanism responsible for their antibacterial activity.<sup>39,40,43,44,56</sup> Specifically, the cationic terminals of the amphiphilic dendrimers interact with the negatively charged bacteria membrane to allow the insertion of the



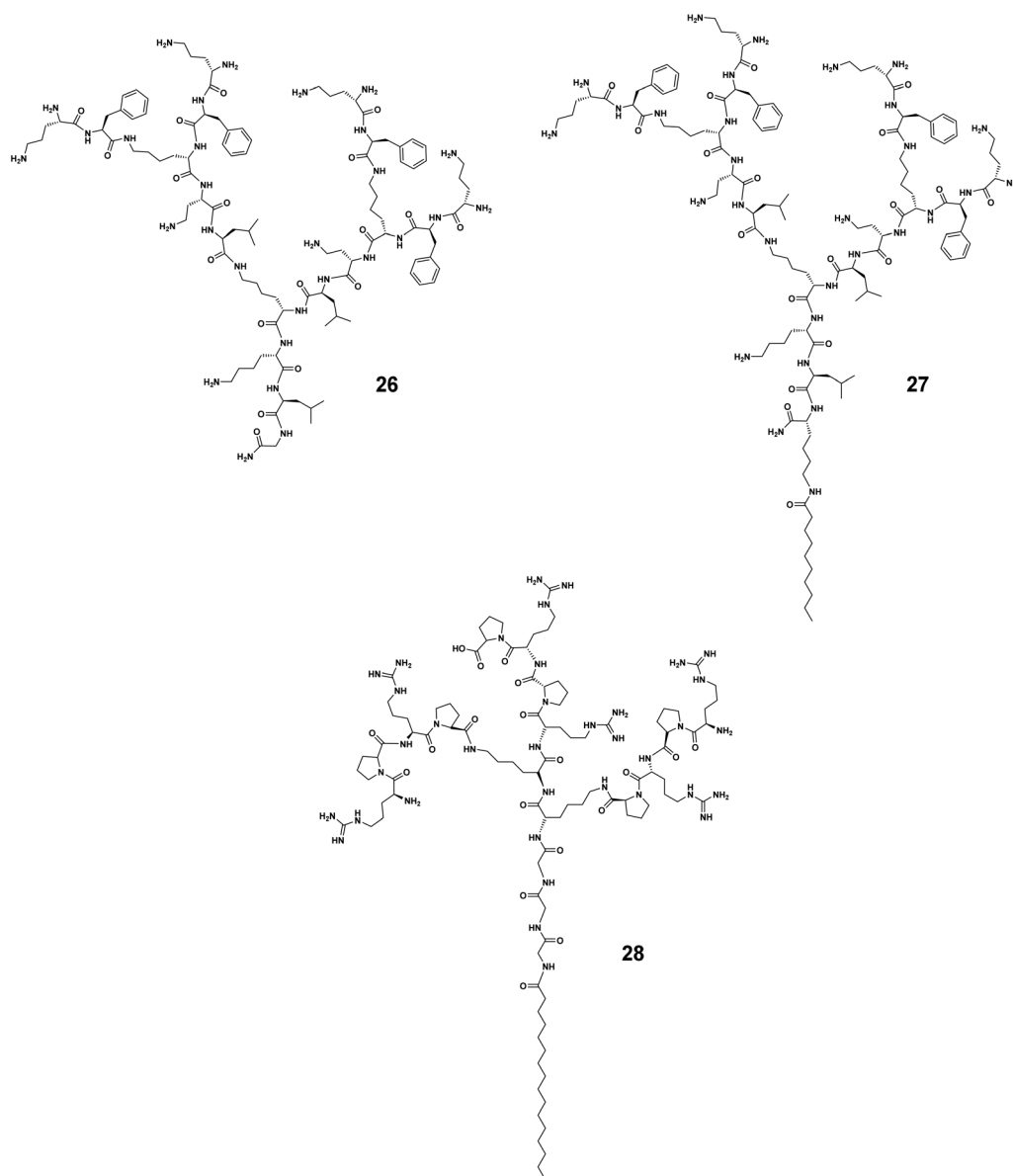


Fig. 6 Amphiphilic dendrimers 26, 27 and 28 with enhanced enzymatic and colloidal stability.

