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# A Novel Glycylcycline, 9-(*N*,*N*-Dimethylglycylamido)-6-Demethyl-6-Deoxytetracycline, Is neither Transported nor Recognized by the Transposon Tn*10*-Encoded Metal-Tetracycline/$H^+$ Antiporter

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**A novel tetracycline derivative, DMG-DMDOT [9-(*N*,*N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline], is one of the glycylcyclines which have a broad antibacterial spectrum, including many tetracycline-resistant bacteria (R. T. Testa, P. J. Petersen, N. V. Jacobus, P.-E. Sum, V. J. Lee, and F. P. Tally, Antimicrob. Agents Chemother. 37:2270–2277, 1993). The mechanism by which DMG-DMDOT overcomes efflux-based tetracycline resistance was investigated. Tetracycline-resistant *Escherichia coli* cells carrying an R plasmid encoding the *tet*(B) gene, which encodes the typical tetracycline efflux pump [TetA(B)] of gram-negative bacteria, were as susceptible to DMG-DMDOT as was the tetracycline-susceptible host. When mid-log-phase cells carrying the *tet*(B) gene were incubated with a subbactericidal concentration of DMG-DMDOT (0.5 μg/ml) for 2 h, a significant amount of the TetA(B) protein was detected in the cell membrane by Western blotting (immunoblotting) with an anti-carboxyl-terminal antibody, similar to the case in which tetracycline was used as the inducer, indicating that the *tet* repressor, TetR, can recognize DMG-DMDOT as an efficient inducer. Everted membrane vesicles prepared from cells producing the TetA(B) protein showed absolutely no transport activity for DMG-DMDOT. Furthermore, the presence of excess DMG-DMDOT had no effect on the tetracycline transport activity of the everted vesicles, indicating that DMG-DMDOT is not recognized as a substrate by the TetA(B) protein.**

Tetracyclines are effective antibiotics having broad antimicrobial activity. However, the spread of microorganisms which have acquired tetracycline resistance has limited the usefulness of tetracyclines (5). The most abundant mechanism for tetracycline resistance throughout gram-negative and -positive bacteria is active drug efflux (6), which is mediated by an exogenous metal-tetracycline/$H^+$ antiporter (7, 16, 19). Although several tetracycline derivatives, such as minocycline and doxycycline, with improved antibacterial activity have been developed, none of them is able to overcome efflux-based resistance. Recently, a new class of semisynthetic tetracyclines, glycylcyclines, was developed (13). They show a high level of antibacterial activity toward organisms carrying tetracycline resistance determinants, including efflux-based resistance, in addition to tetracycline-susceptible organisms (14). Most efflux-based tetracycline resistance determinants are inducible. Thus, there are two possibilities resulting in the high level of antibacterial activity of glycylcyclines against resistant strains, that is, (i) glycylcyclines do not induce the expression of tetracycline efflux protein, and (ii) tetracycline efflux protein cannot export glycylcyclines. Guay et al. (3) reported that mutation of *tetA*(B), which is a typical efflux-based resistance gene in gram-negative bacteria, resulted in increased resistance to a glycylcycline accompanied by a decrease in resistance to tetracycline. Their observations indicated that a change in the substrate specificity of the tetracycline efflux system might affect the antibacterial activity of a glycylcycline; however, the possibility cannot be excluded that the difference between the antibacterial activities of tetracyclines and glycylcyclines toward tetracycline-resistant bacteria is due to a difference between the abilities of these compounds to induce the *tetA* gene until direct measurement of their *tetA* gene expression and the transport activity mediated by tetracycline efflux systems.

In this study, the glycylcycline transport activity of a tetracycline efflux system and its ability to induce the *tetA* gene were directly determined for the first time. Two new glycylcyclines, 9-(*N*,*N*-dimethylglycylamido)-6-demethyl-6-deoxytetracycline (DMG-DMDOT) and 9-(*N*,*N*-dimethylglycylamido)minocycline, were synthesized by Sum et al. (13). They showed similar levels of antibacterial activity toward tetracycline-resistant organisms (2, 14). In this study, DMG-DMDOT was chosen as the substrate for a typical tetracycline efflux protein, TetA(B), in gram-negative bacteria.

