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Mureidomycin A, a New Inhibitor of Bacterial Peptidoglycan Synthesis

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Mureidomycin A (MRD), a novel peptidylnucleoside antibiotic with antipseudomonal activity, inhibited not only peptidoglycan synthesis but also lipid-intermediate formation from UDP-*N*-acetylmuramyl (MurNAc)-pentapeptide and UDP-*N*-acetylglucosamine in an in vitro peptidoglycan-synthesizing system, using ether-treated cells of *Pseudomonas aeruginosa*. Both types of inhibition by MRD disappeared when UDP-MurNAc-pentapeptide was preincubated with ether-treated cells. Moreover, MRD completely inhibited lipid-intermediate I (undecaprenyl-*p*-*p*-MurNAc-pentapeptide) formation at a concentration below the MIC. From these results, it was concluded that the real target of MRD's action was translocase, which catalyzes lipid-intermediate I formation from UDP-MurNAc-pentapeptide and a lipid carrier.

Many new potent beta-lactam antibiotics have been developed, but the increased incidence of resistance of *Pseudomonas aeruginosa* to these antibiotics is still a serious problem. It indicates the importance of evaluating new antibiotics that show no cross resistance with beta-lactam antibiotics for the treatment of *P. aeruginosa* infections.

Mureidomycins, novel peptidylnucleoside antibiotics produced by *Streptomyces flavidovirens*, are selective and potent antipseudomonal antibiotics (3). They are composed of four structurally related components, designated A, B, C, and D (4), that have low toxicity and are effective against *P. aeruginosa* infections in mice (5). In a previous paper, we reported that mureidomycin A (MRD) induced spheroplast formation followed by cell lysis of *P. aeruginosa* at the MIC. In this paper, we report that MRD is a structurally new type of inhibitor of bacterial peptidoglycan synthesis.

MATERIALS AND METHODS

Bacteria. *P. aeruginosa* SANK 75775 was grown in Trypto-soy broth (Eiken) at 37°C with vigorous shaking. Ether-treated cells were prepared from early-log-phase cells by the procedure of Vosberg and Hoffmann-Berling (19).

Preparation of unlabeled and ^{14}C-labeled UDP-MurNAc-pentapeptide. [^{14}C]UDP-MurNAc–L-Ala–D-Glu–*meso*-Dap–D-Ala–D-Ala accumulated in *Bacillus cereus* grown in the presence of L-[^{14}C]alanine by the method of Oka (11) was purified by carbon adsorption chromatography. Unlabeled UDP-*N*-acetylmuramyl (MurNAc)-pentapeptide was also prepared as discussed above, except that bacteria were grown in Trypto-soy broth without chloramphenicol and that the peptide was further purified by using Sephadex G-25 column chromatography.

Assay of peptidoglycan synthesis. A reaction mixture containing 100 μl of 50 mM Tris hydrochloride (pH 8.3), 50 mM NH_4Cl, 20 mM $MgCl_2$, 10 mM ATP, 1 mM dithiothreitol, 0.1 mM UDP-MurNAc-pentapeptide, 5.25 μM UDP-[^{14}C]*N*-acetylglycosamine (GlcNAc) (14 μCi/μmol), and approximately 0.5 mg of protein of ether-treated bacteria was incubated at 37°C for 20 min, transferred onto a Whatman 3MM paper disk, and immersed in ice-cold 5% trichloroacetic acid (TCA) for 30 min. The disks were washed with 5% TCA, ethanol, and ether successively, and radioactivity was counted by an Aloka liquid scintillation counter with a toluene-based scintillation fluid (1).

Assay of lipid-intermediate formation. The same reaction mixture described above except for a small modification (0.25 μM UDP-[^{14}C]GlcNAc [300 μCi/μmol] and 1 mg of protein) was used. It was incubated for 20 min at 37°C, followed by the addition of 6 M pyridinium acetate (pH 4.2) to stop the reaction, and then it was extracted twice with 200 μl of *n*-butanol, followed by washing with water (1). The radioactivity of the solvent-extractable fraction was counted with Picofulor (Packerd).

Assay of phospho-MurNAc-pentapeptide translocase. [^{14}C] UDP-MurNAc-pentapeptide (10 nCi) was incubated without UDP-GlcNAc, and radioactivity incorporated into the *n*-butanol-extractable fraction was measured as discussed above.

Chemicals. Mureidomycins A, B, C, and D were prepared as described previously (3). [^{14}C]alanine (171 mCi/mmol) and UDP-[^{14}C]GlcNAc (300 mCi/mmol) were purchased from Amersham.

