The Vancomycin−Nisin(1−12) Hybrid Restores Activity against Vancomycin Resistant Enterococci[†]

Christopher J. Arnusch,[‡,§] Alexandre M. J. J. Bonvin,[⊥] Anne Marie Verel,[||] Wouter T. M. Jansen,[||] Rob M. J. Liskamp,[§] Ben de Kruijff,[‡] Roland J. Pieters,*[,§] and Eefjan Breukink*[,‡]

Department of Chemical Biology and Organic Chemistry, Institute of Biomembranes, and Department of NMR Spectroscopy, Bijvoet Center for Biomolecular Research, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands, Department of Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands, and Department of Medical Microbiology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands

Received August 25, 2008; Revised Manuscript Received October 27, 2008

ABSTRACT: Lipid II is a crucial component in bacterial cell wall synthesis [Breukink, E., et al. (1999) *Science 286*, 2361−2364]. It is the target of a number of important antibiotics, which include vancomycin and nisin [Breukink, E., and de Kruijff, B. (2006) *Nat. Rev. Drug Discovery 5*, 321−332]. Here we show that a hybrid antibiotic that consists of vancomycin and nisin fragments is significantly more active than the separate fragments against vancomycin resistant entercocci (VRE). Three different hybrids were synthesized using click chemistry and compared. Optimal spacer lengths and connection points were predicted using computer modeling.

Vancomycin and nisin are members of two very different classes of antibiotics that both target the essential cell wall precursor lipid II. The glycopeptide vancomycin binds with high affinity to the tripeptide part (Lys-D-Ala-D-Ala) of lipid II (*3*), which is also present in the immature cell wall prior to cross-linking. The N-terminal fragment (residues 1−12) of the antimicrobial peptide nisin binds the unique pyrophosphate part present on lipid II (*4*). The molecular mechanism of action of both antibiotics is very different but starts in both cases with the aforementioned noncovalent binding to lipid II.

Upon binding, vancomycin mainly prevents the growing oligosaccharide cell wall structure from strengthening through peptide cross-linking. This results in bacterial cell death due to osmotic pressure. Resistance to vancomycin (vanA or vanB type resistance) due to a substitution of D-Lac for the terminal D-Ala residue results in a decreased level of vancomycin binding and therefore decreased efficacy (*5*). One approach for improving or modifying the activity of vancomycin has focused on peripheral synthetic modifications of vancomycin. Hydrophobic appendages have been the most successful modifications and have altered the mode of action of vancomycin (*6, 7*). A second approach has been the synthesis of vancomycin mimics (*8, 9*). This approach has mainly focused on repairing the disrupted ligand binding by modification of the carboxylate binding pocket in small constrained peptides that mimic vancomycin, therefore attempting to restore the original mode of action of vancomycin, although by binding strongly to D-Lac.

The mode of action of nisin is a so-called targeted pore forming mechanism that involves the N-terminal part of nisin [nisin(1−12)]. This part binds to the pyrophosphate portion of the lipid II molecule through five hydrogen bonds. Subsequently, when the C-terminal fragment of nisin inserts into the membrane and associates with other lipid II−nisin complexes, lethal pore formation occurs (*10*). The lantibiotic mutacin 1140, which is structurally very similar, especially the N-terminal part, also binds to lipid II. However, for this antibiotic, the subsequent pore formation is not observed (*11*). High-affinity binding to lipid II, whether or not it leads to pore formation, is thus effective in disrupting the cell wall synthesis pathway. Nevertheless, the peptide fragment nisin(1−12) alone has lost most of its antibiotic activity (*12*). However, this fragment is still able to inhibit the pore forming activity of nisin in model systems (*13*), indicating that it maintains the ability to bind lipid II.

Within this context, we were interested in combining the unique binding ability of vancomycin and nisin(1−12) for the peptide and pyrophosphate part of lipid II. Via combination of both moieties in a single molecule, simultaneous lipid II binding of the two parts could lead to increased affinity through a bivalency or chelate effect. Multivalency has been shown to be a design principle in which the combination of multiple weak interactions has led to very strong affinities (*14*). We hypothesized that enhanced interaction with lipid II could restore antibiotic efficacy against resistant strains such as VRE. The extra affinity gained through pyrophosphate binding due to the nisin(1−12) fragment should compensate for the decreased affinity of vancomycin for the altered lipid II peptide sequences.