**Materials.** DMG-DMDOT and [$^{14}$C]DMG-DMDOT were kindly provided by Lederle (Japan), Ltd. Tetracycline was obtained from Wako Pure Chemical Industries, Ltd., and Sigma. [7-$^3$H]tetracycline was obtained from Du Pont New England Nuclear. Other chemicals were of reagent grade and were from commercial sources.

**Bacterial strain and plasmid.** *Escherichia coli* W3104 (20) was used as the host strain for low-copy-number plasmid pLGT2 (15), which contains the entire *tetR* and *tetA* genes of transposon Tn*10*.

**Measurement of bacterial drug resistance.** Bacterial resistance to DMG-DMDOT and tetracycline was measured by the agar dilution method (17) and expressed as the MIC.

**Induction of the *tet* gene and immunoblot analysis.** *E. coli* W3104/pLGT2 cells were grown in 10 ml of 2 × YT broth until the mid-log phase. When the optical density at 610 nm reached 0.4, different concentrations of DMG-DMDOT, tetracycline, and minocycline were added to the cultures, which were incubated for 2 h. The cells were then harvested and disrupted by sonication with a Branson Sonifier 200 for 1.5 min. After removal of undisrupted cells by low-speed centrifugation, membrane fractions were collected by ultracentrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the cell

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membranes was followed by electroblotting of the proteins. The TetA(B) protein was detected by means of an enzyme-linked immunosorbent assay involving an anti-carboxyl-terminal peptide antibody and an Express blot assay kit (Bio-Rad) as described in a previous report (17).

**Transport assays.** Everted membrane vesicles were prepared by disrupting cells with a French pressure cell as previously described (18). [$^3$H]tetracycline or [$^{14}$C]DMG-DMDOT uptake by everted vesicles was assayed in the presence of 10 μM [$^3$H]tetracycline or 10 μM [$^{14}$C]DMG-DMDOT, respectively, in 50 mM morpholinepropanesulfonic acid (MOPS)-KOH buffer (pH 7.0) containing 0.1 M KCl and 500 μM $CoCl_2$.

**Measurement of dissociation constants for chelation complexes of tetracyclines with $Co^{2+}$.** The dissociation constants of chelation complexes were determined in the presence of 10 μM tetracyclines and various concentrations (5 to 1,000 μM) of $CoCl_2$ as described previously (19).

**Antimicrobial activity of DMG-DMDOT toward *E. coli* carrying an R plasmid encoding the *tetA*(B) gene.** The transposon Tn*10*-encoded *tetR* and *tetA*(B) genes were subcloned into low-copy-number plasmid pLG339 (Km$^r$) (12). *E. coli* W3104 cells carrying the resultant plasmid, pLGT2 (15), showed a high level of tetracycline resistance (MIC, 200 μg/ml), while the host strain was susceptible to tetracycline (MIC, 0.8 μg/ml). A novel tetracycline derivative, DMG-DMDOT, which exhibits a high degree of antibacterial activity toward a wide variety of microorganisms, including tetracycline-resistant ones, similar to another glycylcycline, 9-(*N*,*N*-dimethylglycylamido)minocycline (14), showed a MIC of 0.8 μg/ml for *E. coli* W3104 host cells, which was the same as that of tetracycline. As expected, DMG-DMDOT showed a high degree of antibacterial potency against *E. coli* W3104/pLGT2. The MIC for *E. coli* W3104/pLGT2 was 0.8 μg/ml, which was also the same as that for host cells having no plasmid. In other words, the *tetA*(B) gene had absolutely no effect on the antibacterial activity of DMG-DMDOT.

**Induction of *tetA*(B) gene expression by DMG-DMDOT.** *tetA*(B) gene expression induced by the tetracycline derivatives was measured by Western blotting (immunoblotting) with an anti-carboxyl-terminal antibody as described above. Various concentrations of DMG-DMDOT, tetracycline, and minocycline were added to mid-log-phase cultures of *E. coli* W3104/pLGT2 cells, which were then incubated for an additional 2 h. Membrane vesicles were then prepared after cell disruption by sonication. The TetA(B) protein was detected by Western blotting with carboxyl terminus-specific antiserum after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 1, a significant amount of TetA protein was induced by at least 0.05 μg of DMG-DMDOT per ml. Induction of the TetA protein by DMG-DMDOT was somewhat less than the induction by minocycline; however, it was higher than induction by tetracycline (Fig. 1). This observation indicates that the *tet* repressor, TetR, was able to recognize DMG-DMDOT as an efficient inducer. Thus, the high potency of DMG-DMDOT for tetracycline-resistant bacteria is not due to lack of induction of the resistance gene.

