J|A|C|S
COMMUNICATIONS

Published on Web 11/01/2002

Rethinking Ramoplanin: The Role of Substrate Binding in Inhibition of Peptidoglycan Biosynthesis

Jeremiah S. Helm, Lan Chen, and Suzanne Walker*

Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Received August 16, 2002

The emergence of bacteria resistant to clinically used antibiotics is a leading public health issue. As the clinical incidence of resistance increases, there is a corresponding increase in the need for new antibiotics with distinct modes of action. This need led us to investigate ramoplanin (Figure 1), a cyclic depsipeptide that was proposed in 1990 to inhibit the MurG step of peptidoglycan biosynthesis (Figure 2).[1−3] Ramoplanin was proposed to block the enzyme by binding to its substrate, Lipid I,[1d,e] but there was no direct evidence for this. Furthermore, there were no direct assays to monitor MurG activity and thus no way to test the hypothesis that inhibition is caused by substrate binding.[4] We have developed a direct assay to monitor the activity of *E. coli* MurG using synthetic Lipid I analogues[5] and report here that ramoplanin does not inhibit peptidoglycan synthesis by the proposed mechanism.

Inhibitors that block enzymatic activity by substrate depletion alone yield velocity versus substrate concentration curves that are sigmoidal, with almost no enzymatic activity at low substrate concentrations and no inhibition at high substrate concentrations.[6] Ramoplanin inhibition cannot be overcome with additional substrate, a result inconsistent with a mechanism involving substrate depletion (Figure 3a). This result implies that ramoplanin interacts directly with MurG.

To determine whether ramoplanin inhibits MurG alone or as a complex with Lipid I, we synthesized the alanine-modified derivatives **2** and **3**.[7] Each compound was tested for binding using a previously described NMR assay,[4a,8] and for MurG inhibition.[5] Compound **2** binds to Lipid I analogue **4**, but compound **3** does not, indicating that ornithine 10 is essential for recognition of peptidoglycan intermediates. Both **2** and **3** were found to inhibit MurG (IC_{50} = 20−25 μM). Because **3** is unable to bind Lipid I but inhibits MurG comparably to ramoplanin (IC_{50} = 20 μM), it is reasonable to conclude that ramoplanin also inhibits without binding substrate.

We prepared the fluorescein-labeled derivative **6** to confirm that ramoplanin binds directly to MurG. Like ramoplanin itself, **6** inhibits MurG at micromolar concentrations. We monitored the change in polarization of **6** with increasing concentrations of MurG in the absence of Lipid I (Figure 3b). The binding isotherm obtained fits a 1:1 binding mode with a K_d of 4 × 10^{-6} M, a value reasonably consistent with the IC_{50} value.[9]

Because ramoplanin has been shown to bind *both* Lipid I and Lipid II, we compared the binding of ramoplanin to analogues **4** and **5** to determine whether the antibiotic discriminates between these peptidoglycan intermediates (Figure 4). In the case of the Lipid II analogue **5**, the ramoplanin signals disappear before a full equivalent of the ligand has been added, whereas with the Lipid I analogue **4**, more than 2 equiv are required to achieve the same effect. Furthermore, resonances for both ramoplanin and **4** coexist throughout the titration. Although the NMR titrations provide only qualitative information, it is apparent that ramoplanin interacts more strongly with **5** than with **4**.

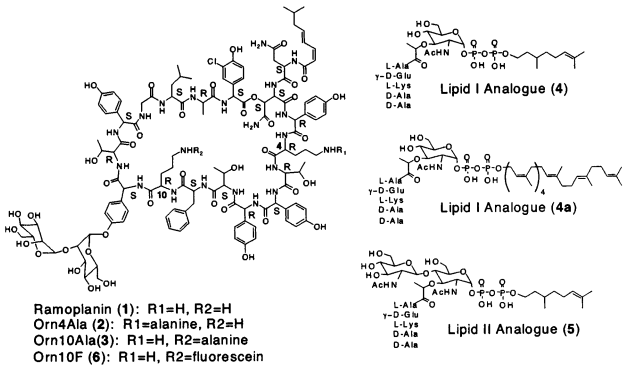

Figure 1. Structures of compounds referred to in the text.

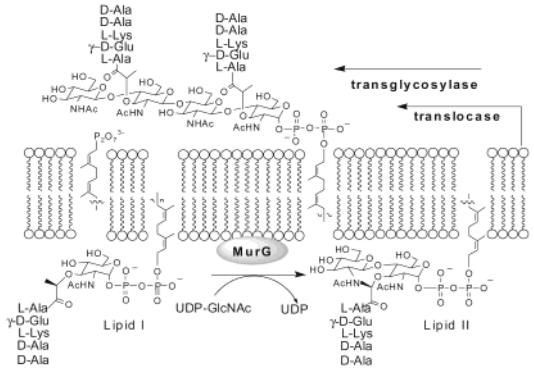

Figure 2. Latter stages of peptidoglycan biosynthesis.

