ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1994, p. 1658–1660
0066-4804/94/$04.00+0

Vol. 38, No. 7

Inhibition of Protein Synthesis Occurring on Tetracycline-Resistant, TetM-Protected Ribosomes by a Novel Class of Tetracyclines, the Glycylcyclines

BETH A. RASMUSSEN,* YAKOV GLUZMAN, AND FRANCIS P. TALLY

Department of Molecular Biology, American Cyanamid Company, Pearl River, New York 10965

Received 7 February 1994/Returned for modification 29 March 1994/Accepted 18 April 1994

One of the two major mechanisms of tetracycline resistance is ribosomal protection. Of this resistance type, *tet*(M) is the best characterized. Although the mechanism of *tet*(M) resistance has not yet been fully elucidated, it has been demonstrated that ribosomes isolated from a *tet*(M) strain are resistant to inhibition of protein synthesis by tetracycline. A new generation of tetracycline compounds, the glycylcyclines, that are able to inhibit protein synthesis occurring on tetracycline-resistant, TetM-protected ribosomes, as well as wild-type, tetracycline-sensitive ribosomes, have been identified.

The high incidence of tetracycline resistance among clinically important microorganisms imposes a severe limitation on the use of tetracyclines in the treatment of bacterial infections. Two major mechanisms of tetracycline resistance exist: tetracycline efflux, active pumping out of the tetracycline decreasing the intracellular concentration, and ribosomal protection, by which the protein synthesis machinery is rendered resistant to inhibition of protein synthesis (1, 2, 10). Resistance mediated via *tet*(M) is of the latter class. The exact mechanism by which TetM functions is unknown. The TetM protein shares strong homology with and has many of the attributes of elongation factor G, such as the ability to hydrolyze GTP in the presence of ribosomes (2). Whether this homology is directly related to TetM's ability to permit protein synthesis in the presence of tetracycline is unclear. However, it has been demonstrated that ribosomes isolated from *tet*(M) cells are resistant to inhibition of protein synthesis by the currently marketed tetracycline compounds (1–3).

A new generation of tetracycline compounds, the glycylcyclines, including DMG-MINO (*N,N*-dimethylglycylamido minocycline) and DMG-DMDOT (*N,N*-dimethylglycylamido 6-demethyl-6-deoxytetracycline) have been identified (11). The glycylcyclines are capable of inhibiting protein synthesis occurring on wild-type ribosomes. In addition, they are able to inhibit protein synthesis occurring on TetM-protected, tetracycline-resistant ribosomes. This is the first demonstration of a tetracycline compound that is able to inhibit protein synthesis occurring on TetM-protected ribosomes. Two methods have been used to demonstrate the ability of the glycylcyclines to specifically inhibit protein synthesis occurring on either tetracycline-sensitive, wild-type ribosomes, or tetracycline-resistant, TetM-protected ribosomes. The first is an in vitro protein synthesis system. The second is an in vivo assay for inhibition of macromolecular synthesis, assessing the incorporation of protein, DNA, or RNA precursors into macromolecules.

Inhibition of protein synthesis in vitro. The ability of the glycylcyclines to inhibit protein synthesis in vitro was demonstrated by using a DNA-directed protein synthesis system similar to that originally described by Zubay (12). The system used in these studies was as described by Collins (6). The assay utilized pUC119 as the DNA template. S30 extracts were prepared from *Escherichia coli* MRE600 (4) or the TetM-expressing strain MRE600(pAT182), in which pAT182 harbors the *tet*(M) gene from the streptococcal conjugative transposon Tn*1545* (7). Each reaction mixture contained 3.5 μl of low-molecular-weight mix (6); 1.0 μl of template plasmid (1 μg/μl); 1.0 μl of 19-amino-acid mix (without methionine; 3 mM each); 1.0 μl of [^{35}S]methionine (10 mCi/ml; translation grade); 1.0 μl of test compound, dissolved and diluted in dimethyl sulfoxide; and 2.5 μl of S30 extract. The reaction mixtures were incubated for 30 min at 37°C. A fraction of the reaction mixture (2.5 μl) was then removed and incubated with 0.5 ml of 1 N NaOH for 15 min at 37°C to destroy any labeled [^{35}S]methionine-charged tRNA. Macromolecules were precipitated by the addition of 3 to 4 ml of 25% trichloroacetic acid (TCA). Tetracycline or minocycline standards were included with every experiment. The inhibition of protein synthesis was quantitated as the percent incorporation of [^{35}S]methionine into TCA-precipitable material relative to a dimethyl sulfoxide control reaction.