**Transport of DMG-DMDOT by everted membrane vesicles prepared from *E. coli* cells producing the TetA(B) protein.** Everted membrane vesicles were prepared from *E. coli* W3104/pLGT2 cells after 2 h of induction of *tetA* gene expression with heat-inactivated chlorotetracycline as described above. Respiration-driven [$^{14}$C]DMG-DMDOT uptake by the everted vesicles was measured in the presence of 10 μM [$^{14}$C]DMG-DMDOT under the same conditions as for [$^3$H]tetracycline uptake. As shown in Fig. 2B, no DMG-DMDOT uptake was observed in the vesicles under conditions in which significant

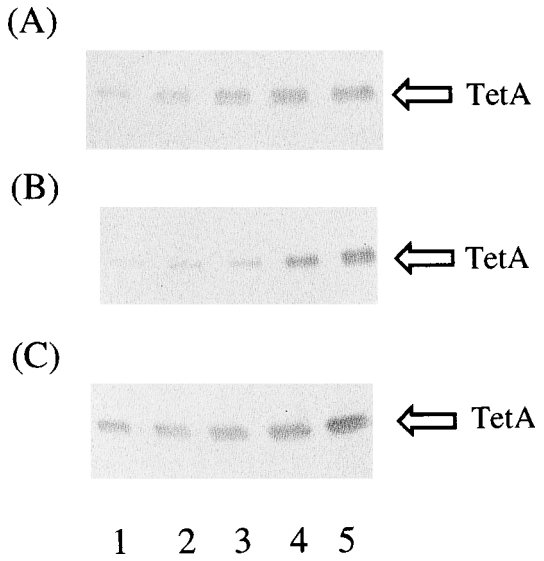


FIG. 1. Immunoblot analysis of membrane fractions prepared from *E. coli* W3104/pLGT2 cells. Each lane contained 5 μg of total proteins. Panels: A, B, and C, induction by DMG-DMDOT, tetracycline, and minocycline, respectively. The final drug concentrations used for induction in lanes 1 to 5 were 0.05, 0.1, 0.20, 0.50, and 1.00 μg/ml, respectively.

tetracycline uptake was detected (Fig. 2A). Thus, it was revealed that the antibacterial potency of DMG-DMDOT for tetracycline-resistant *E. coli* cells carrying the *tetA*(B) gene is based on the inability of the TetA(B) protein to transport DMG-DMDOT.

**Effect of DMG-DMDOT on tetracycline uptake by everted membrane vesicles containing the TetA(B) protein.** The question remaining is whether the inability to transport is due to failure of the TetA(B) protein to recognize DMG-DMDOT or whether TetA(B)-bound DMG-DMDOT is not translocated across the membrane and thus fails to be released into the medium on the opposite side. It is difficult to measure the binding of [$^{14}$C]DMG-DMDOT to the TetA(B) protein directly, since DMG-DMDOT is dissolved in the lipid bilayer region of the membrane, similar to other tetracycline derivatives. Therefore, the affinity of DMG-DMDOT for TetA(B) protein binding was determined by measuring the competitive inhibition of tetracycline uptake by DMG-DMDOT in everted vesicles. [$^3$H]tetracycline uptake was scarcely affected in the

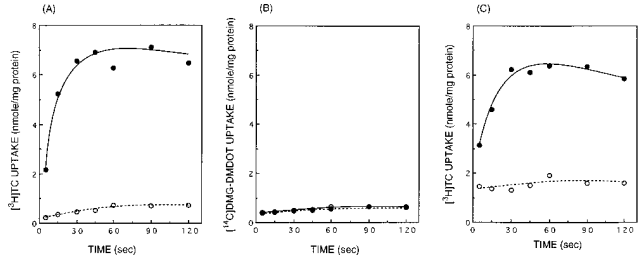


FIG. 2. Uptake of tetracycline (TC) (A) and DMG-DMDOT (B) by everted membrane vesicles prepared from *E. coli* W3104 cells harboring plasmid pLGT2 carrying the entire *tetR* and *tetA* genes encoded by transposon Tn*10* and effect of DMG-DMDOT on [$^3$H]tetracycline uptake (C). The assays were carried out as described in the text. Symbols: ● and ○, drug uptake in the presence and absence of NADH, respectively. The uptake assay for NADH-energized vesicles was performed in duplicate. For panel C, tetracycline uptake was measured in the presence of 10 μM [$^3$H]tetracycline and 100 μM nonlabeled DMG-DMDOT.

