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Reversal of Tetracycline Resistance Mediated by Different Bacterial Tetracycline Resistance Determinants by an Inhibitor of the Tet(B) Antiport Protein

MARK L. NELSON[1,2] AND STUART B. LEVY[1,2,3]*

Center for Adaptation Genetics and Drug Resistance[1] and Departments of Molecular Biology and Microbiology[2] and of Medicine,[3] Tufts University School of Medicine, Boston, Massachusetts 02111

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Active efflux is a useful strategy by which bacteria evade growth inhibition by antibiotics. Certain semisynthetic tetracycline (TC) analogs, substituted at the 13th carbon at C-6 on ring C of the TC molecule, blocked TC efflux as revealed in everted membrane vesicles from class B TC-resistant (Tc^r) *Escherichia coli* (M. L. Nelson, B. H. Park, J. S. Andrews, V. A. Georgian, R. C. Thomas, and S. B. Levy, J. Med. Chem. 36:370–377, 1993). A representative C-13-substituted analog, 13-cyclopentylthio-5-OH-TC (13-CPTC), was shown to competitively inhibit TC translocation by the Tet(B) protein, blocking the uptake of TC into vesicles and therefore the efflux of TC from whole cells. Against Tc^r *E. coli*, 13-CPTC, when used in combination with doxycycline, produced synergistic inhibition of growth. 13-CPTC was shown to increase the uptake of [^{3}H]TC into the resistant cells. 13-CPTC alone was a potent growth inhibitor against TC-susceptible (Tc^s) and Tc^r *Staphylococcus aureus* and enterococci specifying class K or class L efflux-dependent TC resistance mechanisms or, unexpectedly, the class M ribosomal protection mechanism. These findings indicate that derivatives of TC, identified by their ability to block the Tet(B) efflux protein, can restore TC activity against Tc^r bacteria bearing either of the two known resistance mechanisms. Blocking drug efflux and increasing intracellular drug concentrations constitute an effective approach to reversing TC resistance and may be generally applicable to other antibiotics rendered ineffective by efflux proteins.

Resistance to tetracyclines (TCs) is a major obstacle to the use of these drugs in the treatment of a variety of bacterial diseases of the respiratory, urinary, and digestive tracts (5, 18, 20). More than 20 different TC resistance determinants have been identified and given letter designations (9, 18). They mediate resistance by two different mechanisms: active efflux or ribosomal protection. Class A to E, G, and H determinants among *Enterobacteriaceae* and other gram-negative bacilli and classes K and L among gram-positive bacteria specify an active-efflux mechanism for TCs that enables the bacteria to thrive in the presence of therapeutic TC levels (6, 11). This mechanism is mediated by a related family of integral inner membrane antiport proteins which efflux a TC-cation complex in exchange for a proton (23). The carrier proteins, designated Tet proteins (8), are proton motive force (PMF) dependent (4, 11), capable of expelling TCs by using PMF created from electron transport substrates such as lactate or from ATP hydrolysis (11). TC resistance classes M, O, and S among *Streptococcus* spp., *Staphylococcus* spp., and *Listeria* spp. and class Q among *Bacteroides* species impart resistance via the expression of a family of related cytoplasmic proteins which protect ribosomes from the inhibitory action of TCs (1). The class P resistance determinant from *Clostridium perfringens* contains two overlapping resistance genes, one for an active-efflux protein and one for a ribosomal protection-type cytoplasmic protein (19).

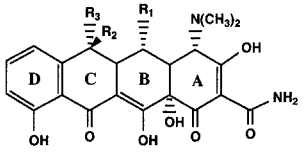
The relative binding affinities of substrates and potential inhibitors of the Tet(B) efflux protein were assessed by using everted inner membrane vesicles from *Escherichia coli* bearing the Tet(B) protein. After cell lysis in a French pressure cell (15), the orientation of the inner membrane bearing the efflux protein is reversed, leading to accumulation of [^{3}H]TC in vesicles instead of antibiotic efflux from the whole cell (11). Vesicles serve as an efficient biological screen for compounds which can interact with the active site of Tet(B) and inhibit the accumulation of [^{3}H]TC in vesicles. Previously, we identified a series of semisynthetic TC derivatives, the C-13-substituted thiol derivatives of methacycline, which had pronounced inhibitory effects on the accumulation of [^{3}H]TC in everted membrane vesicles (16). Based on C-13 substituent STERIMOL values (21), a subset of derivatives with molecular dimensions of *L* of 4.4 to 6.2 Å (maximum length) and *B4* of 3.0 to 4.2 Å (maximum width) combined with favorable substituent lipophilicity parameters (octanol/H_2O partition coefficient = 1.0 to 2.7) were identified as the most effective inhibitors of TC accumulation in vesicles (16, 17).

