Enhanced Membrane Pore Formation by Multimeric/Oligomeric Antimicrobial Peptides†

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ABSTRACT: The pore-forming antibacterial peptide magainin 2 was made divalent, tetravalent, and octavalent via a copper(I)-mediated 1−3 dipolar cycloaddition reaction ("click" chemistry). This series of pore-forming compounds was tested in vitro for their ability to form pores in large unilamillar vesicles (LUVs). A large increase in the pore-forming capability was especially observed with the tetravalent and octavalent magainin compounds in the LUVs consisting of DOPC, and the octavalent magainin compound showed a marked increase with the DOPC/DOPG LUVs. Activity was observed in the low nanomolar range for these compounds.

One of the biggest threats that humanity will face in the coming decade is the increasingly frequent appearance of bacteria resistant to multiple or even all antibiotics. While this process is urging the development of novel antibiotics (*1*), eventually these will also lead to resistant bacteria. In contrast, antimicrobial peptides (AMPs)[1] have remained effective antibacterials without any development of resistance toward them. These peptides, which are found throughout the animal and plant kingdoms, are positively charged and specifically target the bacterial membranes. This is due to a fundamental difference in the composition of the membranes of microbes and multicellular animals (*2*). Bacterial membranes are organized in such a way that they contain relatively large amounts of exposed anionic lipids, while the outer leaflet of the membranes of plants and animals is composed mainly of lipids with no net charge. The majority of the AMPs, of which the best-known member is the peptide magainin (*3*), kill by permeabilizing the membrane of their target, mainly by forming pores. As a result, vital ion gradients are dissipated and small essential molecules (e.g., ATP) leak out of the cell.

Unfortunately, these AMPs are inefficient in their mode of killing. Therefore, high doses are needed to reach sufficient activity levels of these AMPs in the bloodstream. This high dose is also toxic to human cells. Consequently, their use and development has been limited. There are two interdependent reasons for this inefficiency. First, AMPs have a relatively low (micromolar) affinity for their target membranes. Second, pore formation by the AMPs requires that several peptides come into the proximity of one another. This requires a high local concentration at the membrane surface, which in turn requires a high bulk concentration due to their relatively low affinity for the membrane.

While certain AMPs such as nisin have found a solution for the low membrane affinity by specifically binding to a membrane component (Lipid II) (*4, 5*), the assembly hurdle remains a barrier for most AMPs. Our aim is to increase the potency by removing this assembly hurdle by covalently linking a number of AMPs together. Such a preassembled pore should exhibit greatly enhanced potency in membrane pore formation. There are indications in the literature that hint at the feasibility of this approach, while its full potential does not seem to have been reached. In one report, a disulfide dimerized magainin analogue was found to be 1 order of magnitude more active in a vesicle leakage assay, which correlated also with antimicrobial activity (*6*). In another report, short antimicrobial tetra- and octapeptides were linked to lysine dendrimers up to the octavalent form and exhibited broad antimicrobial activity (*7*). Furthermore, linking an antimicrobial tetrapeptide to a polymer lead to a 10-fold increase in antimicrobial potency (*8*).

Covalently linking bioactive compounds to a scaffold or core molecule has proven to be a successful strategy for large affinity enhancement of especially weak carbohydrate ligands

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[1] Abbreviations: Ac, acetyl; AMP, antimicrobial peptide; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; DMF, *N*,*N*-dimethylformamide; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; EDT, 1,2-ethanedithiol; Et, ethyl; Fmoc, fluorenylmethoxycarbonyl; HPLC, high-performance liquid chromatography; *i*Pr, isopropyl; LUV, large unilamellar vesicle; MS, mass spectrometry; MTBE, methyl *tert*-butyl ether; NaAsc, sodium ascorbate; NMP, *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TLC, thin layer chromatography; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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in their binding to bacterial toxins (*9*−*11*) and bacterial surfaces (*12*). Such a multivalency approach has recently become synthetically more facile due to the advent of "click" chemistry (*13*, *14*). In this reaction, a chemo- and regioselective coupling of an alkyne moiety to an azide group yields a 1,4-disubstituted 1,2,3-triazole-linked system. Protecting groups are not needed, and besides carbohydrates (*15*), peptide sequences can be attached (*16*). We here greatly surpassed the length and valency of previous multivalent peptides, by synthesizing an up to octavalent system based on the 23-amino acid magainin peptide. The obtained multivalent AMPs were tested for their ability to form pores in membranes of vesicles. Indeed, an enhanced pore forming action of almost 600-fold was observed.