Many different vancomycin−nisin(1−12) conjugates could be envisioned due to at least three synthetically available connection points on vancomycin, and at least three on the nisin(1−12) peptide structure. For vancomycin, these include the vancosamine NH_2 group and the C- or N-terminus. For

[†] These investigations were supported by the council for Chemical Sciences of The Netherlands-Organization for Scientific Research (CW-NWO).

* To whom correspondence should be addressed. R.J.P.: phone, +31302536944; fax, +31-302536655; e-mail, r.j.pieters@uu.nl. E.B.: phone, +31-302533523; fax, +31-302533969; e-mail, e.j.breukink@uu.nl.

[‡] Institute of Biomembranes, Utrecht University.

[§] Utrecht Institute for Pharmaceutical Sciences, Utrecht University.

[⊥] Department of NMR Spectroscopy, Utrecht University.

[||] University Medical Center Utrecht.

10.1021/bi801597b CCC: $40.75
Published on Web 11/07/2008

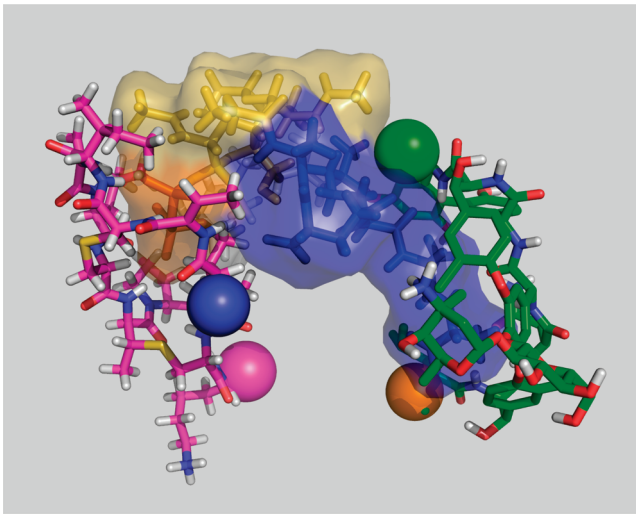

FIGURE 1: Model of the the ternary complex of nisin(1−12) (pink), vancomycin (green), and lipid II (transparent surface representation with the pyrophosphate group to which nisin binds colored orange and the peptide moiety to which vancomycin binds colored blue). The blue and pink spheres represent the N- and C-termini of nisin(1−12), respectively, and the green and orange spheres the C- and N-termini of vancomycin, respectively.

nisin(1−12), the C- or N-terminus and the NH_2 group of Lys(12) were available. The number of possibilities can be further compounded by the type and length of available linkers. Computer modeling was used to investigate the feasibility of simultaneous binding of the two components and to predict suitable connection points. Data-driven docking with HADDOCK generated a model of a ternary complex between lipid II and unconnected vancomycin and nisin(1−12) (*15*). The model (Figure 1) revealed that both nisin and vancomycin can bind simultaneously to lipid II since their target sites are sufficiently separated. The linkages that were judged to be the most suitable were those from the C-terminus of nisin(1−12) to either the N- or C-terminus of vancomycin. From the ensemble of generated low-energy structures, an average distance of 8 ± 1 or 12 ± 1 Å was calculated for each connection, respectively. The N-terminus of nisin(1−12) was not chosen to be modified due to previous work that showed that modification at this site on full-length nisin caused activity loss (*16, 17*). On the basis of this information, suitable hybrid antibiotics were synthesized with chosen actual spacer lengths corresponding to 26, 13, and 11 chemical bond lengths for compounds **5**, **6**, and **7**, respectively. Tethers with similar chemical composition and size (9−15 bond lengths) have been reported to have mean lengths of 10−14 Å (*18*). The long linker in compound **5** contains three PEG repeats and may have more of a PEG-like character for which the length can be difficult to predict. Therefore, we also used the Flory equation to estimate the length of this linker (*19*). Using this equation to calculate the length of a similarly sized (in chemical bond length) PEG spacer (PEG 370) resulted in a length of 12.2 Å (see the Supporting Information). Since PEG 370 contains eight PEG units while our compound **5** contains only three, the length of 12.2 Å would be the minimum length for the linker in compound **5** and thus well suited to cover the estimated distance.