While several of these TC analogs also showed growth-inhibitory activity against efflux-based Tc^r bacteria, we chose one of the most potent inhibitors, 13-cyclopentylthio-5-OH-TC (13-CPTC) (Fig. 1) (16), for further studies of its activity against different Tc^r bacteria, its mechanism of antiport inhibition, and its effect on TC accumulation in both everted vesicles and whole cells possessing the Tet(B) protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. The Tc^s *E. coli* strain ML308-225 (*lacI lacZ*) or its derivative possessing plasmid R222 (designated strain *E. coli* D1-209), which is resistant to TC due to the production of the Tet(B) efflux protein, were used in the studies of vesicle function and in antiport studies. Other strains used were *E. coli* ML308-222 bearing plasmid pIP15 [D1-299 (Tet A)] and the lipopolysaccharide (LPS)-deficient *E. coli* D31m4 (14) with or without plasmid pHCM1 specifying the constituitively expressed Tet(B) protein (3). The gram-positive strains *Staphylococcus aureus* Tc^s RN450 and Tc^r RN4250 (the latter bearing the Tet K determinant on plasmid pT181) were obtained from R. Novick (New York, N.Y.). *Enterococcus hirae* ATCC 9790 (previously classified as *E.*

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: slevy@opal.tufts.edu.



	R_1	R_2	R_3
Tetracycline	H	OH	CH_3
Doxycycline	OH	H	CH_3
13-CPTC	OH	H	CH_2-S

FIG. 1. Structures of TC, doxycycline, and the Tet(B) efflux protein inhibitor 13-CPTC.

faecalis or *E. faecium*), with and without the Tet L determinant on plasmid pMV158 or the Tet M determinant on plasmid pAM211, was obtained from V. Burdett (Duke University, Durham, N.C.).

Media and chemicals. Minimal medium A (11) supplemented with 0.25% glucose and 0.0001% vitamin B_1 was used for the growth of bacteria used in the assay experiments. TC was added for resistant strains only (gram-negative strains, 2 μg/ml; gram-positive strains, 5 μg/ml). Doxycycline hydrochloride was a gift from Pfizer Laboratories (Groton, Conn.), while TC hydrochloride and minocycline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). 13-CPTC HCl was synthesized as previously described (16) and stored as a dry yellow powder at room temperature. Solutions of the TC compounds were prepared in water prior to their use. [7-^{3}H]TC (0.9 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.), as were the [^{3}H]uridine (24 Ci/mmol), [^{35}S]methionine (1,175 Ci/mmol), and [^{3}H]thymidine (20 Ci/mmol) used in the macromolecular synthesis experiments. Solutions of the radiolabeled compounds were aliquoted and stored at −70°C prior to use.

Preparation of vesicles and assay procedures. Everted membrane vesicles were prepared as previously described (11, 16). Cells grown in medium A from an A_{530} of 0.1 to 0.8 were isolated by centrifugation, washed once in cell wash buffer (100 mM K_2HPO_4-KH_2PO_4, 10 mM Na_2EDTA, pH 6.6) and lysed in a cold French pressure cell (11, 15). The viscous solution obtained was first centrifuged at 21,000 × *g* (10 min, 4°C), and the supernatant was then centrifuged at 150,000 × *g*) 1 h, 4°C) to pellet everted membrane vesicles. Protein determinations were done by the method of Lowry et al. (10), and aliquots were stored in vesicle storage buffer (50 mM K_2HPO_4-KH_2PO_4, pH 6.6) at −70°C until use.

