ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1993, p. 980–983
0066-4804/93/050980-04$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 37, No. 5

Selective Inhibition of the Bacterial Translocase Reaction in Peptidoglycan Synthesis by Mureidomycins

MASATOSHI INUKAI,[1]* FUJIO ISONO,[1] AND AKIRA TAKATSUKI[2]

Fermentation Research Laboratories, Sankyo Co., Ltd., 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140,[1] and Animal and Cellular Systems Laboratory, The Institute of Physical and Chemical Research, 2-1, Hirosawa, Wako-shi, Saitama 351-01,[2] Japan

Received 22 September 1992/Accepted 12 February 1993

Mureidomycins (MRDs) A and C inhibited strongly the formation of undecaprenyl pyrophosphoryl *N*-acetylmuramyl-pentapeptide (lipid intermediate I), which is an intermediate in bacterial peptidoglycan synthesis (50% inhibitory concentration [IC_{50}] of MRD A, 0.05 μg/ml). However, they did not inhibit the formation of dolichyl pyrophosphoryl *N*-acetylglucosamine (Dol-p-p-GlcNAc), dolichyl phosphoryl glucose, or dolichyl phosphoryl mannose, the precursors for mammalian glycoprotein synthesis, or the formation in *Bacillus subtilis* of lipid-linked *N*-acetylglucosamine for teichoic acid synthesis (IC_{50}s, >100 μg/ml). In contrast, tunicamycin (TCM) inhibited strongly the formation of Dol-p-p-GlcNAc (IC_{50}, 0.03 μg/ml) but inhibited weakly the formation of bacterial lipid intermediate I (IC_{50}, 44 μg/ml). When the effects of MRDs A and C and TCM on the growth of mammalian cells were compared, MRDs did not show any toxicity, even at 1,000 μg/ml, whereas TCM inhibited the growth of BALB/3T3 cells at 10 μg/ml. On the basis of these results, it was concluded that MRDs are the first specific and potent inhibitors of the translocase reaction in bacterial peptidoglycan synthesis, showing a high level of toxicity against bacteria and a low level of toxicity against mammalian cells. A specific inhibitor of translocase could be a potent antibiotic with highly selective toxicity.

The novel peptidylnucleoside antibiotics mureidomycins (MRDs) A and C (Fig. 1), are structurally new types of bacterial peptidoglycan synthesis inhibitors (3–7). They are produced by *Streptomyces flavidovirens* and have selective and potent in vitro and in vivo activities against *Pseudomonas aeruginosa*. The MICs of MRD C are as follows: *Staphylococcus aureus* FDA 209P JC-1, >200 μg/ml; *Escherichia coli* NIHJ JC-2, >200 μg/ml; *P. aeruginosa* 1046, 1.56 μg/ml; and *P. aeruginosa* NCTC 10490, 0.4 μg/ml (6). MRD A has almost the same MICs as MRD C, although the MICs of MRD A are a little higher. MRDs A and C have a low level of toxicity and can protect mice from *P. aeruginosa* infections (6). The reason for the low level of toxicity of MRDs in mice is very interesting. MRDs inhibit peptidoglycan synthesis through the inhibition of translocase, which catalyzes the formation of undecaprenyl pyrophosphoryl *N*-acetylmuramyl-pentapeptide (lipid intermediate I) from UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and undecaprenyl phosphate (4). Three antibiotics have been reported to inhibit this translocase. They are tunicamycin (TCM; Fig. 1) (14), amphomycin (APM) (9), and liposidomycin (LSM) (10). Among them, TCM and APM are toxic against mammalian cells. They inhibit not only bacterial translocase but also mammalian enzymes that catalyze the formation of lipid-linked saccharides (8, 13), through which membrane and secreted proteins are glycosylated. There is no report on LSM. Therefore, the inhibition of translocase by MRDs is not sufficient to explain their selective toxicity. In this report, we show that MRDs have a low level of toxicity against mammalian cells because they inhibit neither mammalian nor bacterial lipid-linked saccharide formation but inhibit bacterial translocase.

* Corresponding author.

