Glycylcyclines Bind to the High-Affinity Tetracycline Ribosomal Binding Site and Evade Tet(M)- and Tet(O)-Mediated Ribosomal Protection

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Received 2 February 1996/Returned for modification 22 April 1996/Accepted 25 June 1996

N,N-dimethylglycylamido (DMG) derivatives of 6-demethyl-6-deoxycyclin and doxycycline bind 5-fold more effectively than tetracycline to the tetracycline high-affinity binding site on the Escherichia coli 70S ribosome, which correlates with a 10-fold increase in potency for inhibition of E. coli cell-free translation. The potencies of DMG-doxycline and DMG-6-demethyl-6-deoxycycline were unaffected by the ribosomal tetracycline resistance factors Tet(M) and Tet(O) in cell-free translation assays and whole-cell bioassays with a conditional Tet(M)-producing E. coli strain.

The antibiotic tetracycline binds to a single high-affinity site on the prokaryotic ribosome, causing inhibition of peptide synthesis (8, 16). Tetracycline use has been severely restricted because of the emergence of resistance, which has been credited primarily to ribosomal protection or efflux mechanisms, in all almost relevant pathogens (4, 15). Noncovalent association of either Tet(M) or Tet(O), the best-characterized ribosomal protection factors (2, 3, 9, 10, 20, 21), with the ribosome restores polypeptide synthesis in cell-free translation systems inhibited by tetracycline (3, 10).

A new class of tetracycline derivatives, the glycylcyclines, which bear an N,N-dimethylglycylamido (DMG) moiety at position 9 of the tetracycline nucleus, has potent antimicrobial activity versus tetracycline-resistant strains expressing either ribosomal protection or efflux determinants (1, 5, 22). The DMG derivative of 6-demethyl-6-deoxycyclin (DMDOT) inhibits tetracycline-insensitive, Tet(M)-protected in vitro translation (11). The experiments presented in this paper examine glycylcycline binding to the ribosome and the effect of Tet(M) and Tet(O) on glycylcycline activity.

Antibiotics. DMG-DMDOT and DMG-doxycline (DOX) were synthesized on site as described previously (1, 19). All other antibiotics were obtained from commercial sources. (This work was presented in poster form at the First International Conference on Antibiotic Resistance: Impact on Discovery, Englewood, Colo., September 1994, and at the 96th General Meeting of the American Society for Microbiology, New Orleans, La., May 1996.)

Ribosome binding studies. [3H]Tetracycline (New England Nuclear) binding to Escherichia coli BL21(DE3) (17) 70S ribosomes (prepared as described in reference 24, except that the NH4Cl washes were excluded) was performed by a nito-cellulose filter binding assay (9). Background filter binding was determined for each [3H]tetracycline concentration in reactions lacking ribosomes, and [3H]tetracycline reaction mix concentrations were accurately determined by scintillation counting of unfiltered reaction mixtures. The binding of [3H]tetracycline to E. coli ribosomes in the absence of competitor was analyzed by the Scatchard analysis (12), which revealed a high-affinity binding site for tetracycline at concentrations at or below 10 μM (data not shown), which is in agreement with published data (16).

A fixed concentration of 3.0 μM [3H]tetracycline, which should bind primarily to the high-affinity binding site, produced an adequate signal (400 cpn [20:1, signal to background]) in competitive binding assays. The concentration of unlabeled competitor reducing [3H]tetracycline binding by 50% (IC50) for unlabeled tetracycline was 3.5 μM (Table 1), which is consistent with the predicted value 3.0 μM. The IC50s of both DMG-DMDOT and DMG-DOX were 0.7 μM (Table 1). Thus, tetracycline and the glycylcyclines appear to share a common ribosomal binding site, and the glycylcyclines bind five times more effectively to this site.

Expression and partial purification of Tet(M) and Tet(O). High-level Tet(M) and Tet(O) production was induced in cultures of E. coli BL21(DE3)(pSH52) (3) and BL21(DE3) (pNB703) (21), respectively, by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) as described previously (18). The Tet(M) and Tet(O) proteins were partially purified from crude cell lysate by successive passes through Q Sepharose, G-25M, and Mono S HR 10/10 anion-exchange columns (Pharmacia). Tet(M)- and Tet(O)-containing fractions were detected by Western blotting (immunoblotting) (23), with antipeptide antibodies raised against the N-terminally acetylated synthetic peptides representing the C-terminal residues of the proteins being used. The activities of these fractions were confirmed by assessing their abilities to rescue tetracycline-inhibited, poly(U)-directed, cell-free translation (3). Tet(M) and Tet(O) were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to constitute approximately 60% of the total protein (200 μg/ml) in their respective Mono S HR 10/10 active fraction pools. The Tet(M) and Tet(O) pools were exchanged into standard buffer and stored as described previously (3).

MS2-directed polypeptide synthesis inhibition assay. A cell-free translation system using S-30 derived from tet(M)-competent E. coli has been described previously (11). However, the addition of Tet(M) or Tet(O) directly into translation reaction mixtures allowed the normalization of cell lysate and relative quantitation of resistance factors. A system replacing S-30 with 1.25 A260 units per ml of ribosomes washed three times with...
0.5 M ammonium chloride (24) and reconstituted with 400 μg of S150 (ribosome-free) cell lysate per ml was used in these studies (see reference 6 for general methods). The addition of partially purified Tet(M) or Tet(O) fractions at the indicated levels to this system did not alter total peptide synthesis (data not shown).