EXPERIMENTAL PROCEDURES

General Remarks. Chemicals and resins were obtained from commercial sources and were used without further purification unless stated otherwise. Electrospray ionization (ESI) mass spectrometry was carried out using a Shimadzu LCMS QP-8000 single-quadrupole benchtop mass spectrometer (*m*/*z* range of <2000), coupled with a QP-8000 data system. Analytical HPLC was performed on a Shimadzu Class-VP automated HPLC system using an analytical reversed-phase column (Alltech Adsorbosphere C18, 300 Å, 5 μm, 250 mm × 4.6 mm; Alltech Adsorbosphere C8, 90 Å, 5 μm, 250 mm × 4.6 mm; or Alltech Alltima C8, 90 Å, 5 μm, 250 mm × 4.6 mm) and a UV detector operating at 220 and 254 nm. Preparative HPLC was performed on a Gilson automated HPLC apparatus using a preparative reversed-phase column (Alltech Adsorbosphere C18 or C8, 10 μm, 250 mm × 22 mm) and a UV detector operating at 220 and 254 nm. Unless otherwise stated, elution was effected using an appropriate gradient from 0.1% TFA in H_2O to 0.1% TFA in CH_3CN/H_2O (95/5, v/v) using a flow rate of 1 mL/min (analytical) or 11.5 mL/min (preparative). Microwave reactions were performed in an Initiator reactor from Biotage. SDS−PAGE was performed using the Bio-Rad mini protean II system. Fluorescence measurements were performed on an SLM-Aminco SPF-500 C fluorimeter.

5(6)-Carboxyfluorescein Leakage Experiments. Experiments were performed as previously described (*4*, *17*). In short, large unilamellar vesicles (LUVs) composed of DOPC and a DOPC/DOPG mixture (1/1, molar ratio) containing 5(6)-carboxyfluorescein were made from multilamellar vesicles in 5(6)-carboxyfluorescein [50 mM, 10 mM Tris (pH 7), and 150 mM NaCl] by extruding these 10 times through a polycarbonate membrane filter (0.2 μm). The LUVs were separated from the extravesicular 5(6)-carboxyfluorescein solution using Sephadex G50. The lipid concentration in the LUVs was determined after destruction of the vesicles and measurement of the P_i level according to Rouser (*18*). An experiment consisted of adding vesicles to 1 mL of 10 mM Tris (pH 7) and 150 mM NaCl, resulting in a lipid concentration of 25 μM in a fluorescence cuvette. The change in fluorescence (excitation 492 nm, emission 515 nm) was measured upon injection of the peptide over 3 min. The percent leakage was determined at 80 s, and the vesicles were destroyed with 0.2% Triton X-100 detergent for maximum obtainable leakage at 160 s.

*Fmoc-Lys(N_3)-OH (**5**)*. **4** (1.38 g, 5.1 mmol) was dissolved in CH_2Cl_2 (20 mL), and TFA (20 mL) was added. The reaction was monitored with TLC. After the complete disappearance of the starting material, the reaction mixture was evaporated in vacuo and coevaporated with EtOH (twice). The residue was used with no further purification. The residue was dissolved in H_2O (20 mL) and the pH adjusted to 8.5 with Et_3N. Fmoc-OSu (1.7 g, 5.1 mmol) in CH_3CN (20 mL) was added, and the pH was kept at 8.5 with Et_3N. The reaction was neutralized with HCl, and the acetonitrile was removed in vacuo. $KHSO_4$ (1 M, 50 mL) was added and the mixture extracted with EtOAc. The residue was purified via column chromatography (eluents, 100/0.5 CH_2Cl_2/AcOH, gradient to 95/5/0.5 CH_2Cl_2/CH_3OH/AcOH) to give an oil (1.52 g, 76%): $R_f = 0.31$ (eluent, 95/5/1 CH_2Cl_2/CH_3OH/AcOH). ^1H NMR and MS confirmed its identity.

General Solid-Phase Synthesis Procedure. All washing and deprotection steps were performed with 3 volumes of the swelled resin: Fmoc deprotection, 20% piperidine in NMP (3 × 8 min); washing, NMP and then CH_2Cl_2 (3 × 2 min each). A Kaiser test was performed after each coupling and deprotection step. All coupling steps were performed with 4 equiv of amino acid, 4 equiv of BOP, and 8 equiv of iPr$_2$NEt, followed by washing. Coupling and deprotection procedures were performed twice as needed.