Click chemistry (*20, 21*) was used for the conjugation of the two components; thus, each antibiotic fragment was

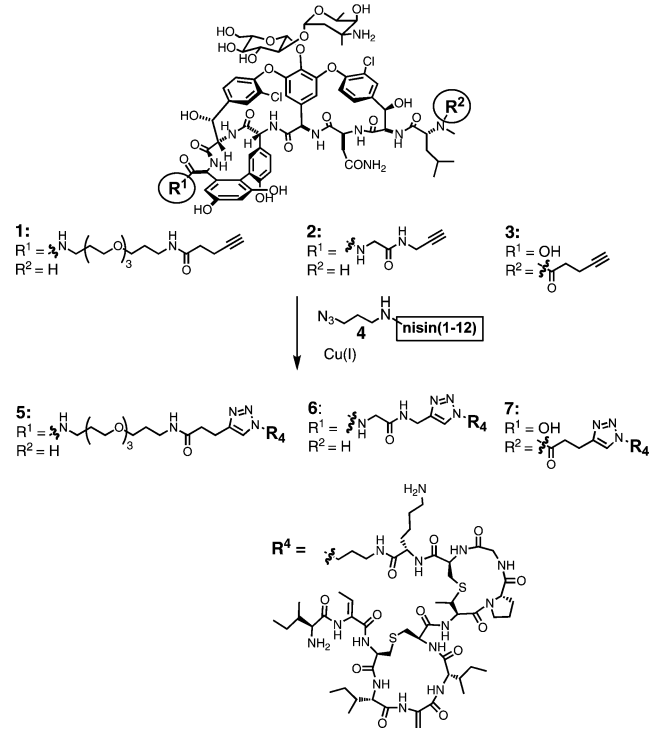

FIGURE 2: Structure of vancomycin alkyne derivatives **1**−**3**, nisin(1−12) azide derivative **4**, and the preparation of vancomycin−nisin(1−12) conjugates **5**−**7**.

outfitted with either an alkyne or an azide group. The C-terminally modified vancomycin alkyne derivatives **1** and **2** (Figure 2) were prepared by standard solution-phase peptide synthesis using BOP or EDC/HOAt as the coupling reagents and purified using preparative HPLC (see the Supporting Information). The N-terminally modified vancomycin derivative **3** was synthesized using PyBroP according to a published procedure which enables selective coupling (*22*). Tandem mass spectroscopy (MS/MS) clearly confirmed the product identities and showed the alkyne linkers to be part of the peptide portion of the molecule. The nisin(1−12) fragment bearing an azido group linked to the C-terminus, compound **4**, was synthesized starting with a trypsin digestion of nisin and purification of the nisin(1−12) fragment using preparative HPLC. This product was treated with a large excess of 1-amino-3-azidopropane with BOP and DIPEA in a small volume of DMF for 15 min. HPLC purification afforded compound **4**, and MS/MS confirmed the identity of the product, which showed that the azide was successfully coupled to the C-terminus of the nisin(1−12) fragment. More dilute reactions, longer reaction times, or fewer equivalents of 1-amino-3-azidopropane resulted in lower yields. Vancomycin−nisin conjugates **6** and **7** were obtained by mixing the appropriate vancomycin alkyne derivative and compound **4** in a ratio of 1:1 in H_2O containing a small amount of DMF, and heating in a microwave at 80 °C in the presence of $CuSO_4$ and sodium ascorbate for 5−10 min. Compound **5** was synthesized by mixing **1** with **4** under similar conditions. Purification was achieved by using HPLC, and MALDI confirmed the identity of the products.