presence of 100 μM DMG-DMDOT, although the concentration was 10 times higher than that of tetracycline (Fig. 2C). The increase in the energy-independent background uptake of [$^3$H]tetracycline on the addition of DMG-DMDOT might be due to perturbation of the membrane structure on dissolution of DMG-DMDOT in the lipid bilayer region. A similar nonspecific background increase was also observed in the presence of high concentrations of other tetracycline derivatives. Thus, DMG-DMDOT is not bound, at least to the tetracycline-binding site of TetA(B). This was unexpected because TetR, which also binds to a metal-tetracycline chelation complex similar to TetA(B) (4), can recognize DMG-DMDOT as an inducer. The lack of binding of DMG-DMDOT to TetA(B) may ensure the potency of this drug against organisms overproducing the TetA(B) protein.

**Formation of chelation complexes of DMG-DMDOT with divalent cations.** The lack of binding of DMG-DMDOT to TetA(B) might be due to the lack of formation of chelation complexes of this drug with divalent cations, since tetracycline derivatives are transported as a form of metal chelation complex by TetA(B) (19). The chelation complex formation of tetracycline derivatives can be detected as the bathochromic effect of a divalent cation on a tetracycline derivative (11). Thus, we examined whether or not the absorption spectrum (300 to 450 nm) of DMG-DMDOT changes on addition of $CoCl_2$. With tetracycline, the absorption maximum at 360 nm in the absence of a divalent cation gradually shifted to a longer wavelength, 380 nm, with an increase in the concentration of $CoCl_2$ (19). The dissociation constant for the $Co^{2+}$-tetracycline chelation complex calculated from the bathochromic shift was 23 μM (19). As for DMG-DMDOT, a similar bathochromic shift from 350 nm in free DMG-DMDOT to 380 nm in the presence of excess $CoCl_2$ was observed (data not shown). The dissociation constant calculated from the bathochromic shift was 17 μM, which was similar to that of tetracycline. Therefore, DMG-DMDOT forms a chelation complex with a divalent cation, like other tetracycline derivatives. This is also reasonable for its recognition by TetR., since TetR recognizes the chelation complex (4). Thus, the lack of recognition of DMG-DMDOT by TetA(B) could be due to the glycyl moiety of DMG-DMDOT.

Tetracycline derivatives hitherto developed are classified into two categories on the basis of their modes of action (8): (i) bacteriostatic, classical (typical) tetracyclines, including tetracycline, chlorotetracycline, minocycline, doxycycline and so on, and (ii) bactericidal, atypical tetracyclines, such as chelocardin, anhydrotetracycline, 6-thiatetracycline, anhydrochlortetracycline, and 4-epianhydrochlortetracycline. The latter group of derivatives have modifications in their basic structures. The primary target of the latter tetracycline derivatives is not the ribosome but the cytoplasmic membrane (9, 10). Chelation of tetracycline derivatives with a divalent cation is important for avoiding cytotoxic perturbation of the cytoplasmic membrane by tetracycline derivatives accumulated in cells. DMG-DM DOT belongs to the first category on the basis of its chemical structure, except for the *N*,*N*-dimethylglycylamido group at carbon 9 (13), and forms a chelation complex with a divalent cation. This is the first compound with a substituent introduced at carbon 9 without alteration of the basic structure of classical tetracyclines, although chelocardin, an atypical tetracycline, has a methyl group at carbon 9 (1). Our observations revealed that a modification at carbon 9 is useful for avoiding recognition by a tetracycline efflux protein [TetA(B)] without recognition by the TetR protein being affected. These observations also indicated the possibility of further development of new tetracycline analogs by means of modification at carbon 9.

Guay et al. (3) reported that the Trp-231→Cys or Leu-308→Ser mutation in TetA(B) resulted in moderately increased resistance to 9-(*N*,*N*-dimethylglycylamido)minocycline. Our attempt to isolate DMG-DMDOT-resistant TetA(B)-containing strains was unsuccessful. Therefore, the appearance of glycylcycline-resistant mutants seems to be very rare.

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