General Synthesis of Magainin−*Azide Derivatives (**6** and **7**) and Magainin 2 Amide (**8**)*. These compounds were synthesized using general SPPS procedures on an Argogel resin outfitted with a Rink linker (*19*). The amino acids used were N^{α}-Fmoc-protected, and the side chains were protected with an acid labile group. For azide-containing peptides, 3 equiv of Boc-Lys(N_3)-OH (**4**) or Fmoc-Lys(N_3)-OH (**5**) was used with 3 equiv of BOP and 6 equiv of iPr$_2$NEt for a reaction time of 2 h. Cleavage from the resin and deprotection of side chain protecting groups were achieved by treatment of the resin with a TFA/H_2O/TIS/EDT mixture (85/8.5/2/4.5) for 3 h. Under these cleavage conditions negligible azide reduction was observed. The acidic cleavage/deprotection mixture containing magainin or its azide derivatives was added dropwise to MTBE/hexane (1/1, −20 °C); the precipitate was centrifuged and washed with cold MTBE/hexane (1/1, four times). Preparative HPLC was performed, and peptides were analyzed by MS and pure according to HPLC and were isolated as TFA salts. Lyophilization afforded white powders. **6**: retention time, 17.78 min; MS *m*/*z*, 874.2 $[M + 3H]^{3+}$, 1310.4 $[M + 2H]^{2+}$. **7**: retention time, 18.23 min; MS *m*/*z*, 874.6 $[M + 3H]^{3+}$, 1311.1 $[M + 2H]^{2+}$, 1746.7 $[2M + 3H]^{3+}$. **8**: MS *m*/*z*, 823.2 $[M + 3H]^{3+}$, 1233.4 $[M + 2H]^{2+}$, 1644.2 $[2M + 3H]^{3+}$.

*N-Terminal Divalent Magainin (**9**)*. To **6** (20 mg, 6.3 μmol) was added a 3/1 H_2O/DMF mixture (400 μL). **1** (128 μL, 4.5 mg/mL DMF) was added, and subsequently, $CuSO_4$ (7 μL, 0.1 M) and NaAsc (100 μL, 2.9 mg/mL H_2O) were added. The mixture was subjected to microwave heating for 20 min at a constant temperature of 80 °C. The solvents were removed in vacuo and coevaporated with toluene (2 × 2 mL). The crude mixture was subjected to preparative HPLC, and **9** was purified and lyophilized to give 5.3 mg of a white powder in a yield of 33%. The identity of **9** was confirmed with MS: retention time, 17.7 min; MS *m*/*z*, 915.0 $[M + 6H]^{6+}$, 1098.3 $[M + 5H]^{5+}$, 1371.2 $[M + 4H]^{4+}$, 1828.1 $[M + 3H]^{3+}$.

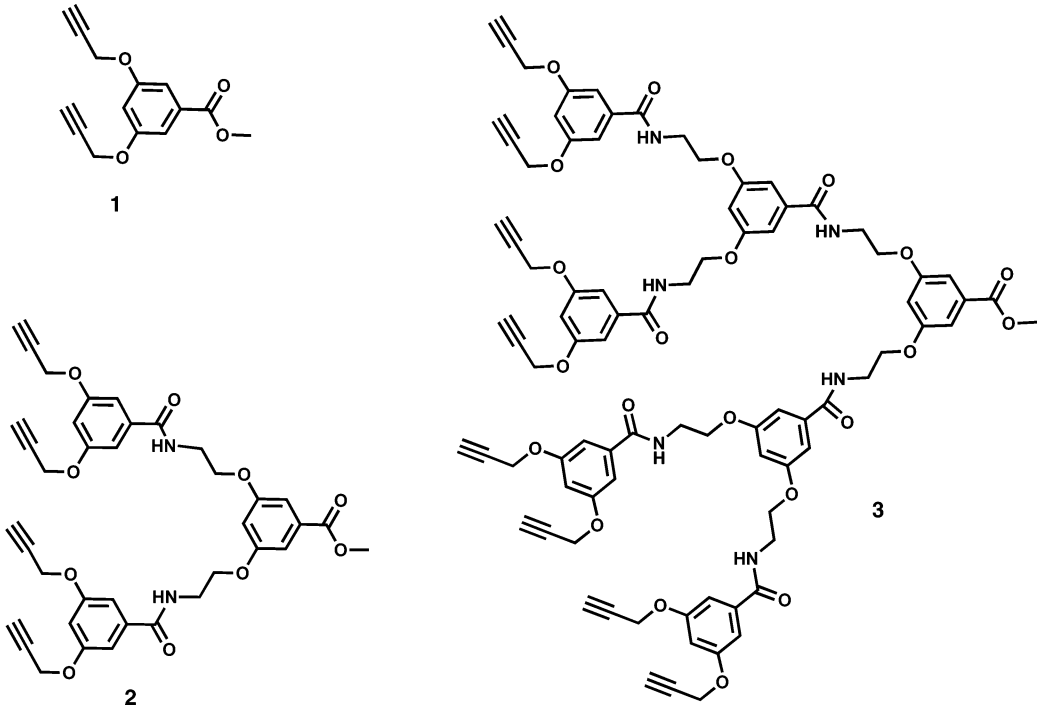

FIGURE 1: Structures of alkyne-functionalized dendrimers **1**−**3**.