Determination of K_ms, K_is, and IC_{50}s of TC derivatives by using everted membrane vesicles. Dilutions of the test compounds as well as the control compound methacycline hydrochloride were placed in test tubes and incubated in a water bath at 30°C. The negative control used carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) (Sigma Chemical Co.), an agent that collapses the PMF, whereas the positive control and test solutions received only Li lactate (12 mM), the energy source used to achieve electron transfer and thereby PMF. At the beginning of each experiment, vesicles were added to vesicle assay buffer (50 mM K_2HPO_4-KH_2PO_4, 10 mM $MgSO_4$, pH 7.5) to a final concentration of 0.5 mg of protein per ml. Approximately 300 μl was used for each experiment with each compound examined. At zero time, Li lactate was added and the vesicles were incubated with shaking at 30°C. The vesicles were then added to the individual drug concentrations and incubated for 30 s before [^{3}H]TC was added to a final concentration of 3 to 4 μM. Every 30 s, 50 μl of mixture was removed, diluted in 10 ml of dilution buffer, filtered through presoaked Gelman GN6 filters (0.45 μm pore size), and rinsed once with dilution buffer (4 ml). Filters were dried, and the radioactivity was assayed with a scintillation counter. The background due to filter trapping was subtracted but represented <10% of the radioactivity measured. Calculations of uptake of [^{3}H]TC per milligram of protein resulted in the determination of the apparent K_i and the apparent 50% inhibitory concentration (IC_{50}) by use of the Cheng-Prusoff equation (2). Using Mg^{2+} as the dicationic species, our laboratory finds a K_m range of 6 to 20 μM for TC among various vesicle preparations.

Fluorescence assay of TC transport and inhibition. Everted membrane vesicles from *E. coli* ML308-225 (Tc^s) and *E. coli* D1-209 (Tc^r) were added to 2 ml of vesicle assay buffer to a final concentration of 0.5 mg/ml in a cuvette containing a stir bar. Acridine orange (AO) (Sigma Chemical Co.), the fluor of which is an indicator of ΔpH, was added (2 μM) and equilibrated for 2 min (15, 22). Lithium lactate was added (20 mM), and the fluorescence was monitored (Hitachi model F2000 spectrometer) with an excitation wavelength set at 490 nm while the emission wavelength was observed at 530 nm. At various times after vesicle activation, TC or 13-CPTC was added, and the change in the relative amount of fluorescence was observed. Where indicated, the pH gradient was collapsed by using CCCP (50 μM).

Determination of MICs and synergy MICs. The bacterial strains used were grown in Luria broth overnight at 37°C. Tc^r *E. coli* D1-299 (class A, pIP15), D31m4* (class B, bearing pHCM1), and D1-209 (class B, R222) were grown with 2 μg of TC HCl per ml. *S. aureus* RN4250 (class K, pT181) and *E. hirae* ATCC 9790 (class L, pMV158, or class M, pAM211) were grown with 5 μg of TC HCl per ml. Cultures were inoculated into Luria broth and grown at 37°C to an A_{530} of 0.6 to 0.8. All TCs and analogs were soluble in water and/or <2% dimethyl sulfoxide. Test compound dilution and bacterial inoculations were performed with a 96-well microtiter plate format on a programmable Perkin-Elmer Cetus Propette, where each sterile well received serial dilutions of compounds beginning at 40 or 50 μg/ml and bacteria (2×10^5 to 5×10^5 cells/ml) in Luria broth. After incubation at 37°C for 18 to 24 h, the microtiter plates were read at 530 nm on a Bio-Rad Microtiter Plate Reader and scored by using Luria broth as a blank (A_{530} = 0.00). Synergy MICs were determined and generated by the summation of the lowest MICs of each compound alone and then in combination. Compounds contributing additively to bioactivity possessed a fractional inhibitory concentration of ≥0.5, whereas synergistic inhibition of growth was defined as a fractional inhibitory concentration of ≤0.5.

Accumulation of [7-^{3}H]TC. Cells were grown in medium A supplemented with 0.25% glucose and 0.0001% vitamin B_1 (*E. coli* ML308-225) and 2 μg of TC HCl per ml (*E. coli* D1-209) to an A_{530} of 0.8, washed once with assay medium (50 mM K_2HPO_4-KH_2PO_4, 10 mM $MgSO_4$, pH 6.0), and pelleted (6,000 rpm, 5 min). The cells were diluted to an A_{530} of 4.0 with assay medium, 0.2% glucose was added, and the cells were incubated for 1 min at 30°C. At various times after the addition of 4 to 6 μM [^{3}H]TC or [^{3}H]TC plus 13-CPTC, 50-μl samples were removed, mixed in 10 ml of 0.1 M LiCl–0.1 M KPO_4 buffer, filtered through a Gelman GN6 0.45-μm-pore-size membrane, and rinsed once with 4 ml of buffer. The filters were dried, and the radioactivity was assayed in Liquifluor scintillation liquid. Background radioactivity caused by the filters alone was subtracted, the counts were normalized, and the results were plotted as picomoles of [^{3}H]TC per A_{530} unit. For [^{3}H]TC uptake experiments in the presence of increasing concentrations of unlabeled TC, minocycline, or 13-CPTC, cells were prepared and incubated for 20 min at 30°C in the presence of compound and treated as described above.