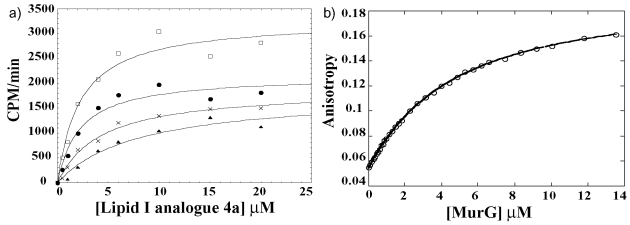

Figure 3. (a) Velocity versus [**4a**] curves for MurG at different concentrations of ramoplanin: 0 μM (□); 1 μM (●); 2 μM (×); and 5 μM (▲). (b) Anisotropy of **6** as a function of [MurG].

To evaluate whether substrate binding plays *any* role in biological activity, we measured the minimum inhibitory concentrations (MICs) of **2** and **3** against two representative Gram-positive bacterial strains.[10] Compound **2**, which is capable of binding peptidoglycan intermediates, is biologically active (MIC = 0.8 μg/mL), but compound **3**, which cannot bind, has almost no activity (MIC =

* To whom correspondence should be addressed. E-mail: swalker@princeton.edu.

10.1021/ja021097n CCC: $22.00

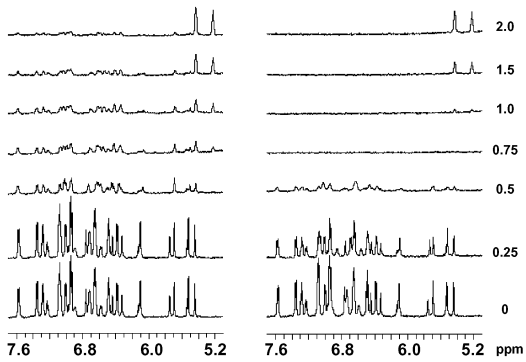

Figure 4. 600 MHz ^{1}H NMR spectra of downfield regions of ramoplanin (0.1 mM) in D_2O (pD = 6.9) at the indicated ratios of Lipid I analogue **4** (left) or Lipid II analogue **5** (right).

100 μg/mL). These results suggest that substrate recognition, while unnecessary for MurG inhibition, is important for in vivo activity.

It has been widely accepted that ramoplanin inhibits MurG by binding to the Lipid I substrate.[1d,e,4,11] The results described above disprove this hypothesis. However, the ability of ramoplanin to bind peptidoglycan intermediates does correlate with biological activity. Because ramoplanin binds better to Lipid II than to Lipid I, Lipid II is probably the relevant substrate to consider. There are two ways in which Lipid II binding could be involved in ramoplanin's biological activity. First, docking of ramoplanin to Lipid II on the external surface of the bacterial membrane could facilitate entry of the antibiotic into the bacterial cell where MurG is located. It is believed that several other antibiotics, including nisin and some vancomycin derivatives, combine an ability to bind Lipid II with a second mechanism of action.[12] Alternatively, ramoplanin could kill bacterial cells by blocking enzymes that use Lipid II as a substrate. We have previously shown that ramoplanin does, in fact, inhibit the bacterial transglycosylases,[4a,8] supporting this hypothesis. Furthermore, there is a good correlation between the ability of ramoplanin to inhibit transglycosylation in vitro and the amounts of compound required to inhibit bacterial cell growth (IC_{50} = 0.25 μM; MIC = 0.1 μM). In contrast, the dissociation constant of labeled ramoplanin for MurG (4 μM) is more than 10 times higher than the concentration of ramoplanin (**1**) required to inhibit growth. These data suggest that MurG inhibition is not the mechanism that leads to bacterial cell death. However, the studies on MurG were done in the absence of membranes, and it is conceivable that membranes influence the affinity of ramoplanin for MurG. The role of MurG inhibition, if any, in the activity of ramoplanin remains to be established.

In summary, the results presented in this paper show that Lipid I binding is not involved in MurG inhibition by ramoplanin and suggest that Lipid I plays no role in ramoplanin's activity. Instead, Lipid II is implicated as the relevant peptidoglycan intermediate targeted by ramoplanin. Experiments to characterize the mode of transglycosylase inhibition and to probe the in vivo mechanism further are underway.

Acknowledgment. This work was supported by NIH grant R01AI50855. We thank IntraBiotics for a generous gift of ramoplanin and Yanan Hu for the preparation of MurG used in the binding experiments.