hydrophobic entity which causes membrane disruption, depolarization, and subsequent cell lysis. Although amphiphilic dendrimers can disrupt both Gram-negative and -positive bacterial membranes, they usually show higher potency against Gram-negative bacteria. This is because the outer membrane of Gram-negative bacteria differs from that of Gram-positive bacteria by an extra outer layer comprising negatively charged lipopolysaccharides (LPS). The amphiphilic dendrimers being positively charged preferably interact with the negatively charged LPS layer *via* electrostatic adsorption.

One study on the amphiphilic peptide dendrimers 27 demonstrated the importance of the hydrophobic chain added to the peptide dendrimer for crossing the LPS layer of the bacteria.<sup>39</sup> For example, the peptide dendrimer 1 and its peptide dendrimer analogs were all inactive against *P. aeruginosa* wild-

type strains but showed activity against *P. aeruginosa* mutants lacking LPS. In contrast, the amphiphilic dendrimer 27 was active against all strains regardless of LPS composition of the bacterial outer membrane. These results highlighted the importance of the alkyl chain in allowing the amphiphilic dendrimers to cross the bacterial membrane and thereby express their antibacterial activity. This is in line with a similar reported effect of the N-terminal fatty acid chain in the structure of polymyxin B.<sup>57</sup> Furthermore, time-dependent bacteria-killing experiments showed a rapid bactericidal effect against *P. aeruginosa* within 30 minutes upon addition of 27.<sup>39</sup> Such rapid killing kinetics often implies a membrane-disruptive mechanism of action.

To further demonstrate that membrane disruption is indeed the primary mechanism underlying the antibacterial



activity of the amphiphilic dendrimers, we performed a series of experimental studies alongside computer modeling on the amphiphilic PAMAM dendrimer **8**.<sup>44</sup> We first examined the action of **8** on both Gram-negative and -positive bacteria using a live/dead cell staining assay with the fluorescent dyes SYTO9 and propidium iodide (PI). SYTO9 is a universal dye that crosses intact membranes and stains nucleic acids of all live cells green, whereas PI can only cross compromised membranes, emitting a red fluorescence once bound to nucleic acids. Both Gram-negative bacteria, such as *E. coli*, and Gram-positive bacteria, such as MRSA, displayed a prominent red fluorescence upon treatment with **8** implying a mechanism of action associated with membrane disruption. This was confirmed by scanning electron microscopy studies that revealed obvious membrane deformities such as blebbing and release of intracellular content at the cell surface of bacteria treated with **8**. Further membrane permeabilization and depolarization assays confirmed that **8** interacted with the bacterial membrane, causing significant outer membrane permeation in *E. coli* and rapid inner membrane depolarization in both *E. coli* and MRSA.