MATERIALS AND METHODS

Assay of translocase. UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-Ala ([^{14}C]UDP-MurNAc-pentapeptide) was prepared from *Bacillus cereus* in the presence of ristocetin by the method of Oka (11) with D-[^{14}C]Ala instead of *meso*-[^{14}C]Dap. For enzyme preparation, *P. aeruginosa* SANK 75775 grown in Trypto-soy broth (Eiken) was ether treated by the procedure of Vosberg and Hoffmann-Berling (16). A reaction mixture containing 100 μl of 50 mM Tris hydrochloride (pH 8.3), 50 mM NH_4Cl, 20 mM $MgCl_2$, 10 nCi of [^{14}C]UDP-MurNAc-pentapeptide, approximately 0.5 mg of protein from ether-treated bacteria, and various concentrations of MRD A, MRD C, or TCM was incubated without UDP-*N*-acetylglucosamine (UDP-GlcNAc), and radioactivity incorporated into the *n*-butanol-extractable fraction was counted with Picofluor (Packard).

Assay of Dol-p-p-GlcNAc. For the assay of dolichyl pyrophosphoryl *N*-acetylglucosamine (Dol-p-p-GlcNAc) formation, microsomes were prepared from 8-week-old Donryu rat livers (13) and suspended in 10 mM Tris-maleate buffer (pH 7.1) containing 0.1 M KCl. A reaction mixture containing microsomes, Tris-maleate buffer, 0.1 M KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.1 μCi of [^{3}H]UDP-GlcNAc per ml, and various concentrations of MRD A, MRD C, or TCM was incubated at 28°C for 12 min. Labeled lipids were then extracted and processed as described previously (13).

Assay of lipid-linked GlcNAc formation in *Bacillus subtilis*. Cells of *B. subtilis* NA64 grown in Trypto-soy broth at 37°C were collected at the late log phase of growth by centrifugation at 10,000 × *g* for 15 min at 4°C and washed twice with 50 mM Tris-maleate buffer (pH 7.1) containing 2 mM dithiothreitol. They were then suspended in the same buffer containing 0.5 M sucrose, 5 mM $MgCl_2$, and 300 μg of lysozyme per ml and allowed to stand at 37°C for 50 min to make protoplasts. After protoplasts were collected by centrifugation at 8,000 × *g* for 10 min, they were burst by

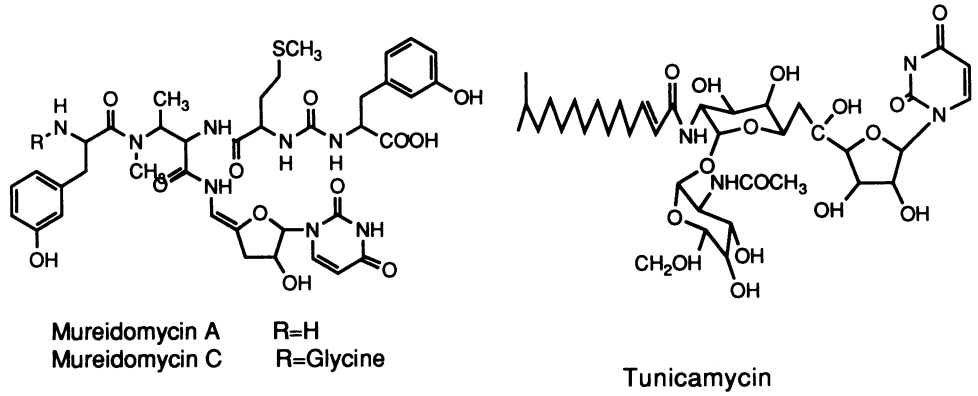

FIG. 1. Structures of MRD A, MRD C, and TCM.

suspension in the same buffer containing 50 μg of DNase I per ml at 0°C for 10 min. A membrane fraction was obtained by centrifugation at 100,000 × *g* for 2 h and homogenized for use as the enzyme source for lipid-linked *N*-acetylglucosamine (GlcNAc) formation. Lipid-linked GlcNAc formation was measured as described previously (13).