Supporting Information Available: Experimentals and spectral data for **2**, **3**, and **6**; procedures for enzyme assays and binding; NMR titrations for **2** and **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Collins, L. A.; Eliopoulos, G. M.; Wennersten, C. B.; Ferraro, M. J.; Moellering, R. C., Jr. *Antimicrob. Agents Chemother.* **1993**, *37*, 1364−1366. (b) Cavalleri, B.; Pagani, H.; Volpe, G.; Selva, E.; Parenti, F. *J. Antibiot. (Tokyo)* **1984**, *37*, 309−317. (c) Ciabatti, R.; Kettenring, J. K.; Winters, G.; Tuan, G.; Zerilli, L.; Cavalleri, B. *J. Antibiot. (Tokyo)* **1989**, *42*, 254−267. (d) Somner, E. A.; Reynolds, P. E. *Antimicrob. Agents Chemother.* **1990**, *34*, 413−419. (e) Reynolds, P. E.; Somner, E. A. *Drugs Exp. Clin. Res.* **1990**, *16*, 385−389.

(2) The total synthesis of ramoplanin aglycone has recently been reported by Boger and co-workers. See: (a) Jiang, W.; Wanner, J.; Lee, R. J.; Bounaud, P. Y.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 5288−5290. (b) Boger, D. L. *Med. Res. Rev.* **2001**, *21*, 356−381.

(3) For recent reviews on peptidoglycan biosynthesis, see: (a) van Heijenoort, J. *Nat. Prod. Rep.* **2001**, *18*, 503−519. (b) van Heijenoort, J. *Glycobiology* **2001**, *11*, 25R−36R.

(4) It has recently been shown, however, that ramoplanin binds to Lipid I analogues. See: (a) Lo, M.-C. Ph.D. Thesis, Princeton University, 2000. (b) Cudic, P.; Kranz, J. K.; Behenna, D. C.; Kruger, R. G.; Tadesse, H.; Wand, A. J.; Veklich, Y. I.; Weisel, J. W.; McCafferty, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7384−7389. (c) Cudic, P.; Behenna, D. C.; Kranz, J. K.; Kruger, R. G.; Wand, A. J.; Veklich, Y. I.; Weisel, J. W.; McCafferty, D. G. *Chem. Biol.* **2002**, *9*, 897−906.

(5) (a) Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1998**, *120*, 2484−2485. (b) Ha, S.; Chang, E.; Lo, M.; Men, H.; Park, P.; Ge, M.; Walker, S. *J. Am. Chem. Soc.* **1999**, *121*, 8415−8426. (c) Chen, L.; Men, H.; Ha, S.; Ye, X. Y.; Brunner, L.; Hu, Y.; Walker, S. *Biochemistry* **2002**, *41*, 6824−6833.

(6) Segel, I. H. *Enzyme Kinetics*; John Wiley & Sons: New York, 1975.

(7) Several studies indicate that ramoplanin recognizes the pyrophosphate portion of the substrate. See ref 4. This suggests that electrostatic interactions with one or both ornithines could be important for binding. A recent NMR structure of a ramoplanin dimer shows that the ornithine amines flank separate clefts, suggesting that only one of them can contact the pyrophosphate. See: Lo, M. C.; Helm, J. S.; Sarngadharan, G.; Pelczer, I.; Walker, S. *J. Am. Chem. Soc.* **2001**, *123*, 8640−8641. Therefore, we reasoned that acylating with alanine might eliminate a specific interaction with the substrate while maintaining the net charge on the molecule. The dialanine derivative was also synthesized and behaved similarly to compound **3**.

(8) Lo, M.; Men, H.; Branstrom, A.; Helm, J.; Yao, N.; Goldman, R.; Walker, S. *J. Am. Chem. Soc.* **2000**, *122*, 3540−3541.

(9) The binding isotherm was fit by nonlinear least-squares regression. See: Reid, S. L.; Parry, D.; Liu, H.-H.; Connolly, B. A. *Biochemistry* **2001**, *40*, 2484−2494.

(10) MICs were measured against *E. faecium* strain L19624 and *E. faecalis* strain Z9212 according to standard methods. See: *Methods for Dilution Anti Microbial Susceptibility Tests for Bacteria that Grow Aerobically* (approved standard, NCCLS Document M7-A4, National Committee for Clinical Laboratory Standards, Wayne, PA, ed. 4, 1997).

(11) See, for example: (a) Baptista, M.; Depardieu, F.; Courvalin, P.; Arthur, M. *Antimicrob. Agents Chemother.* **1996**, *40*, 2291−2295. (b) Ritter, T. K.; Wong, C. H. *Angew. Chem., Int. Ed.* **2001**, *40*, 3508−3533.

(12) (a) Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D. *Science* **1999**, *284*, 507−511. (b) Breukink, E.; Wiedemann, I.; van Kraaij, C.; Kuipers, O. P.; Sahl, H.; de Kruijff, B. *Science* **1999**, *286*, 2361−2364. (c) Pag, U.; Sahl, H. G. *Curr. Pharm. Des.* **2002**, *8*, 815−833.

JA021097N