RESULTS

Inhibition of peptidoglycan synthesis and lipid-intermediate formation. As it was supposed that MRD would inhibit peptidoglycan synthesis of *P. aeruginosa* from the results reported in a previous study (5), an in vitro peptidoglycan-synthesizing system was constructed, using ether-treated *P. aeruginosa* cells as an enzyme source. It is well-known that precursors of peptidoglycan synthesis, such as UDP-MurNAc-pentapeptide and UDP-GlcNAc, are permeable in ether-treated cells (9, 10). The UDP-MurNAc-pentapeptide-dependent incorporation of UDP-[^{14}C]GlcNAc into a 5% TCA-insoluble fraction was increased in a time-dependent manner and inhibited by tunicamycin, ristocetin, and ampicillin, indicating that peptidoglycan synthesis could be monitored by this system (Table 1). In this system, MRD also inhibited the incorporation of [^{14}C]GlcNAc into a 5% TCA-insoluble fraction at concentrations less than those required to cause cell lysis or spheroplast formation in the organism. Approximately 0.5 μg of MRD per ml was required for 50% inhibition, although the MIC of MRD for this organism was 1.56 μg/ml. Mureidomycins B, C, and D also inhibited peptidoglycan synthesis to almost the same extent.

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TABLE 1. Inhibition of peptidoglycan synthesis by antibiotics in ether-treated cells of *P. aeruginosa*

Antibiotic	Concn (μg/ml)	dpm[a]	Inhibition (%)
None		2,662	
MRD	10	0	100
	1	150	94
	0.1	1,851	31
	0.01	2,796	0
Tunicamycin	100	1,266	52
	10	2,186	18
	1	2,862	0
Ristocetin	100	183	93
	10	595	78
	1	2,514	6
Ampicillin	100	361	86
	10	427	84
	1	693	74

[a] Radioactivity incorporated into the TCA-insoluble fraction was determined as described in Materials and Methods.

To measure the effect on lipid-intermediate formation, the reaction mixture was extracted at pH 4.2 with *n*-butanol. The incorporation of UDP-[^{14}C]GlcNAc into a solvent-extractable fraction was inhibited by MRD and tunicamycin to the same extent as that of the 5% TCA-insoluble fraction (Table 2). Ristocetin also inhibited this incorporation at a high concentration, but in contrast, a low concentration of ristocetin and ampicillin did not inhibit but rather increased incorporation into the solvent-extractable fraction. These results were in accordance with the known mechanism of actions of antibiotics; that is, tunicamycin inhibits the transfer of MurNAc-pentapeptide (17, 20), ristocetin transglycosylation (2), and ampicillin cross linkage formation (14). It is also supposed that MRD would inhibit the transfer of MurNAc-pentapeptide to an endogenous lipid carrier or the addition of GlcNAc to the lipid-bound MurNAc-pentapeptide.

Inhibition of phospho-MurNAc-pentapeptide translocase. Transfer of MurNAc-pentapeptide from UDP to a lipid carrier is catalyzed by phospho-MurNAc pentapeptide translocase (15). The effect of MRD on this enzyme was directly examined by using UDP-MurNAc-[^{14}C]Ala-pentapeptide as the substrate. MRD inhibited the incorporation of labeled precursor into a solvent-extractable fraction by 93 and 87% at concentrations of 1 and 0.1 μg/ml, respectively (Table 3). Tunicamycin also inhibited this reaction, but neither ristocetin nor ampicillin inhibited it, even at a concentration of 100 μg/ml. The concentration of MRD required for 50% inhibition was calculated to be about 0.05 μg/ml. Therefore, it was suggested that translocase was the primary target of MRD.

TABLE 2. Effect of antibiotics on lipid-intermediate formation in ether-treated cells of *P. aeruginosa*

Antibiotic	Concn (μg/ml)	dpm[a]	% of control
None		1,896	100
MRD	10	256	13
	1	303	16
	0.1	989	52
Tunicamycin	200	979	52
	20	1,554	82
Ristocetin	100	727	38
	10	2,725	144
Ampicillin	100	2,625	138
	10	2,616	138

[a] Radioactivity incorporated into the *n*-butanol-extractable fraction was determined as described in Materials and Methods.

TABLE 3. Effect of antibiotics on phospho-MurNAc-pentapeptide translocase[a]

Antibiotic	Concn (μg/ml)	Inhibition[b] (%)
MRD	10	94
	1	93
	0.1	87
	0.01	11
Tunicamycin	100	70
	10	14
Ristocetin	100	0
Ampicillin	100	0

[a] Assay of phospho-MurNAc-pentapeptide translocase was done as described in Materials and Methods.

[b] In this assay, there were 708 dpm for the control and 39 cpm for background.