All of the tetracycline and glycylcycline compounds tested were effective at inhibiting protein synthesis when the S30 extract used was prepared from the wild-type strain and contained tetracycline-sensitive ribosomes (Fig. 1A). Tetracycline was slightly less effective at inhibiting protein synthesis with an IC_{50} (the concentration of compound needed to reduce the incorporation of [^{35}S]methionine by 50%) of approximately 0.06 mg/ml. Minocycline and the two glycylcyclines exhibited IC_{50}s of <0.03 mg/ml. However, when the S30 extract was prepared from the tetracycline-resistant strain and contained TetM-protected ribosomes, tetracycline and minocycline were significantly less effective at inhibiting protein synthesis (Fig. 1B). The IC_{50}s for tetracycline and minocycline were 0.25 and 0.12 mg/ml, respectively. In contrast, the two glycylcycline compounds, DMG-MINO and DMG-DMDOT, were highly effective at inhibiting protein synthesis occurring on TetM-protected ribosomes. The inhibition of incorporation of methionine was nearly identical to that seen when the tetracycline-sensitive ribosome S30 extract was used. Both glycylcycline compounds had IC_{50}s of <0.03 mg/ml. The glycylcyclines were more than four- to eightfold better at inhibiting protein synthesis occurring on TetM-protected ribosomes than either tetracycline or minocycline. Unlike tetracycline and minocycline, DMG-MINO and DMG-DMDOT were equally effective at inhibiting protein synthesis in vitro whether

* Corresponding author. Mailing address: American Cyanamid Company, Building 205, Room 214, Pearl River, NY 10965. Phone: (914) 732-4569. Fax: (914) 732-2480.