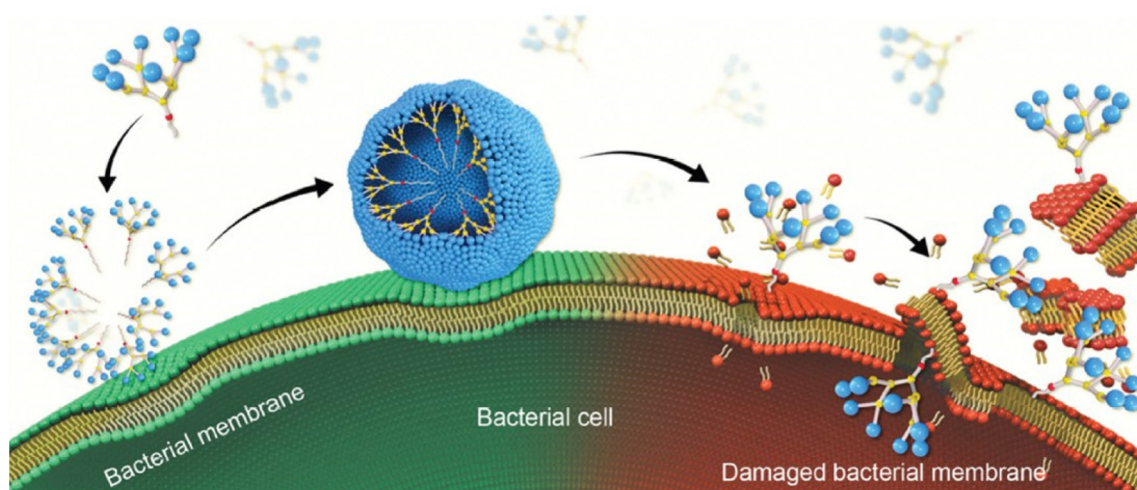
In addition, the self-assembling properties of **8** played an additional crucial role in the mechanism of action, inducing the adsorption and accumulation of **8** on the bacterial membrane *via* electrostatic interaction, leading to a high local concentration of **8** at the bacterial surface. The enriched **8** on the bacterial surface then formed nanomicelles *via* self-assembly for more effective and stronger binding with the bacterial membrane *via* cooperative multivalent binding. Once bound on the bacterial surface, these dendrimer nanomicelles coercively interacted with and inserted into the bacterial membrane with additional hydrophobic interactions, producing profound membrane disruption and hence potent antibacterial activity (Fig. 7).<sup>44</sup>

Similarly, Lai *et al.* demonstrated that their amphiphilic peptide dendrimers self-assembled into nanoparticles.<sup>40</sup>

These nanoparticles not only interacted with the bacterial membrane to exert a killing effect but also involved other mechanisms that addressed intracellular targets (see the section below). In addition, Gide *et al.* came to the same conclusion using the amphiphilic dendrimer **6** (Fig. 2), which formed nanoparticles to interact with and compromise the bacterial membrane to exert its antibacterial activity.<sup>41</sup> These different studies provide support for the important contribution of the self-assembling of the amphiphilic dendrimers to create localized high concentrations on the bacterial surface membrane for strong binding and subsequent membrane disruption, ultimately inducing lysis and killing of the bacteria.

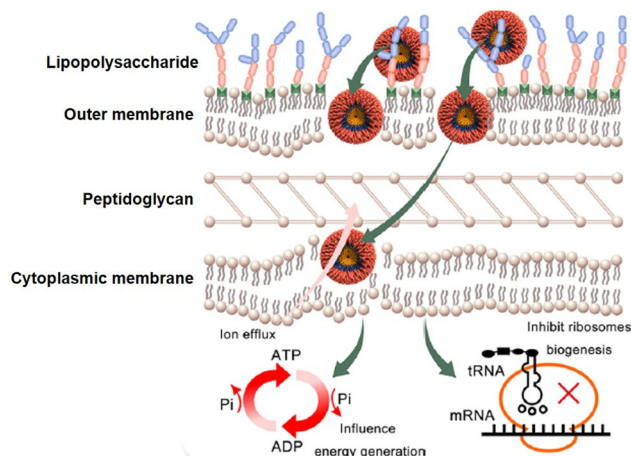
## Amphiphilic dendrimers also act on intracellular targets