*C-Terminal Divalent Magainin (**10**).* To **7** (15 mg, 4.7 μmol) was added a 3/1 H_2O/DMF mixture (400 μL). **1** (138 μL, 3.2 mg/mL DMF) was added, and subsequently, $CuSO_4$ (10 μL, 0.1 M) and NaAsc (72 μL, 2.9 mg/mL H_2O) were added. The mixture was subjected to microwave heating for 20 min at a constant temperature of 80 °C. The solvents were removed in vacuo and coevaporated with toluene (2 × 2 mL). The crude mixture was subjected to preparative HPLC. **10** was purified and lyophilized to give 4.8 mg of a white powder in a yield of 41%. The identity of **10** was confirmed with MS: retention time, 18.8 min; MS *m*/*z* 687.6 $[M + 8H]^{8+}$, 784.6 $[M + 7H]^{7+}$, 914.9 $[M + 6H]^{6+}$, 1098.5 $[M + 5H]^{5+}$, 1371.6 $[M + 4H]^{4+}$, 1828.3 $[M + 3H]^{3+}$.

*N-Terminal Tetravalent Magainin (**11**).* To **6** (2.4 mg, 0.75 μmol) was added a 1/1 H_2O/*t*BuOH mixture (3 mL). **2** (67 μL, 1.58 mg/mL DMF) was added, and subsequently, $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol) and NaAsc (0.5 mL, 1/1 8 mg/mL H_2O/*t*BuOH). The mixture was subjected to microwave heating for 10 min at a constant temperature of 80 °C. After the mixture had been heated, the precipitate was collected by centrifugation and washed with a mixture of H_2O (2 mL) and HPLC buffer A (95/5/0.1 H_2O/CH_3CN/TFA, 1 mL). The white precipitate was collected and lyophilized for 1.2 mg of a white powder (57%). The product was analyzed using SDS−PAGE (18%), with a stacking gel of 4%. The voltage was set to 30 V for 30 min and then to 200 V for 40 min. A Coomassie blue stain was used to visualize the product, in which the main product corresponded to a MW of ∼11000. There was also a minor product visible at a MW of ∼22000.

*N-Terminal Octavalent Magainin (**12**).* To **6** (4.7 mg, 1.47 μmol) was added a 1/1 H_2O/*t*BuOH mixture (3 mL). **3** (117 μL, 2.05 mg/mL DMF) was added, and subsequently, $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol) and NaAsc (0.5 mL, 1/1 8 mg/mL H_2O/*t*BuOH) were added. The mixture was subjected to microwave heating for 10 min at a constant temperature of 80 °C. After the mixture had been heated, the precipitate was collected by centrifugation and washed with a mixture of H_2O (5 mL) and HPLC buffer A (95/5/0.1 H_2O/CH_3CN/TFA, 2 mL). The white precipitate was collected and lyophilized to give 3.2 mg of a white powder (93%). The product was analyzed using SDS−PAGE (18%), with a stacking gel of 4%. The voltage was set to 30 V for 30 min and then to 200 V for 40 min. A Coomassie blue stain was used to visualize the product, in which the main product corresponded to a MW of ∼22000. Other product bands corresponded to MWs of ∼44000 and ∼88000.

RESULTS

One of the most active magainin peptides, magainin 2 (*3*), was made divalent, tetravalent, and octavalent via a copper-mediated 1−3 dipolar cycloaddition reaction ("click" chemistry) (*13*). This chemistry was previously explored in our hands and proved to be convenient for the conjugation of both carbohydrates and peptide structures (*11*, *15*, *16*). Dendrimers based on the 3,5-bis(2-aminoethoxy)benzoic acid repeating unit (*20*) were outfitted with alkyne groups as described previously (*15*,*16*) to yield compounds **1**−**3** (Figure 1). With these alkyne-functionalized dendrimers in hand, magainin peptides outfitted with an azide functionality at their N- or C-terminus were prepared using standard solid-phase synthesis. The azido amino acid Boc-Lys(N_3)-OH **4** (*21*) was linked to the N-terminus of the magainin peptide, while the Fmoc-protected derivative Fmoc-Lys(N_3)-OH **5** was used for incorporation on the C-terminal side of the magainin peptide. The azide-functionalized magainins **6** and **7** as well as magainin 2 amide **8** were purified with preparative HPLC, and mass spectrometry confirmed their identity (Figure 2).