Assay of cytotoxicity. BALB/3T3 cells were suspended in Dulbecco-modified Eagle's medium (5×10^5 cells per ml) containing 10% fetal bovine serum, plated into 96-well microplates, and grown in a humidified atmosphere of 95% air–5% carbon dioxide at 37°C. After 24 h, MRD A, MRD C, or TCM dissolved in growth medium was added, and the cells were incubated for a further 48 h. The cell number was then determined colorimetrically.

Chemicals. MRDs A and C were prepared as described previously (3). [^{14}C]alanine (171 mCi/mmol) was purchased from Amersham. [^{3}H]UDP-GlcNAc (10 Ci/mmol) was obtained from New England Nuclear. Other chemicals were commercially available.

RESULTS AND DISCUSSION

When the inhibitory effects of MRD A and TCM against *P. aeruginosa* translocase, which catalyzes the formation of lipid intermediate I, were compared, MRD A inhibited this enzyme much more potently than TCM; the 50% inhibitory concentrations (IC_{50}s) of MRD A and TCM were 0.05 and 44 μg/ml, respectively (Fig. 2). MRD C was as potent as MRD A. Kimura et al. (10) reported that LSM was more potent than TCM against the *E. coli* enzyme; the IC_{50}s were 0.038 and 12 μg/ml, respectively. The IC_{50} of APM against *Bacillus megaterium* translocase is about 20 μg/ml (15). Therefore, the MRDs and LSM seem to be much more potent inhibitors of translocase than TCM and APM.

TCM and APM have been shown to inhibit mammalian enzymes that catalyze the formation of lipid-linked saccharides from nucleotide sugars and dolichyl phosphate. Although the substrates are somewhat different from those of bacterial translocase, mammalian lipid-linked saccharide formation seems to be catalyzed by a mechanism similar to that of bacterial translocase. TCM (13) and APM (1) contain a long fatty acid side chain that is supposed to be necessary for their inhibitory activity not only against bacterial translocase but also against mammalian enzymes. LSM (10) also contains fatty acids, but there has been no report on its activity against mammalian enzymes. Since MRDs are unique in that they consist of only nucleoside and peptide moieties (Fig. 1), we were interested in testing whether MRDs would inhibit mammalian lipid-linked saccharide formation.

Labeled nucleotide sugars were incubated with microsomes prepared from rat liver, and incorporation into the lipid fraction was measured as described previously (13). As shown in Fig. 3, MRDs A and C inhibited the synthesis of Dol-p-p-GlcNAc in a dose dependent manner only at high concentrations. Dolichyl p-mannose formation and dolichyl p-glucose formation were not affected by MRD A or MRD C, even at 4 mg/ml (data not shown). The IC_{50} of MRD A