Effect of compounds on macromolecular synthesis. Cells at 10^7/ml were inoculated in Luria broth without or with increasing amounts of 13-CPTC as well as a radiolabeled precursor ([^{35}S]methionine, [^{3}H]thymidine, or [^{3}H]uridine [New England Nuclear]). At different times, 50-μl aliquots were removed and spotted onto 4-cm^2 squares of Whatman no. 1 filter paper. After incubation in ice-cold 10% trichloroacetic acid (TCA) for 10 min, washing in 5% TCA for 10 min, and then rinsing in ethanol, the filter papers were dried at 37°C for 2 h and the radioactivity was assayed in a scintillation counter.

RESULTS

Effect of 13-CPTC on accumulation of [^{3}H]TC by everted vesicles containing Tet(B) protein. In Tet(B)-containing everted vesicles, accumulation of [^{3}H]TC is saturable, with a K_m in the range of 6 to 20 μM (11, 13, 17). Increasing amounts of [^{3}H]TC were added to vesicles along with two different concentrations of 13-CPTC, and the amount of lactate-dependent [^{3}H]TC accumulation at equilibrium (2.5 min) (16) was

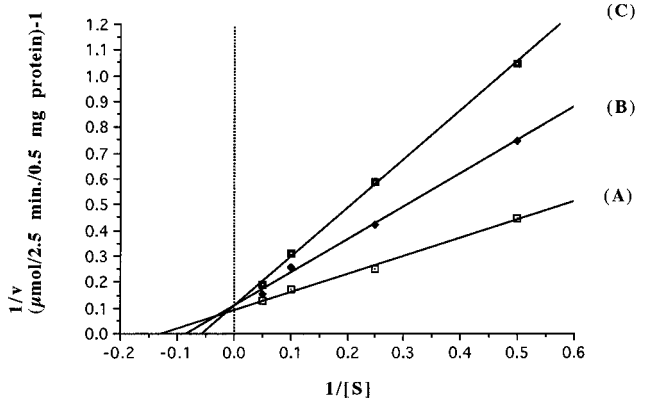


FIG. 2. Lineweaver-Burke plot of inhibition of [^{3}H]TC accumulation in *E. coli* D1-209 everted membrane vesicles by 13-CPTC. Everted vesicles from *E. coli* D1-209 were incubated with different concentrations of [^{3}H]TC ([S] = 2.5, 5.0, 10.0, and 20 μM) in the absence and presence of different concentrations of 13-CPTC. Samples were taken at 2.5 min. (A) No 13-CPTC; (B) 0.9 μM 13-CPTC; (C) 3.7 μM 13-CPTC.

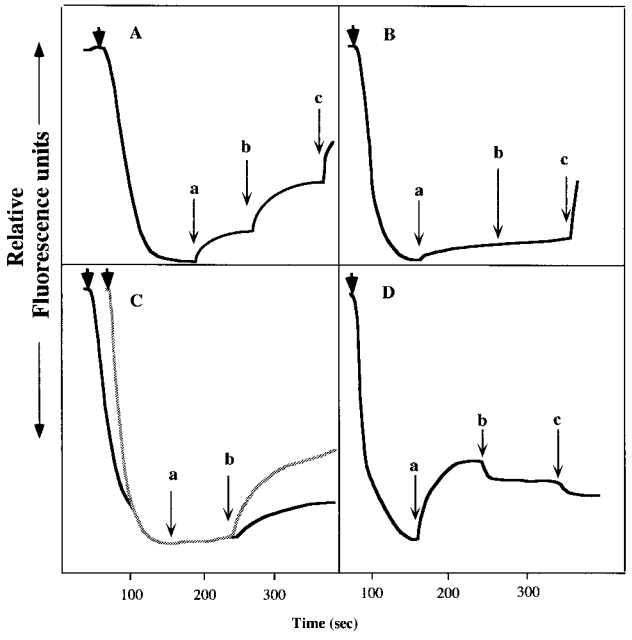


FIG. 3. Effect of TC and 13-CPTC on pH gradient formation in *E. coli* D1-209 everted membrane vesicles observed by AO (2.5 μM) fluorescence. Vesicles were energized with Li lactate (20 mM, arrows) and TC or 13-CPTC added at different times. (A) Addition of 7.0 μM TC (a), 20 μM TC (b), and 50 μM CCCP (c). (B) Addition of 22 μM 13-CPTC (a), 20 μM TC (b), and 50 μM CCCP (c). (C) Grey tracing, Li-lactate-energized vesicles treated with control buffer (a) followed by 2.0 μM TC (b). Black tracing, Li-lactate-energized vesicles treated with 0.2 μM 13-CPTC (a) followed by 2.0 μM TC (b). (D) Addition of 20 μM TC (a) followed by addition of 22 μM 13-CPTC (b) and further addition of 22 μM 13-CPTC (c).