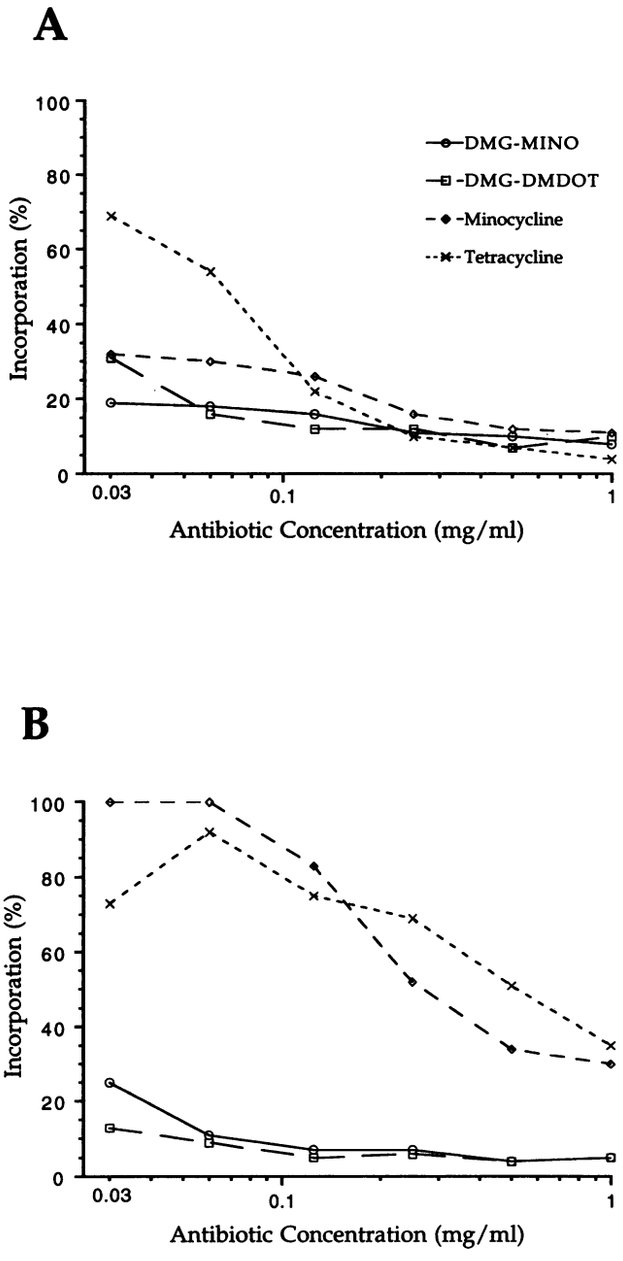

FIG. 1. Inhibition of protein synthesis in vitro. The results are plotted as the percent incorporation of radiolabeled [^{35}S]methionine into TCA-precipitable material compared with control reaction values. (A) S30 extract prepared from MRE600 and containing tetracycline-sensitive, wild-type ribosomes (100% = 110,000 cpm). (B) S30 extract prepared from MRE600(pAT182) and containing tetracycline-resistant, TetM-protected ribosomes (100% = 118,000 cpm).

occurring on tetracycline-sensitive, wild-type ribosomes or tetracycline-resistant, TetM-protected ribosomes.

Inhibition of protein synthesis in vivo. The in vitro translation assay system employed was a coupled transcription-translation reaction (6, 12). Therefore, compounds that inhibit protein synthesis, as well as those affecting mRNA synthesis, score as protein synthesis inhibitors in this assay. To demonstrate that the glycylcyclines selectively inhibit protein synthesis, these compounds were tested for their effect on macromolecular synthesis in vivo. *E. coli* MC4100 (5) or MC4100 (pAT182) (7) cells were grown at 37°C on M63 glucose minimal medium (8) with aeration. The test compound was added to an exponentially growing culture (optical density at 550 nm, 0.4 to 0.6) to a final concentration of 10 μg/ml. At 0 min (immediately prior to the addition of the test compound) and at 15 and 30 min postaddition of the compound, 1-ml aliquots of the culture were removed and incubated for 4.5 min with radiolabeled protein, RNA, or DNA precursors: 0.25 μCi of ^{14}C uniformly labeled L-amino acid mixture, 1 μCi of [^{3}H]uracil, or 1 μCi of [^{3}H]thymine, respectively. Termination of the incorporation of radiolabel and precipitation of macromolecules was accomplished by the addition of 1 ml of cold 10% TCA followed by placement on ice. TCA-precipitable material was then collected by filtration onto Whatman GF/C glass fiber filters. The radiolabel retained on the filters was quantitated by liquid scintillation counting. The percent incorporated radioactivity relative to the zero time point for the same culture was determined.

TABLE 1. Inhibition of protein synthesis by tetracycline and the glycylcyclines, using tetracycline-sensitive and tetracycline-resistant, *tet*(M), *E. coli* isolates

Compound	Incorporation of radiolabeled precursors (%) in isolate					
	Tetracycline-sensitive MC4100 at:			Tetracycline-resistant MC4100(pAT182) at:		
	0 min	15 min	30 min	0 min	15 min	30 min
DMSO control[a]	100	101	99	100	99	109
Tetracycline	100	10	9	100	60	58
DMG-MINO	100	7	7	100	8	7
DMG-DMDOT	100	5	4	100	6	5

[a] DMSO, dimethyl sulfoxide.