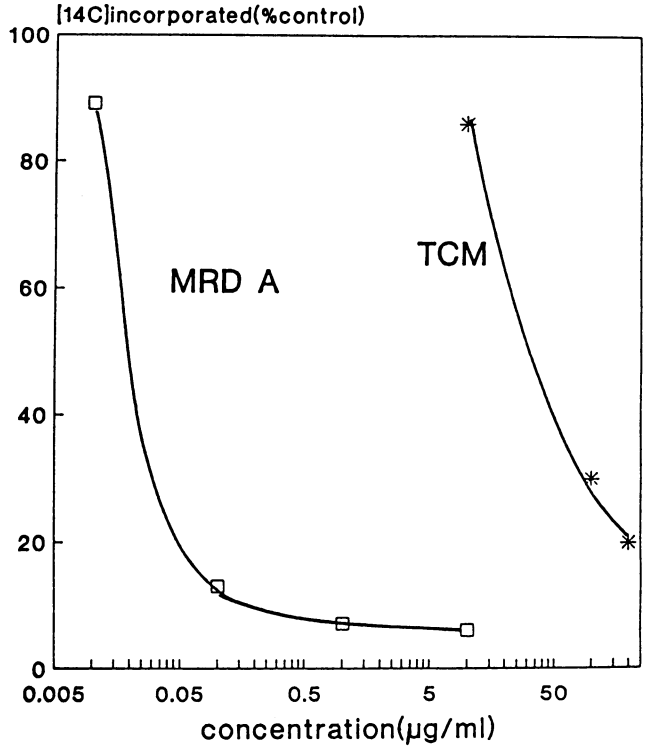

FIG. 2. Effects of MRD A and TCM on the translocase of *P. aeruginosa*. [^{14}C]UDP-MurNAc-pentapeptide (10 nCi) prepared from *B. cereus* was incubated with ether-treated *P. aeruginosa* SANK 75775 and various concentrations of MRD A or TCM but without UDP-GlcNAc as described in Materials and Methods. Radioactivity incorporated into the *n*-butanol-extractable fraction was counted with Picofluor.

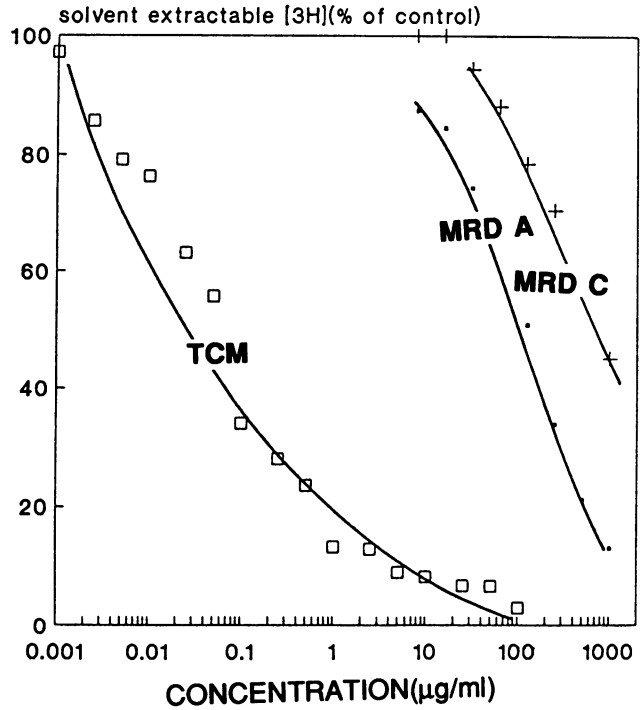

FIG. 3. Effects of MRD A, MRD C, and TCM on Dol-p-p-GlcNAc formation in rat liver microsomes. Microsomes prepared from 8-week-old Donryu rats were suspended in 10 mM Tris-maleate buffer (pH 7.1) containing 0.1 M KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.1 μCi of [^{3}H]UDP-GlcNAc per ml, and various concentrations of MRD A, MRD C, or TCM. Labeled lipids were extracted after incubation at 28°C for 12 min and processed as described previously (12).

against the mammalian enzyme was ca. >2,500 times higher than that against bacterial translocase, a result in clear contrast to the inhibitory activities of TCM and APM. TCM inhibited Dol-p-p-GlcNAc formation preferentially, as shown in Fig. 3. APM has been reported to inhibit both dolichyl phosphoryl mannose formation and Dol-p-p-GlcNAc formation at nearly the same concentration as that needed to inhibit bacterial translocase (8, 9).

Another step of glycoconjugate synthesis in bacteria in which lipid-linked saccharides participate is the synthesis of teichoic acid. As shown in Table 1, MRD A did not inhibit lipid-linked GlcNAc formation in the *B. subtilis* membrane, even at a concentration of 100 μg/ml, although TCM inhibited this process nearly completely at the same concentration. The IC_{50} of TCM was about 0.8 μg/ml.

Thus, MRDs are the first specific inhibitors of the UDP-MurNAc-pentapeptide translocase, which catalyzes the first step of the lipid cycle in bacterial peptidoglycan synthesis. MRDs did not significantly affect mammalian or bacterial lipid-linked saccharide formation, except in peptidoglycan synthesis.

As each MRD consists of four or five amino acids and a pseudouridine nucleoside, the MRDs would be recognized as analogs of UDP-MurNAc-pentapeptide, a distinctive substrate of translocase. In contrast, TCM and APM would mimic in part lipid and/or nucleotide moieties of substrates or intermediates of both mammalian and bacterial reactions. This mimicry may explain why these two antibiotics inhibit nonselectively both bacterial and mammalian enzymes.