Although membrane disruption is the primary mechanism employed by amphiphilic dendrimers to exert their antibacterial activity, recent studies report that amphiphilic dendrimers may interact with intracellular targets.<sup>40</sup> For example, Lai *et al.* used continuous-scanning fluorescence imaging to demonstrate that FITC-labeled **3** localized within bacteria. Further transcriptome studies revealed the downregulation in expression of essential genes involved in the synthesis of peptidoglycan and ribosome subunits upon treatment with **3**.<sup>40</sup> In addition, the negatively impacted genes were involved in pathways associated with basic metabolic processes, including cellular carbohydrate metabolism as well as glycolysis/gluconeogenesis and oxidative phosphorylation. Therefore, **3** exerted antibacterial activity through a multimodal mechanism primarily involving membrane integrity disruption and affected bacterial energy production and metabolism (Fig. 8).<sup>40</sup> The finding of amphiphilic dendrimers interacting with multiple targets is encouraging with regards the desired prevention of target bacteria developing resistance.



**Fig. 7** Cartoon illustration of the mechanism underlying the antibacterial activity of the amphiphilic dendrimer **8** *via* membrane adsorption, self-assembly, interaction, insertion, disintegration and disruption. Reproduced from ref. 44 with permission from Royal Society of Chemistry.





**Fig. 8** Multimodal mechanism for the antibacterial activity of the amphiphilic peptide dendrimer **3**. Reproduced from ref. 40 with permission from ACS Publications, copyright 2023.

## Amphiphilic dendrimers prevent bacteria from acquiring drug resistance

Acquired drug resistance occurs when bacteria gain the ability to resist the activity of antibacterial agents by evolutionary processes. Recent studies demonstrate that amphiphilic dendrimers can circumvent acquired bacterial resistance. For example, Lai *et al.* studied the acquired resistance of *E. coli* to the amphiphilic dendrimer **3** (MIC of 1.0  $\mu\text{M}$ ) at sub-MICs for up to 30 days.<sup>40</sup> No notable acquired resistance was observed against **3**, whereas *E. coli* quickly generated effective resistance upon treatment with sub-MICs of ciprofloxacin after 6 days. Notably, the genes encoding cationic antimicrobial peptide resistance that are primarily involved in bacterial membrane synthesis were significantly upregulated in *E. coli* upon treatment with **3**. This upregulation may have decreased the affinity for **3** by increasing the positive charge on the outer bacterial membrane as well as by altering the metabolism of amino acids and sugars within the inner membrane. However, despite upregulation of resistance genes, the multimodal mechanism of **3** ensured a maintained strong bactericidal effect. Notably, following treatment of *E. coli* with **3**, 1479 genes exhibited significant differential expression (out of a total of 5130 genes), that was upregulated in 809 genes and downregulated in 670 genes. These differentially expressed genes were mostly associated with ribosomal function, valine, leucine, and isoleucine biosynthesis, and C5-branched dibasic acid metabolism. These results indicate that **3** acted differently from most antibiotics, which have only a very limited number of targets, and that the actions of **3** are more likely to cause comprehensive changes to multiple functions and structural features of bacteria. Therefore, **3** may have crossed the bacterial membrane after initially damaging it to generate a comprehensive response involving DNA transcription and translation, protein

synthesis, energy production, transmembrane transport, and other processes in *E. coli*.

Guo *et al.* also reported the lack of drug resistance induced in either *S. aureus* or *E. coli* to amphiphilic dendrimer.<sup>42</sup> In particular, the MIC value of their reported amphiphile remained unchanged despite repeat exposure of both *S. aureus* and *E. coli* at sub-MIC levels. These results further support the encouraging findings of the inherent difficulty for bacteria to develop resistance to cationic amphiphilic dendrimers which target not only bacterial membrane but also internal biosynthetic and metabolic processes.