Conjugation of magainin building blocks **6** and **7** to the dendrimers was achieved using a number of different reaction conditions (Figure 3). In general, organic/aqueous solvent mixtures were used to facilitate the solubility of both the

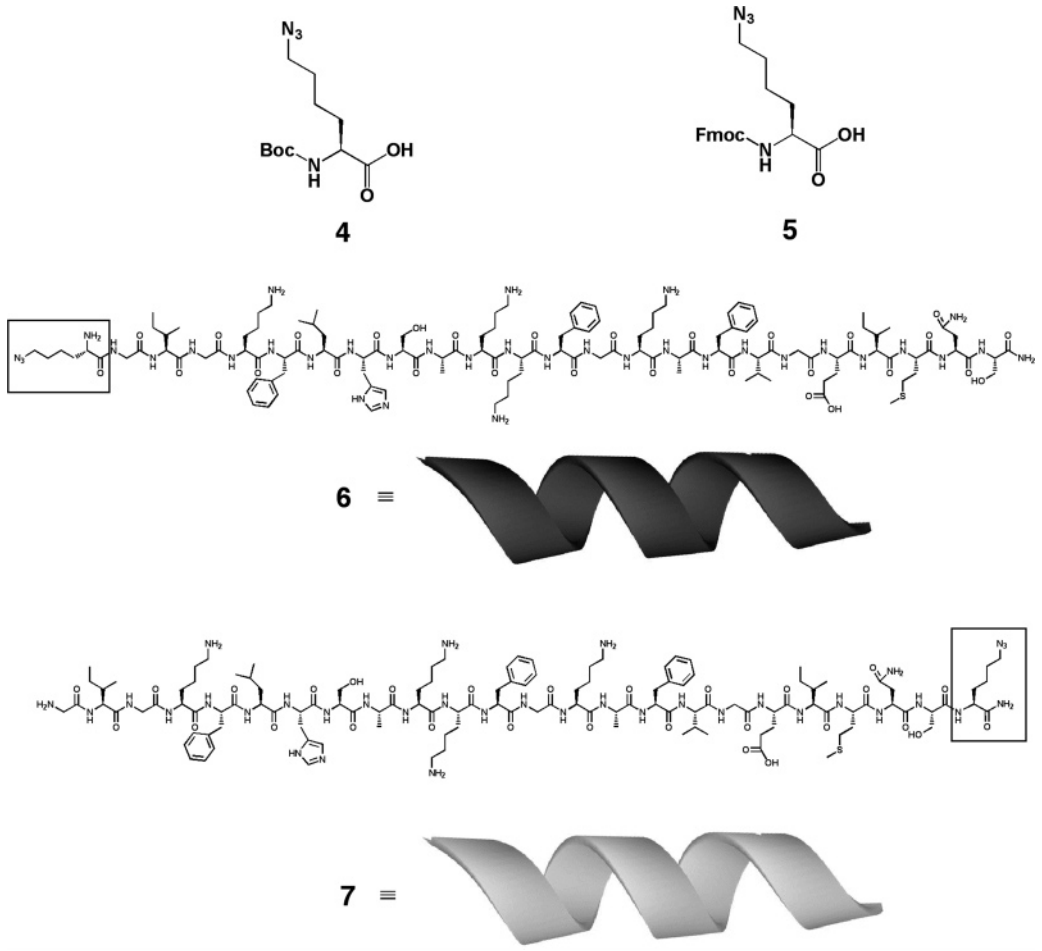

FIGURE 2: Structures of modified magainin peptides **6** and **7** represented by helices and lysine azide compounds **4** and **5**.