The translation IC₅₀ values (the concentration of the inhibitor reducing polypeptide synthesis by 50%) are presented in Table 1. DMG-DMDOT and DMG-DOX are 10-fold more potent than tetracycline at inhibiting polypeptide synthesis, which correlates with their greater degree of ribosomal binding.

Tet(M) and Tet(O) at estimated concentrations of 1.0 μM decreased tetracycline potency by 10-fold but did not reduce DMG-DMDOT potency. Similarly, 0.22 μM Tet(M) and 0.25 μM Tet(O) decreased tetracycline potency by 5-fold and 2.5-fold, respectively, but did not reduce DMG-DOX potency. Thus, the in vitro activities of the glycycyclines during Tet(M)- and Tet(O)-containing translation reactions correlate with their favorable whole-cell activities versus tetracycline-resistant strains harboring these ribosomal resistance factors (1, 22).

**Agar disk diffusion bioassay.** It is possible that the glycycyclines overcome the mechanism of ribosomal protection only by virtue of their greater cell-free potency (reference 11 and see above). To explore this possibility, the potencies of tetracyclines and glycycyclines against an *E. coli* strain harboring an inducible tet(M) construct were evaluated by bioassay under conditions promoting various levels of tet(M) production.

The methods for the agar disk diffusion bioassay have been described previously (7). The indicator organism, a spontaneous mutant of BL21(DE3)(pSH52) (which grew poorly following induction), retained inducible tetracycline resistance (MICs of 3.25 μg/ml for IPTG-free broth and 25 μg/ml for IPTG-containing broth [the MIC method is discussed in reference 6]) and grew equally well induced or uninduced. Paper disks containing 30 μg of test antibiotics were prepared, and plate-to-plate variation was standardized with 10-μg disks of ciprofloxacin (13). Tet(M) production by the indicator organism, as appropriate, was induced by the addition of 0.4 mM IPTG (18). An initial broth inoculum (M9 medium [17] with 100 μg of ampicillin per ml) of the indicator organism was split into two cultures with optical densities at 595 nm of 0.6, and one of these cultures was induced. After the cultures reached an optical density at 595 nm of 1.0, 1-liter portions of the IPTG-containing and IPTG-free cultures in M9-ampicillin with 1.5% agar were inoculated with 1.0 ml of the induced and uninduced broth cultures (for a total of 4 liter-volumes) and used in the preparation of bioassay pour plates. By means of this tet(M) induction scheme, the level of tetracycline resistance expressed by the indicator organism was varied in accordance with the relative number of bacterial generations producing Tet(M).

Bioassay data are expressed as the differences in the diameters of the zones of inhibition (Δmm) for uninduced cultures and broth-agar-induced cultures (Table 1). For example, the diameters of the zones of tetracycline inhibition measured 20.0 mm uninduced, 19.0 mm broth induced, 12.2 mm agar induced, and 10.1 mm broth and agar induced (Δmm = 9.9 mm). Similarly, minocycline and doxycycline potencies decreased with the increasing stringency of tet(M) expression (Δmm = 8.8 and 9.3 mm, respectively). DMG-DMDOT and DMG-DOX potencies were unaffected by increased Tet(M) production (Δmm = 0.4 and 0.2 mm, respectively).

These results correlate with the cell-free translation data and suggest that glycycycline evasion of ribosomal resistance is not the result of a mere increase in vitro potency. Alternatively, the indicator organism, which appears to avoid the toxic side effects associated with the T7/pET system (observed in the parent strain and explained in reference 18) by attenuating tet(M) expression (data not shown), may not produce enough Tet(M), even under stringent induction, to impair glycycycline activity. Thus, it is unlikely that the T7/pET expression system can be used to fabricate an appropriate indicator organism which produces greater quantities of Tet(M).

**Tet(A/B)-mediated efflux of DMG-DOX.** DMG-DOX has been shown not to be a substrate for the Tet(A/B) tetracycline efflux antiporter (14). Using similar methods, we have confirmed that DMG-DMDOT and additionally DMG-DOX do not compete with tetracycline for Tet(A/B)-mediated transport at the levels tested (Table 1).

**References**


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**TABLE 1. Summary of data for in vitro and whole-cell assays**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Ribosomal binding</th>
<th>Tet(A/B) effluxa</th>
<th>Translation</th>
<th>Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (μM)b</td>
<td>No resistance factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 μM Tet(M)</td>
<td>1.0 μM Tet(O)</td>
<td>0.42 μM Tet(M)</td>
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<tr>
<td>Tetracycline</td>
<td>3.5</td>
<td>25</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>DMG-DMDOT</td>
<td>0.7</td>
<td>&gt;40</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>DMG-DOX</td>
<td>0.7</td>
<td>&gt;40</td>
<td>0.15</td>
<td>ND</td>
</tr>
<tr>
<td>Minocycline</td>
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<td>2</td>
<td>0.45</td>
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<td>Doxycycline</td>
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<td>ND</td>
<td>1.4</td>
<td>ND</td>
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</tbody>
</table>

*ND, not determined.

b IC₅₀, for uptake of 5 μM [³H] tetracycline into Tet(A/B) containing everted membrane vesicles.