## Conclusion and future perspectives

The alarming situation of AMR in bacteria imposes the rapid development of novel antibacterial agents that are effective and stable while inducing no toxicity or resistance development. Amphiphilic dendrimers are emerging as a promising solution to combat bacterial AMR. They are composed of distinct hydrophobic and hydrophilic entities bearing dendritic structures, which can be precisely designed and synthesized. The primary mechanism underlying their antibacterial activity involves the binding of their positively charged entities with the negatively charged bacterial surface *via* multivalent electrostatic interactions. This is then followed by penetration of their hydrophobic entity into the bacterial cell membrane resulting in membrane disruption and thereby cell death.<sup>44</sup> Additional modes of action of the amphiphilic dendrimers have been reported that involve intracellular targets, such as synthesis of membranes, amino acids and nucleic acids.<sup>40</sup> The potent antibacterial activity achieved by amphiphilic dendrimers appears to involve multimodal mechanisms of action that effectively kill bacteria including multidrug-resistant species.

Notably, the hydrophilic–hydrophobic balance of the amphiphilic dendrimers is crucial to ensure high potency against a broad spectrum of bacteria and to avoid adverse effects on host cells, especially red blood cells. The trade-off between antibacterial activity and toxicity needs careful consideration when designing amphiphilic dendrimers as antibacterial candidates. The precise choice of appropriate dendrimer structure, generation and surface functionalities, as well as hydrophobic entities can impact the level of selectivity that is achieved. Although amphiphilic dendrimers encompass core-shell and dendron-tail structures<sup>30,31</sup> (Fig. 1B), the latter have a more straightforward design allowing a more precise synthesis in accordance with the desired balance between hydrophilicity and hydrophobicity. As such dendron-tail structures are the most studied for their antibacterial activity and in particular to overcome antibiotics resistance.

To date, most studies on antibacterial activity involve amphiphilic peptide dendrimers, PAMAM dendrimers and polyester dendrimers. Many other dendrimers with different scaffolds have been evaluated for use as antibacterial agents in recent years, including carbosilane dendrimers,<sup>58–60</sup> phos-



phorus dendrimers<sup>61</sup> and glycodendrimers dendrimers<sup>62</sup> etc. More investigations are now needed on the antibacterial activity of amphiphilic dendrimers with these different dendrimer scaffolds for combatting AMR.

In parallel, more efforts should be applied to preclinical investigations of potent amphiphilic dendrimers in different animal models of infectious diseases to thoroughly assess their robustness and pave the way towards their clinical application. It should be mentioned that knowledge on amphiphilic dendrimer-AMP mimics that exert antibacterial activity *via* immunomodulatory mechanisms is still lacking, and further studies in this regard are required to accelerate the clinical translation of amphiphilic dendrimers as effective and potent antibacterial candidates. In addition, focus should be given to the development of narrow-spectrum antibiotics, as the use of broad-spectrum antibiotics not only generates drug resistance but also harms beneficial microbial communities inhabiting humans. Narrowing the spectrum of antibacterial agents now appears to represent a feasible perspective for precision antibacterial therapies to combat AMR.

It is to note that amphiphilic dendrimers can self-assemble into nanostructures that can accommodate drug molecules<sup>31,63,64</sup> for combination therapy and simultaneously harness the advantage of the nanotechnology-based drug delivery for specific accumulation at the site of infection and inflammation. Targeted drug delivery can be readily achieved through either passive targeting *via* the enhanced permeability and retention effect or active targeting *via* conjugation of ligands, antibodies or other bacterial cell-specific targeting moieties, enabling effective treatments while reducing adverse effects and toxicities.

Finally, combatting bacterial AMR needs a One Health approach as this is a critical global problem affecting human and animal life as well as the environment.<sup>4</sup> The first step in line with this approach involves improving regulations and policies aimed at halting the excessive use of antimicrobials in agriculture and animal husbandry. Alongside this, antibiotic stewardship programs within the clinical setting group together specialists dedicated to optimizing use of antibiotic therapy in humans. Finally, a community-based surveillance of the migration of individual and animals infected with resistant bacteria would help limit the spread of infections.<sup>65</sup> Such combined efforts should help reduce bacterial infections and enable progress to be made in the fight against antibiotic resistance.

## Conflicts of interest

There are no conflicts of interest to declare.

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