determined, as an approximation of the initial rate of transport. Applying Michelis-Menton kinetics, the data, plotted by using Lineweaver-Burke methods, showed that 13-CPTC acted by competitive inhibition of TC transport (Fig. 2). The calculated K_m for TC (7.75 μM) determined was within the established range for TC, while the K_i determination for 13-CPTC in the same experiment was 1.1 μM. These findings show that the 13-CPTC derivative had greater affinity and was competing with TC for the TC binding site in the Tet(B) protein. The increase in $1/K_m$ apparent upon addition of the inhibitor changes the distribution of available Tet(B) protein from affinity for TC and high TC translocation ability to decreased affinity for TC and low TC translocation.

Competition for TC transport by 13-CPTC determined by fluorescence spectroscopy. TC proton antiport dynamics in Tet(B) protein-containing everted vesicles were also examined in the presence of 13-CPTC by fluorescence spectroscopy with AO emission (13, 15, 23). Upon energization by lactate addition, fluorescence decreased in the vesicles as a pH gradient was established, changing the distribution of fluorescing AO (Fig. 3A). Addition of TC to vesicles from resistant cells, but not susceptible cells, led to increased fluorescence, representing the exchange of a proton for the TC-cation complex mediated by the Tet(B) protein (Fig. 3A) (13, 23). CCCP, a metabolic energy uncoupler, collapsed the pH gradient in vesicles and restored fluorescence.

The coupling of TC transport to AO fluorescence was cumulative; increasing concentrations of TC resulted in larger changes in AO fluorescence (Fig. 3A) up to TC concentrations approximating the K_m of TC (6 to 20 μM) in the everted-vesicle assay. Vesicles treated with 13-CPTC also showed an increase in AO fluorescence but to a much smaller degree than with TC, where greater than 20 μM 13-CPTC was needed to observe an effect similar to that of TC at <5 μM (Fig. 3B). These findings suggest that the inhibitor was transported, but less efficiently than Tc. Vesicles pretreated with 13-CPTC (22 μM) did not show a change in fluorescence when 20 μM TC was added (Fig. 3B). At much lower concentrations of 13-CPTC, when translocation of the analog was not detected by AO fluorescence, the TC response was still inhibited (Fig. 3C). When 13-CPTC was added after vesicle translocation of an equimolar amount of TC, there was a loss of total fluorescence and a gradual reversal of the pH change (Fig. 3D).

These results suggest that 13-CPTC is capable of interfering with TC transport by competitively binding to the Tet(B) antiporter but is itself transported less efficiently than TC, causing less proton translocation.

TABLE 1. Growth-inhibitory effect of 13-CPTC alone or in combination with doxycycline against different TC-susceptible and TC-resistant bacterial strains

Drug	MIC (μg/ml)[a] for:									
	E. coli					*S. aureus* RN450[d]		*E. hirae* ATCC 9790[e]		
	ML308-225[b]			D31m4[c]						
	WT	Tet(A)	Tet(B)	WT	Tet(B)	WT	Tet(K)	WT	Tet(L)	Tet(M)
13-CPTC	20	50	50	0.8	6.25	0.5	1.56	0.8	0.39	3.12
Doxycycline	0.5	50	>50	0.5	12.5	0.25	12.5	0.5	6.25	25
Doxycycline + 13-CPTC	NT[f]	3.12 + 1.56	1.56 + 1.56	NT	1.56 + 0.39	NT	3.12 + 0.78	NT	1.56 + 0.19	6.25 + 1.56
Effect on growth[g]		S	S		S		A		A	A

[a] Concentration of compound which inhibited growth after 18 h in L broth after incubation at 37°C. Values are representative of those from at least three experiments. The MIC of tetracycline was ≥50 μg/ml for all determinants.
[b] Wild type (WT) or containing Tet(A) specified by plasmid pIP15 or Tet(B) specified by plasmid R222.
[c] Wild type or containing Tet(B) specified by plasmid pHCM1.
[d] Wild type or bearing Tet(K) specified by plasmid pT181.
[e] Wild type or bearing Tet(L) specified by plasmid pMV158 or Tet(M) specified by plasmid pAM211.
[f] NT, not tested.
[g] S, synergy; A, additive.