The compounds were tested in an inhibition assay against a panel of bacteria which included VSE (vancomycin susceptible enterococci) and VRE (vancomycin resistant enterococci). Indeed, an activity increase for the hybrid antibiotics was observed. The most significant increase was

Table 1: MIC Values [Micrograms per Milliliter or Micromolar (Bold)] of the Vancomycin−Nisin(1−12) Derivatives

	N[a]	V[b]	**3**	**5**	**6**	**7**
VSE (15A797)	128	0.5	16	2	8	21
	101	**0.3**	**10**	**0.6**	**2.5**	**7.2**
VRE (15A799)	128	128	<256	8	32	43
	101	**86**	**<167**	**2.3**	**9.9**	**14.4**
Moraxella catarrhalis (58L028)	256	32	256	16	32	<91
	202	**22**	**167**	**4.6**	**9.9**	**<30.6**

[a] N represents nisin(1−12). [b] V represents vancomycin.

for compound **5** with an MIC value of 8 μg/mL (2.3 μM) for VRE (Table 1). The hybrid is significantly more active than each of the components separately. The MICs for nisin(1−12) and vancomycin were 128 μg/mL, which corresponds to 101 and 86 μM, respectively. In terms of the MIC expressed in micromolar, the hybrid antibiotic is approximately 40 times more active than the components. Interestingly, compound **5** exhibited an activity of 16 μg/mL against Gram-negative species *Klebsiella pneumoniae* where vancomycin was inactive (see the Supporting Information). Compound **7** showed mostly decreased activity probably due to the derivatization on the N-terminus of vancomycin, which could interfere with vancomycin−ligand binding. Indeed, the alkyne vancomycin fragment **3** also showed decreased antibiotic efficacy compared with vancomycin. Of note is the fact that no improvement was observed in VSE. Compound **5** exhibited similar MIC values with vancomycin (0.6 and 0.3 μM, respectively); however, compounds **6** and **7** show slightly decreased activities. By considering the two ways by which vancomycin can inhibit the bacterial cell wall synthesis, we can provide a possible explanation for this effect. By binding to the D-Ala-D-Ala sequence of newly synthesized peptidoglycan strains, vancomycin inhibits the transpeptidation reaction of the peptidoglycan synthesis. Additionally, by binding to the D-Ala-D-Ala sequence present in lipid II, it also is able to inhibit the transglycosylation reaction (*7*). In vancomycin sensitive strains, the inhibition of the transpeptidation reaction is likely to be dominant due to the relative abundance of binding sites for vancomycin on the newly synthesized peptidoglycan strands (that are not cross-linked yet) versus the amount of binding sites that is available on the single lipid II molecules. Thus, the inhibition of the vancomycin portion of the hybrid is dominating the activity of this molecule in case of vancomycin sensitive bacteria, resulting in similar MICs. However, this situation is reversed in VRE where the vancomycin part of the hybrid has lost most of its affinity for the available D-Ala-D-Lac binding sites, while the presence of the nisin part makes sure that the hybrid still has affinity for lipid II in these strains. This results in an increased MIC for VRE strains with respect to vancomycin alone.

Multivalency is often a good way to increase binding affinity (provided that there is more than one binding site available) (*23*−*25*). Here we have built a hybrid antibiotic from two existing ones that have the same target but bind to different sites. The anticipated heterobivalent interaction was supported by molecular modeling. The most effective of the three synthesized hybrids was compound **5**, which contained a long flexible linker and was ∼40 times more active than its components against VRE.