TABLE 1. Effects of MRD A and TCM on the formation of lipid-linked GlcNAc in the *B. subtilis* membrane[a]

Drug concn (μg/ml)	% Inhibition by:	
	MRD A	TCM
100	15.2	99.1
50	13.6	97.7
25	9.3	96.9
1.6	0	62.6
0.8	0	48.4
0.4	0	12.2

[a] Cells of *B. subtilis* NA64 were allowed to stand at 37°C for 50 min in Tris-maleate buffer containing 300 μg of lysozyme per ml to make protoplasts. After protoplasts were collected, they were burst by suspension in Tris-maleate buffer containing 50 μg of DNase I per ml at 0°C for 10 min. The membrane fraction was homogenized, and lipid-linked GlcNAc formation was measured as described in Materials and Methods.

It is well known that TCM selectively blocks the glycosylation of membrane and secreted proteins in mammalian cells (2), thereby preventing their normal functioning. As a result, TCM is toxic for mammalian cells. In fact, TCM inhibited the growth of BALB/3T3 fibroblasts (Fig. 4). On the contrary, MRDs did not affect the growth of mammalian cells, even at the highest concentration tested. As reported previously, MRDs did not show any toxic effects against mice and protected them from *P. aeruginosa* infections (6). These results are consistent with data showing that MRDs preferentially inhibit the bacterial translocase reaction in peptidoglycan synthesis, while TCM inhibits both bacterial and mammalian lipid-linked saccharide formation.

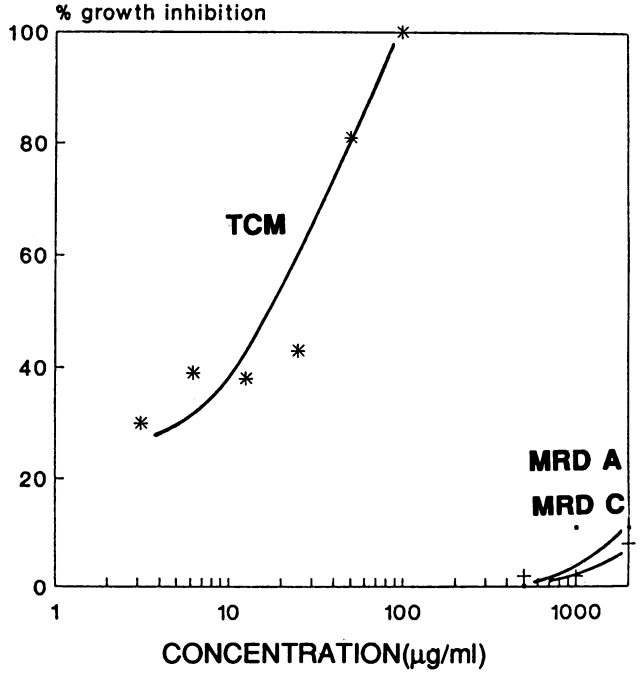

FIG. 4. Effects of MRD A, MRD C, and TCM on the growth of mammalian cells. BALB/3T3 cells were suspended in Dulbecco-modified Eagle's medium containing 10% fetal bovine serum, plated into 96-well microplates, and grown in a humidified atmosphere of 95% air–5% carbon dioxide at 37°C. After 24 h, MRD A, MRD C, or TCM was added. The cell number was determined after 48 h of incubation.

The mechanisms of the selective antipseudomonal activities of MRDs are not yet elucidated. In in vitro experiments, MRDs inhibited translocases from *P. aeruginosa*, *E. coli*, and *S. aureus* at nearly the same magnitude. This result means that translocases of almost all bacteria are commonly sensitive to MRDs. The selective antipseudomonal activities of MRDs could involve different membrane permeabilities in each bacterium. We have not yet detected any degradative activity for MRDs in MRD-resistant bacteria, such as *E. coli*, *S. aureus*, or a resistant mutant of *P. aeruginosa*.