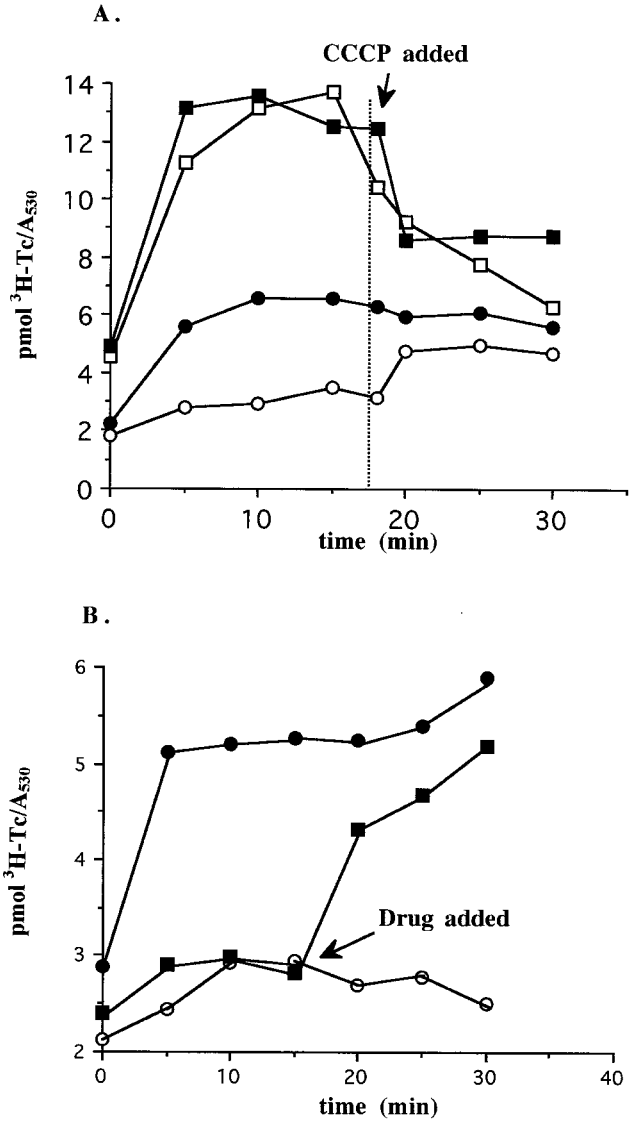


FIG. 4. (A) [^{3}H]TC accumulation in Tc^s *E. coli* ML308-225 alone (□) and with 40 μM 13-CPTC (■) compared to accumulation in Tc^r *E. coli* D1-209 expressing the Tet(B) protein without (○) or with (●) 40 μM 13-CPTC. At 18 min (vertical line) CCCP was added. (B) Effect of 13-CPTC on [^{3}H]TC accumulation in *E. coli* D1-209 alone (○) or with 13-CPTC (40 μM) added prior to [^{3}H]TC addition (●) or added 15 min after [^{3}H]TC addition (■).

Antibacterial activity of 13-CPTC, with or without doxycycline, against TC-susceptible and -resistant bacteria. The antibacterial activity of 13-CPTC was assayed alone or in combination with a clinically used TC, doxycycline, against Tc^r efflux-dependent *E. coli*, *S. aureus*, and *E. hirae*. 13-CPTC was relatively inactive alone against wild-type *E. coli* ML308-225 with or without the Tet(A) or Tet(B) protein (Table 1). However, the analog was more active against an LPS-deficient mutant, *E. coli* D31m4 (14). This finding demonstrates the effect of the outer cell wall of *E. coli* on the activity of this analog. In *E. coli* D31m4* expressing Tet(B) (class B determinant on plasmid pHCMI), 13-CPTC was more active than TC or doxycycline (Table 1). Additionally, 13-CPTC in combination with doxycycline exhibited synergy against all three Tc^r *E. coli* strains expressing either the Tet(A) or Tet(B) protein regardless of membrane LPS content, lowering the MICs by ≥2 serial dilutions for both doxycycline and 13-CPTC (Table 1).