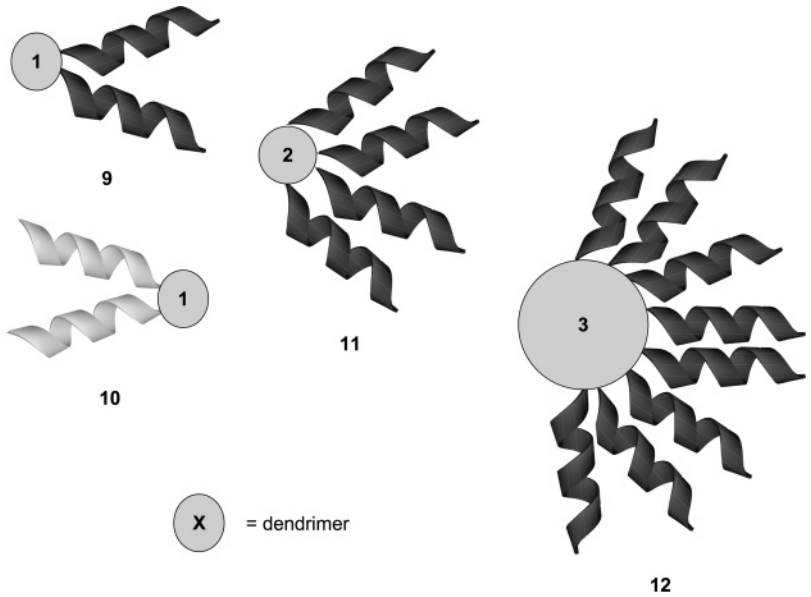

FIGURE 3: Schematic representations of multivalent magainin compounds **9**−**12**.

dendrimers and the peptides. The peptides were added in excess to ensure complete reactions, and varying amounts of $CuSO_4$ and sodium ascorbate were used for generation of the Cu(I) in situ. The reactions were performed in the microwave at a constant temperature of 80 °C for reaction times ranging from 5 to 20 min. Divalent peptides, derived from the N-linked peptide **6** and C-linked peptide **7**, were synthesized using 1.3 equiv of the magainin azide per alkyne and afforded magainin dimers **9** and **10**. The divalent compounds were purified with preparative HPLC, and their identities were confirmed with mass spectrometry. Remarkably, with tetravalent **11** and octavalent **12**, a white precipitate resulted after microwave heating of the reaction mixtures. The precipitate was collected and identified as the product, whereas the excess reagents were observed to stay in the supernatant solution. These products could be visualized

Table 1: Leakage Results for 5(6)-Carboxyfluorescein from Mixed DOPC/DOPG Vesicles (25 μM, 1/1)

compound	L_{50}[a] (nM)	relative potency (per peptide)	molecular mass (kDa)	real potency (μg/mL)
octavalent (**12**)	14	107 (13.4)	22.5	0.31
tetravalent (**11**)	130	11.5 (2.9)	11.2	1.49
divalent (**10**)	230	6.5 (3.25)	5.48	1.26
divalent (**9**)	250	6 (3)	5.48	1.37
magainin (**8**)	1500	1 (1)	2.47	3.7

[a] The L_{50} value represents the concentration at with 50% leakage was observed.

Table 2: Leakage Results for 5(6)-Carboxyfluorescein from DOPC Vesicles (25 μM)

compound	L_{50}[a] (nM)	relative potency (per peptide)	molecular mass (kDa)	real potency (μg/mL)
octavalent (**12**)	17	588 (73)	22.5	0.38
tetravalent (**11**)	22	454 (113)	11.2	0.25
divalent (**10**)	250	40 (20)	5.48	1.37
divalent (**9**)	370	27 (13.5)	5.48	2.03
magainin (**8**)	10000	1	2.47	24.7

[a] The L_{50} value represents the concentration at with 50% leakage was observed.

using an 18% SDS−PAGE gel and Coomassie staining, and the bands were visible at the expected position according to their size (not shown).

Leakage Experiments with the Multivalent Magainin Peptides. Large unilamellar vesicles (LUVs) composed of the neutral phospholipid DOPC or a 1/1 molar mixtue of DOPC with the negatively charged phospholipid DOPG were prepared containing 5(6)-carboxyfluorescein. These LUVs were treated with the multivalent magainin compounds (Tables 1 and 2). The potency of the various compounds was expressed as an L_{50} value, defined as the concentration of the compound required to effect 50% of the maximal obtainable leakage of the 5(6)-carboxyfluorescein from the LUVs of that compound.

In the leakage studies, the magainin peptide that was functionalized with the azide (compound **6**) was slightly less active than the magainin peptide itself. The N-linked dimer **9** and the C-linked dimer **10** exhibited similar potencies in the range of 230−370 nM with both the DOPC vesicles and the mixed 1/1 DOPC/DOPG vesicles. Compared to that of magainin, an increase in activity was observed, even when we corrected for the number of peptides that were present. This corrected increase was much more pronounced with the DOPC vesicles (13.5−20-fold) than with the DOPC/DOPG vesicles (3−3.25-fold).