As reported in this paper, although MRDs may mimic only the UDP-MurNAc-pentapeptide moiety, one of the substrates of translocase, they have strong and highly selective inhibitory activity against translocase. A specific inhibitor of bacterial translocase that does not mimic a lipid moiety could be a potent antibiotic with highly selective toxicity against bacteria. Translocase may become a new target for therapeutic antibiotics, as many beta-lactam antibiotics have been synthesized as inhibitors of transpeptidase.

REFERENCES

1. **Bodanszky, M., G. Sigler, and A. Bodanzsky.** 1973. Structure of the peptido antibiotic amphomycin. J. Am. Chem. Soc. **95:** 2352–2362.
2. **Hickamn, S., and S. J. Kornfeld.** 1978. Effect of tunicamycin on IgM, IgA, and IgG secretion by mouse plasmacytoma cells. Immunology **121:**990–996.
3. **Inukai, M., F. Isono, S. Takahashi, R. Enokita, Y. Sakaida, and T. Haneishi.** 1989. Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. I. Taxonomy, fermentation, isolation and physico-chemical properties. J. Antibiot. **42:**662–666.
4. **Isono, F., and M. Inukai.** 1991. Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. Antimicrob. Agents Chemother. **35:**234–236.
5. **Isono, F., M. Inukai, S. Takahashi, T. Haneishi, T. Kinoshita, and H. Kuwano.** 1989. Mureidomycins A-D, novel peptidylnucleoside antibiotics with spheroplast forming activity. II. Structural elucidation. J. Antibiot. **42:**667–673.
6. **Isono, F., T. Katayama, M. Inukai, and T. Haneishi.** 1989. Mureidomycins A-D, novel nucleoside antibiotics with spheroplast forming activity. III. Biological properties. J. Antibiot. **42:**674–679.
7. **Isono, F., K. Kodama, and M. Inukai.** 1992. Susceptibility of *Pseudomonas* species to the novel antibiotics mureidomycins. Antimicrob. Agents Chemother. **36:**1024–1027.
8. **Kang, M. S., J. D. Spencer, and A. D. Elbein.** 1978. Amphomycin inhibits the incorporation of mannose and GlcNAc into lipid-linked saccharides by aorta extracts. Biochem. Biophys. Res. Commun. **82:**568–574.
9. **Kang, M. S., K. J. Spencer, and A. D. Elbein.** 1978. Amphomycin inhibition of mannose and GlcNAc incorporation into lipid-linked saccharides. J. Biol. Chem. **253:**8860–8866.
10. **Kimura, K., N. Miyata, G. Kawanishi, Y. Kamio, K. Izaki, and K. Isono.** 1989. Liposidomycin C inhibits phospho-N-acetylmuramylpentapeptide transferase in peptidoglycan synthesis of Escherichia coli Y-10. Agric. Biol. Chem. **53:**1811–1815.
11. **Oka, T.** 1976. Mode of action of penicillins in vivo and in vitro in *Bacillus megaterium*. Antimicrob. Agents Chemother. **10:** 579–591.
12. **Takatsuki, A., K. Arima, and G. Tamura.** 1971. Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. J. Antibiot. **24:**215–223.
13. **Takatsuki, A., K. Kohno, and G. Tamura.** 1975. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. Agric. Biol. Chem. **39:**2089–2091.
14. **Tamura, G., T. Sasaki, M. Matsuhashi, A. Takatsuki, and M. Yamasaki.** 1976. Tunicamycin inhibits the formation of lipid intermediate in cell-free peptidoglycan synthesis of bacteria. Agric. Biol. Chem. **40:**447–449.
15. **Tanaka, H., R. Oiwa, S. Matsukura, and S. Omura.** 1979. Amphomycin inhibits phospho-N-acetylmuramyl-pentapeptide translocase in peptidoglycan synthesis of Bacillus. Biochem. Biophys. Res. Commun. **86:**902–908.
16. **Vosberg, H. P., and H. Hoffmann-Berling.** 1971. DNA synthesis in nucleotide permeable Escherichia coli cells. I. Preparation and properties of ether-treated cells. J. Mol. Biol. **58:**739–753.