Against Tc^r *S. aureus* and *E. hirae*, 13-CPTC possessed antibacterial activity alone, showing similar MICs against both Tc^s and Tc^r *S. aureus* and *E. hirae* (Table 1). The Tc^r cells bore either the Tet(K) or Tet(L) efflux protein, which are only distantly related to the Tet(B) protein (6, 7). 13-CPTC also inhibited the growth of *E. hirae* bearing a different resistance determinant and protein, Tet(M), which specifies a ribosomal protection mechanism (1). In contrast to the case for the gram-negative strains, only additive growth inhibition was seen when 13-CPTC was combined with doxycycline against the three Tc^r gram-positive strains examined (Table 1).

Uptake of TC with and without 13-CPTC. The accumulation of [^{3}H]TC in Tc^s *E. coli* ML308-225 and Tc^r D1-209 whole cells expressing the Tet(B) protein in the presence and absence of 13-CPTC was examined. Resistant D1-209 cells accumulated approximately fivefold less TC than did Tc^s ML308-225 cells. TC accumulation in Tc^s cells was not affected by the presence of 13-CPTC (Fig. 4A). In contrast, pretreatment of resistant cells with 13-CPTC increased TC accumulation to approximately that observed in sensitive cells after energy deprivation by CCCP. Thus, in these cells, the analog had not yet totally blocked efflux to reestablish the active accumulation of Tc^s cells (Fig. 4A). The effect of 13-CPTC on strain D1-209 actively effluxing TC was also examined. Within 5 min after addition of 40 μM 13-CPTC (Fig. 4B), there was a rapid accumulation of [^{3}H]TC, reaching approximately 50% of that found in Tc^s cells. This finding suggests that concentrations of 13-CPTC that do not inhibit growth can increase the cellular levels of TC by efficiently blocking TC efflux by competitive inhibition of the Tet protein. The lack of an effect on energy-dependent TC accumulation in ML308-225, as well as previous data showing no effect of 13-CPTC on everted susceptible vesicles, suggests that the analog acts on the Tet protein only and not on PMF, membrane perturbation, or energized transport processes.

In Tc^r D1-209 whole cells, even maximal concentrations of unlabeled TC (>1,000 μM) did not saturate the Tet(B) efflux

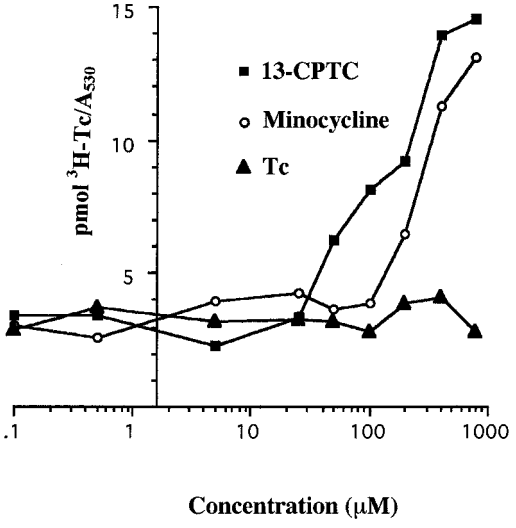


FIG. 5. Accumulation of [^{3}H]TC (4 μM) in the presence of increasing concentrations of 13-CPTC, minocycline, or unlabeled tetracycline. Tc^r *E. coli* D1-209 cells were incubated for 20 min and then assayed for accumulation of [^{3}H]TC by filtration of samples onto 0.45-μm-pore-size nitrocellulose membranes. The background counts were subtracted, and the counts were normalized.

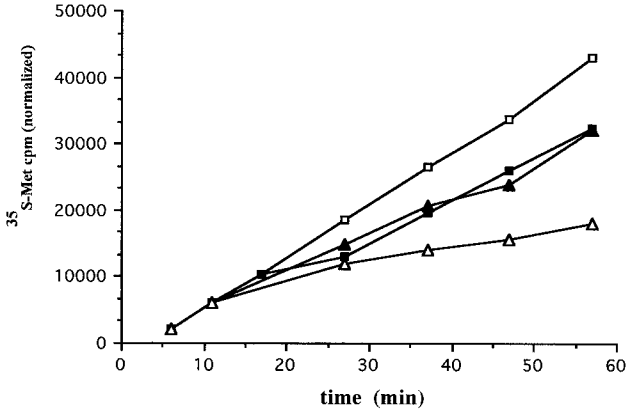


FIG. 6. [^{35}S]methionine (^{35}S-Met) (10 μCi) incorporation by mid-log phase Tc^r *E. coli* D1-209 cells in Luria broth alone (□) and in the presence of doxycycline (3 μg/ml) (■), 13-CPTC (24 μg/ml) (▲), or a combination of doxycycline (3 μg/ml) and 13-CPTC (24 μg/ml) (△). Macromolecular synthesis was stopped by using TCA, and the radioactivity was assayed, normalized, and reported as counts per minute.