With the DOPC LUVs, tetravalent **11** and octavalent **12** were similarly active and were measured to have L_{50} values of 22 and 17 nM, respectively (Figure 4). This represents a 113-fold (per peptide) potency increase over magainin **8** for the tetravalent compound and a 100-fold enhancement when expressed in micrograms per milliliter. With the DOPC/DOPG LUVs, the most potent compound was shown to be octamer **12**, exhibiting an L_{50} activity of 14 nM, a 13.4-fold (per peptide) increase in activity compared to that of magainin **8**.

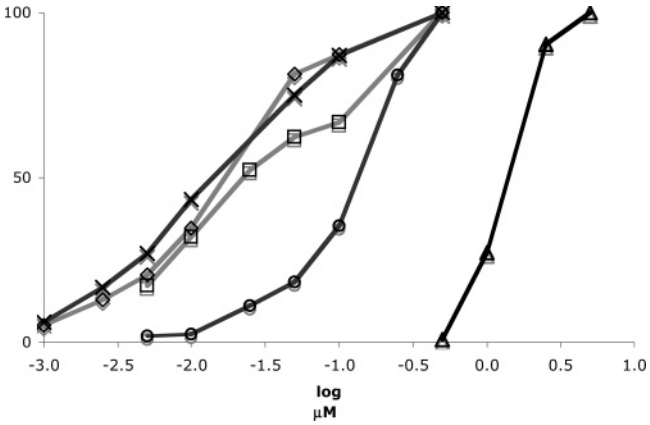

FIGURE 4: Normalized leakage results of compounds **11** and **12** compared with magainin **8** with LUVs consisting of DOPC and a 1/1 mixture of DOPC and DOPG: (□) tetravalent **11** with DOPC, (◇) octavalent **12** with DOPC, (○) tetravalent **11** with DOPC and DOPG, (×) octavalent **12** with DOPC and DOPG, and (△) monovalent **8** with DOPC and DOPG.

DISCUSSION

Our aim of this study was to overcome the assembly barrier antimicrobial peptides like the magainins have in the lipid membrane. The 23-amino acid antimicrobial peptide magainin 2 amide was made multivalent by incorporation of an azide functionality into the peptide sequence and coupling it to dendrimers bearing alkyne moieties via a copper-mediated 1−3 dipolar cycloaddition reaction (click chemistry). These peptidodendrimers provided a means of testing the differences in the membrane disrupting ability of the dimer, tetramer, and octamer of magainin 2. Large increases in pore forming capabilities were observed with especially the tetravalent and octavalent magainin compounds in both the DOPC and DOPC/PG LUVs and caused the model membranes to leak in the low nanomolar range. The number of peptides required to cause a significant improvement in leakage was different for the two membrane types. In the lipid system that consisted of a mixture of neutral lipids (DOPC) and negatively charged lipids (DOPG), the octamer exhibited the most significant improvement. In comparison to the membranes consisting of purely neutral lipids (DOPC), already at the level of the tetramer optimal pore formation was reached. Clearly, the nature of the pore seems to be membrane-dependent. It is intriguing to note that for the neutral DOPC vesicles the enhancement was larger, since magainin itself has little affinity for the neutral membrane but the multivalent derivatives are just as active as on the charged DOPC/DOPG LUVs. The origin of this effect is not yet known, but the lipophilic dendrimer portion of the molecule may contribute to this effect; otherwise, the proximity of the peptide presentation by the dendrimer may facilitate amphipathic helix formation of the peptides and thus enlarge its exposed lipophilic area.

Recent computer modeling studies suggested that four magainin peptides are enough to effectively form a pore in lipid bilayers (*22*). Our results indicate that pore formation can be greatly improved using between four and eight peptide monomer units, depending on the lipid composition. The magainin (tetravalent and octavalent) compounds were found to be the most effective constructs made, which caused leakage of the model membranes at concentrations similar to that of the targeted pore-forming antibiotic nisin when using Lipid II-containing vesicles (data not shown). Addition of a targeting module to these dendrimer systems would

perhaps even further improve the pore forming ability by increasing the affinity of the constructs for the membrane. We are currently pursuing this by attaching different targeting modules. Possible applications for such an "adaptable targeted pore forming" construct may include, besides antimicrobial or antifungal agents, other applications such as antitumor agents.