Tetracycline and the two glycylcycline compounds, DMG-MINO and DMG-DMDOT, were all able to effectively reduce the incorporation of radiolabeled protein precursors by more than 90% within 15 min after their addition to a culture of tetracycline-susceptible cells. In contrast, no significant inhibition of incorporation of radiolabeled DNA or RNA precursors was observed (data not shown). The two glycylcycline compounds were equally effective at inhibiting protein synthesis occurring on TetM-protected ribosomes. When DMG-MINO or DMG-DMDOT was added to a culture of TetM-expressing cells, more than 90% reduction in the incorporation of radiolabeled protein precursors was observed. In contrast, tetracycline inhibited the incorporation of radiolabeled protein precursors by only 40% (Table 1). This level of protein synthesis inhibition was not sufficient to arrest cell growth, as measured by a continued increase in the optical density at 550 nm with time, equating to a doubling time of 4 versus 1.5 h for the control culture. As described above, no significant inhibition of DNA or RNA synthesis was observed following exposure to any of the compounds (data not shown). Thus, the antibacterial activity of the glycylcyclines is mediated through the inhibition of protein synthesis.

With a few exceptions, notably the anhydrotetracyclines whose mode of action is not limited to the inhibition of protein synthesis (9), there have been no tetracycline compounds with antimicrobial activity against TetM-expressing microorganisms. The mechanism by which the glycylcyclines overcome the TetM-mediated resistance has not been fully elucidated. It is possible that the glycylcyclines simply bind more tightly to ribosomes than the tetracyclines and that TetM is not able to

disrupt this tight binding and allow protein synthesis to progress. Alternatively, the glycylcyclines may bind to a ribosome in such a way that TetM is not able to effectively interact with the ribosome and permit protein synthesis to progress. Whatever the mechanism by which the glycylcyclines overcome TetM-mediated tetracycline resistance, it is clear that the glycylcyclines represent a new generation of tetracycline compounds that specifically inhibit protein synthesis and are active at inhibiting protein synthesis occurring on TetM-protected ribosomes. This finding is supported by the low MICs for the glycylcycline compounds when tested against tetracycline-resistant bacteria of a variety of species, with resistance due to either ribosomal protection or tetracycline efflux (11). Like the newer generations of β-lactam antibiotics, the glycylcyclines should breathe new life into the use of tetracycline compounds in antimicrobial chemotherapy.

REFERENCES

1. **Burdett, V.** 1986. Streptococcal tetracycline resistance mediated at the level of protein synthesis. J. Bacteriol. **165**:564–569.
2. **Burdett, V.** 1991. Purification and characterization of Tet(M), a protein that renders ribosomes resistant to tetracycline. J. Biol. Chem. **266**:2872–2877.
3. **Burdett, V., J. Inamine, and S. Rajagopalan.** 1982. Heterogeneity of tetracycline resistance determinants in *Streptococcus*. J. Bacteriol. **149**:995–1004.
4. **Cammack, K. A., and H. E. Wade.** 1965. The sedimentation behavior of ribonuclease-active and -inactive ribosomes from bacteria. Biochem. J. **96**:671–680.
5. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* operon to selected promoters in *Escherichia coli* using bacteriophage λ and Mu. J. Mol. Biol. **104**:541–555.
6. **Collins, J.** 1979. Cell-free synthesis of proteins coding for mobilization functions of ColE1 and transposition functions of Tn3. Gene **6**:29–42.
7. **Martin, P., P. Trieu-Cuot, and P. Courvalin.** 1986. Nucleotide sequences of the *tet*M tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn1545. Nucleic Acids Res. **14**:7047–7058.
8. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Rasmussen, B. A., H. F. Noller, G. Daubresse, B. Oliva, Z. Misulovin, D. M. Rothstein, G. A. Ellestad, Y. Gluzman, F. P. Tally, and I. Chopra.** 1991. Molecular basis of tetracycline action: identification of analogs whose primary target is not the bacterial ribosome. Antimicrob. Agents Chemother. **35**:2306–2311.
10. **Speer, B. S., N. B. Shoemaker, and A. A. Salyers.** 1992. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin. Microbiol. Rev. **5**:387–399.
11. **Testa, R. R., P. J. Petersen, N. V. Jacobus, P.-E. Sum, V. J. Lee, and F. P. Tally.** 1993. In vitro and in vivo antibacterial activities of the glycylcyclines, a new class of semisynthetic tetracyclines. Antimicrob. Agents Chemother. **37**:2270–2277.
12. **Zubay, G.** 1973. In vitro synthesis of protein in microbial systems. Annu. Rev. Genet. **7**:267–287.