Effect on other steps of peptidoglycan synthesis. To determine whether MRD inhibits the steps of peptidoglycan synthesis after translocase, ether-treated cells were preincubated with only UDP-MurNAc-pentapeptide to accumulate lipid-bound MurNAc-pentapeptide, and then peptidoglycan synthesis was measured by adding to the reaction mixture UDP-[^{14}C]GlcNAc in the presence or absence of MRD (18). The rates of incorporation of UDP-[^{14}C]GlcNAc into the solvent-extractable and TCA-insoluble fractions after preincubation were not significantly affected by MRD, even at 10 μg/ml, which inhibited incorporation more than 90% when preincubation was omitted (Table 4). This fact indicated that MRD did not inhibit the other enzymes, such as GlcNAc transglycosylase, peptidoglycan transglycosylase, or transpeptidase.

DISCUSSION

It was suggested by the following results that the mechanism of action of MRD's antipseudomonal activity was the

TABLE 4. Influence of preincubation on lipid-intermediate formation and peptidoglycan synthesis

MRD concn (μg/ml)	Inhibition[a] (%)			
	Lipid-intermediate formation		Peptidoglycan synthesis	
	With preincubation	Without preincubation	With preincubation	Without preincubation
10	6	90	5	91
1	3	77	0	67
0.1	0	22	0	10

[a] Assay of the following steps of translocase was carried out as described by Tanaka et al. (18) with slight modifications. UDP-MurNAc-pentapeptide (0.1 mM) was preincubated with ether-treated cells of *P. aeruginosa* at 37°C for 15 min to form lipid-intermediate I. Then UDP-GlcNAc (0.8 μM, 300 μCi/μmol) and MRD were added and incubated for 2 min. Radioactivity incorporated into the *n*-butanol-extractable fraction or TCA-insoluble fraction was determined as described in Materials and Methods.

inhibition of peptidoglycan synthesis. (i) MRD induced spheroplast formation of susceptible bacteria at the MIC. (ii) Susceptible cells treated with MRD at the MIC increased their turbidity during the beginning and then decreased it through cell lysis. (iii) MRD was bactericidal. (iv) MRD was extremely low in toxicity to animals. In this study, we presented evidence indicating that MRD is a structurally new type of bacterial peptidoglycan synthesis inhibitor and that its target is phospho-MurNAc-pentapeptide translocase. There was no evidence to suggest that other enzymes, such as GlcNAc transglycosylase, peptidoglycan transglycosylase, and transpeptidase, were affected by MRD.

It should be noted that the possibility that lipid carrier recycling was inhibited was ruled out by the following observation. Bacitracin, an inhibitor of lipid carrier regeneration (13), did not strongly inhibit both peptidoglycan and lipid intermediate formation in ether-treated *P. aeruginosa* (data not shown). This fact suggested that there was enough endogenous lipid carrier in the ether-treated cells and that peptidoglycan synthesis proceeded to an extent equivalent to the amount of lipid carrier in the presence of bacitracin. This was consistent with a report by Hammes and Neuhaus (2). In addition, the effect of bacitracin on lipid-intermediate formation was completely abolished by the addition of 10 μg of ristocetin per ml, although that of MRD was not changed at all (data not shown).

Another stage of peptidoglycan biosynthesis is the formation of precursors in the cytoplasm (12) and MRD was concluded not to affect the steps of UDP-MurNAC-pentapeptide formation because in the growing cells labeled with [^{14}C]Ala (8), an enhanced amount of UDP-MurNAc-[^{14}C] pentapeptide was detected on silica gel thin-layer chromatography in the presence of MRD.

From these results, it was concluded that MRD interfered with peptidoglycan synthesis by inhibiting phospho-MurNAc-pentapeptide translocase, the first enzyme of the lipid cycle in peptidoglycan synthesis. Although the precise stereochemistry of MRD has not yet been elucidated, it seems to be structurally related to UDP-MurNAc-pentapeptide and to compete with it at the catalytic site of translocase. Tunicamycin (17, 20), amphomycin (18), and liposidomycin (7) have been reported to inhibit translocase, and they contain lipid moieties that are supposed to relate to their inhibitory activities. In fact, tunicamycin and amphomycin inhibit not only bacterial peptidoglycan synthesis but also mammalian polysaccharide synthesis (6, 16) which is also catalyzed through lipid-intermediate formation. However, MRD does not contain a lipid moiety, and it did not inhibit mammalian polysaccharide synthesis.

It is also interesting to note that MRD inhibited peptidoglycan synthesis in *E. coli*, which was intrinsically resistant to MRD, and in MRD-resistant *P. aeruginosa* mutants which were isolated spontaneously, to the same extent as *P. aeruginosa* in ether-treated system, although they were resistant to MRD concentrations of more than 200 μg/ml. It seems likely that the translocase in *E. coli* and in MRD-resistant mutant of *P. aeruginosa* is sensitive to MRD. The resistance of these bacteria might derive from their permeability deficiency for MRD. The precise mechanism of the selective antipseudomonal activity of MRD remains to be determined.

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