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REFERENCES

1. Leeb, M. (2004) A shot in the arm, *Nature 431*, 892−893.
2. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms, *Nature 415*, 389−395.
3. Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor, *Proc. Natl. Acad. Sci. U.S.A. 84*, 5449−5453.
4. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H., and de Kruijff, B. (1999) Use of the Cell Wall Precursor Lipid II by a Pore-Forming Peptide Antibiotic, *Science 286*, 2361−2364.
5. Hsu, S. T. D., Breukink, E., Tischenko, E., Lutters, M. A. G., de Kruijff, B., Kaptein, R., Bonvin, A. M. J. J., and van Nuland, N. A. J. (2004) The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics, *Nat. Struct. Mol. Biol. 11*, 963−967.
6. Dempsey, E. C., Ueno, S., and Avison, M. B. (2003) Enhanced membrane permeabilization and antibacterial activity of a disulfide-dimerized magainin analogue, *Biochemistry 42*, 402−409.
7. Tam, J. P., Lu, Y.-A., and Yang, J.-L. (2002) Antimicrobial dendrimeric peptides, *Eur. J. Biochem. 269*, 923−932.
8. Liu, Z., Deshazer, H., Rice, A. J., Chen, K., Zhou, C., and Kallenbach, N. R. (2006) Multivalent antimicrobial peptides from a reactive polymer scaffold, *J. Med. Chem. 49*, 3436−3439.
9. Zhang, Z., Merritt, E. A., Ahn, M., Roach, C., Hou, Z., Verlinde, C. L. M. J., Hol, W. G. J., and Fan, E. (2002) Solution and crystallographic studies of branched multivalent ligands that inhibit the receptor-binding of cholera toxin, *J. Am. Chem. Soc. 124*, 12991−12998.
10. Kitov, P. I., Sadowska, J. M., Mulvey, G., Arnstrong, G. D., Ling, H., Pannu, N. S., Read, R. J., and Bundle, D. R. (2000) Shiga-like toxins are neutralized by tailored multivalent carbohydrate ligands, *Nature 403*, 669−672.
11. Pukin, A. V., Branderhorst, H. M., Sisu, C., Weijers, C. A. G. M., Gilbert, M., Liskamp, R. M. J., Visser, G. M., Zuilhof, H., Pieters, R. J. (2007) Strong Inhibition of Cholera Toxin by Multivalent GM1 Derivatives, *ChemBioChem 8*, 1500−1503.
12. Pieters, R. J. (2007) Intervention with Bacterial Adhesion by Multivalent Carbohydrates, *Med. Res. Rev. 27*, 796−816.
13. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A Stepwise Huisgen Cycloaddition Process: Copper-(I)-Catalyzed Regioselective Ligation of Azides and Terminal Alkynes, *Angew. Chem., Int. Ed. 41*, 2596−2599.
14. Tornoe, C. W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on Solid Phase: [1,2,3]-Triazoles by Regiospecific Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions of Terminal Alkynes to Azides, *J. Org. Chem. 67*, 3057−3069.
15. Joosten, J. A. F., Tholen, N. T. H., Ait El Maate, F., Brouwer, A. J., van Esse, G. W., Rijkers, D. T. S., Liskamp, R. M. J., and Pieters, R. J. (2005) High yielding microwave assisted synthesis of triazole-linked glycodendrimers via copper catalyzed [3+2] cycloaddition, *Eur. J. Org. Chem.*, 3182−3185.
16. Rijkers, D. T. S., van Esse, G. W., Merkx, R., Brouwer, A. J., Jacobs, H. J. F., Pieters, R. J., and Liskamp, R. M. J. (2005) Efficient microwave-assisted synthesis of multivalent dendrimeric peptides using cycloaddition reaction (click) chemistry, *Chem. Commun.*, 4581−4583.
17. Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J. C., Kuipers, O. P., and de Kruijff, B. (1997) The C-Terminal Region of Nisin Is Responsible for the Initial Interaction of Nisin with the Target Membrane, *Biochemistry 36*, 6968−6976.
18. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids 5*, 494−496.
19. Rink, H. (1987) Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin, *Tetrahedron Lett. 28*, 3787−3790.
20. Brouwer, A. J., Mulders, S. J. E., and Liskamp, R. M. J. (2001) Convergent Synthesis and Diversity of Amino Acid Based Dendrimers, *Eur. J. Org. Chem.*, 1903−1915.
21. Link, A. J., Vink, M. K. S., and Tirrell, D. A. (2004) Presentation and Detection of Azide Functionality in Bacterial Cell Surface Proteins, *J. Am. Chem. Soc. 126*, 10598−10602.
22. Leontiadou, H., Mark, A. E., and Marrink, S. J. (2006) Antimicrobial Peptides in Action, *J. Am. Chem. Soc. 128*, 12156−12161.

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