protein or affect [^{3}H]TC accumulation (Fig. 5). In contrast, 13-CPTC caused increased accumulation of [^{3}H]TC in a dose-dependent manner and was more potent as an efflux-blocking agent than minocycline, a known Tet(B) ligand (12). Concentrations of 13-CPTC of around 40 μM led to no net active efflux of TC, whereas fivefold higher concentrations of minocycline (200 μM) were needed.

Effect of 13-CPTC on macromolecular synthesis. The site of antibacterial activity and possible cellular target(s) of 13-CPTC were examined at the level of macromolecular synthesis, using radiolabeled [^{35}S]methionine for protein, [^{3}H]uridine for RNA, and [^{3}H]thymidine for DNA. At the MIC of analog or doxycycline, only incorporation of [^{35}S]methionine into protein was inhibited in whole cells; RNA and DNA syntheses were little affected in *S. aureus* or *E. coli* D31m4 cells subjected to levels of analog even fivefold or more higher than the MIC (data not shown). Further studies examined the effect of 13-CPTC, doxycycline, or combinations of both on the incorporation of [^{35}S]methionine into protein by mid-log phase Tc^r *E. coli* expressing Tet(B) efflux protein. Against *E. coli* D1-209 expressing Tet(B) protein, at below the MICs, the compounds were moderately active as protein synthesis inhibitors, with doxycycline demonstrating approximately eightfold greater activity than 13-CPTC. However, when used in combination, at below the individual MICs, protein synthesis was significantly inhibited to a greater degree than was expected from additive amounts of both compounds. This finding suggests an increase in the intracellular doxycycline concentration and its availability for ribosome inhibition (Fig. 6).

DISCUSSION

These findings demonstrate that the TC analog 13-CPTC acts as a potent inhibitor of the Tet(B) protein, where it binds competitively with TC for the putative binding site(s) of the efflux protein. The sixfold-increased affinity of 13-CPTC for this site, compared to TC and doxycycline, demonstrates that chemically modified TCs can modulate the efflux and TC translocation process, reversing the effect of active Tet proteins on the intracellular concentrations of TCs.

13-CPTC is more active than doxycycline against Tc^r strains bearing Tet(K) or Tet(L) efflux proteins as well as a Tet(M) ribosomal protection mechanism. The latter finding demonstrates a further facility of the everted-vesicle assay system to screen and detect compounds which interfere with and modulate TC resistance by two different mechanisms. While 13-CPTC acted by inhibiting protein synthesis in both Tc^s and Tc^r bacteria, it was found to be considerably more potent when used in combination with doxycycline against *E. coli* expressing efflux mechanism (Fig. 6). Although 13-CPTC is less potent than doxycycline as a growth inhibitor or as a protein synthesis inhibitor of Tc^s cells, when used in combination with doxycycline against Tc^r *E. coli*, synergy was observed at well below the individual MICs, rendering the Tc^r cells Tc^s.

We hypothesize that the ability of 13-CPTC and other structurally related efflux inhibitors to affect the growth of Tc^r efflux-dependent bacteria is due to competitive inhibition of the Tet efflux protein, followed either by inhibition of protein synthesis by the analog, in the case of gram-positive bacteria, or by a coadministered TC, in the case of gram-negative bacteria. By combining subinhibitory amounts of compounds, it is possible to demonstrate a pronounced inhibitory effect both on growth in overnight cultures and on protein synthesis. Similarly, the addition of 13-CPTC to bacteria actively exporting TC results in the rapid accumulation of TC, reversing export and drug resistance.

By inhibiting Tet(B) efflux proteins, we have demonstrated the ability to disrupt transport processes and modulate tetracycline resistance, changing the bacterial cell to a state of TC susceptibility by using therapeutically attainable concentrations of TCs. In view of the widespread frequency of Tc^r among bacterial species, this approach offers methods and insights for the development of compounds that may one day be used to intervene pharmacologically to reverse TC drug resistance clinically. More broadly, drug efflux mechanisms, such as Tet proteins, are ideal models for the study of ligand binding, antibiotic structure-activity relationships, and, ultimately, the reversal of drug resistance phenotypes.

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