ACKNOWLEDGMENT

Annemarie Dechesne is gratefully acknowledged for mass spectrometry analysis and Kawe Bitaraf for synthetic work on compound **6**.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures, methods, and modeling details. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H., and de Kruijff, B. (1999) *Science 286*, 2361–2364.
2. Breukink, E., and de Kruijff, B. (2006) *Nat. Rev. Drug Discovery 5*, 321–332.
3. Barne, J. C., and Williams, D. H. (1984) *Annu. Rev. Microbiol. 38*, 339–357.
4. Hsu, S. T., Breukink, E., Tischenko, E., Lutters, M. A., de Kruijff, B., Kaptein, R., Bonvin, A. M., and van Nuland, N. A. (2004) *Nat. Struct. Mol. Biol. 11* (10), 963–967.
5. Arthur, M., and Courvalin, P. (1993) *Antimicrob. Agents Chemother. 37*, 1563–1571.
6. Higgins, D. L., Chang, R., Debabov, D. V., Leung, J., Wu, T., Krause, K. M., Sandvik, E., Hubbard, J. M., Kaniga, K., Schmidt, D. E., Jr., Gao, Q., Cass, R. T., Karr, D. E., Benton, B. M., and Humphrey, P. P. (2005) *Antimicrob. Agents Chemother. 49* (3), 1127–1134.
7. Leimkuhler, C., Chen, L., Barret, D., Panzone, G., Sun, B., Falcone, B., Oberthür, M., Donadio, S., Walker, S., and Khane, D. (2005) *J. Am. Chem. Soc. 127*, 3250–3251.
8. Arnusch, C. J., and Pieters, R. J. (2003) *Eur. J. Org. Chem. 16*, 3131–3138.
9. Jia, Y. X., Ma, N. C., Liu, Z. S., Bois-Choussy, M., Gonzalez-Zamora, E., Malabarba, A., Brunati, C., and Zhu, J. P. (2006) *Chem.—Eur. J. 12* (20), 5334–5351.
10. Hasper, H. E., de Kruijff, B., and Breukink, E. (2004) *Biochemistry 43*, 11567–11575.
11. Smith, L., Hasper, H., Breukink, E., Novak, J., Cerkasov, J., Hillman, J. D., Wilson-Stanford, S., and Orugunty, R. S. (2008) *Biochemistry 47*, 3308–3314.
12. Chan, W. C., Leyland, M., Clark, J., Dodd, H. M., Lian, L. Y., Gasson, M. J., Bycroft, B. W., and Roberts, G. C. K. (1996) *FEBS Lett. 396*, 129–132.
13. Ghalit, N., Reichwein, J. F., Hilbers, H. W., Breukink, E., Rijkers, D. T., and Liskamp, R. M. J. (2007) *ChemBioChem 8*, 1540–1554.
14. Mammen, M., Chio, S.-K., and Whitesides, G. M. (1998) *Angew. Chem., Int. Ed. 37*, 2754–2794.
15. Dominguez, C., Boelens, R., and Bonvin, A. M. J. J. (2003) *J. Am. Chem. Soc. 125*, 1731–1737.
16. Bonev, B. B., Breukink, E., Swiezewska, E., De Kruijff, B., and Watts, A. (2004) *FASEB J. 18*, 1862–1869.
17. Kuipers, O. P., Rollema, H. S., de Vos, W. M., and Siezen, R. J. (1993) *FEBS Lett. 330*, 23–27.
18. Kitov, P. I., Shimizu, H., Hormans, S. W., and Bundle, D. R. (2003) *J. Am. Chem. Soc. 125*, 3284–3294.
19. Flory, P. (1971) *Principles of Polymer Chemistry*, Cornell University Press, Ithica, NY.
20. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) *Angew. Chem., Int. Ed. 41*, 2596–2599.
21. Tornoe, C. W., Christensen, C., and Meldal, M. (2002) *J. Org. Chem. 67*, 3057–3069.
22. Staroske, T., and Williams, D. H. (1998) *Tetrahedron Lett. 39*, 4917–4920.
23. Rao, J., Lahiri, J., Isaacs, L., Weis, R. M., and Whitesides, G. M. (1998) *Science 280*, 708–711.
24. Kramer, R. H., and Karpen, J. W. (1998) *Nature 395*, 710–713.
25. Pukin, A. V., Branderhorst, H. M., Sisu, C., Weijers, C. A. G. M., Gilbert, M., Liskamp, R. M. J., Visser, G. M., Zuilhof, H., and Pieters, R. J. (2007) *ChemBioChem 8*, 1500–1503.

BI801597B