Molecular Requirements for the Inhibition of the Tetracycline Antiport Protein and the Effect of Potent Inhibitors on the Growth of Tetracycline-Resistant Bacteria

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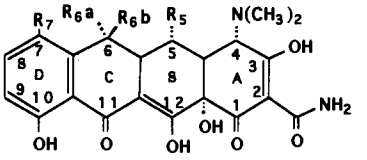
Forty-seven compounds and tetracycline (Tc) structural analogues were tested for their ability to interfere with [^{3}H]Tc uptake in everted inner membrane vesicles derived from Tc-resistant *Escherichia coli* D1-209, bearing the class B tetracycline resistance efflux protein (Tet protein). For effective inhibition of Tc uptake, the molecule had to have an intact ABCD tetracyclic carbon skeleton and a conjugated phenolic β-diketone substructure at positions 10–12a with the subsequent development of keto–enol tautomerization. Molecular variations at carbon positions 2, 4, 5, 6, 7, 8, and 9 did not decrease, and some increased, the inhibitory activity as compared to that of Tc. Among these compounds, the highest inhibition of uptake occurred with certain position 6 and 13 derivatives of 5-hydroxytetracycline. In a group of 13-(propylthio) derivatives of 5-OH-Tc [13-propyl, 13-(3-chloropropyl), and 13-(2-carboxyethyl)] there was a correlation between uptake inhibitory activity and antibacterial activity. The 13-(3-chloropropyl) derivative, with the best efflux inhibitory activity, exhibited synergistic activity when tested in combination with doxycycline against Tc-resistant *E. coli* bearing the class A or B determinant, against *Staphylococcus aureus* bearing class K, and against *Enterococcus faecalis* bearing the class L determinant. The 13-propyl analogue also showed high transport blocking activity and showed synergistic antibacterial activity against *E. coli* bearing the class A determinant and additive activity against the other Tc-resistant bacteria. The synergistic antibacterial activity of these compounds was not shown by the 13-[(2-carboxyethyl)thio] homologue, whose efflux blocking activity was 70-fold less. These findings suggest that multiple sites on the Tc molecule are available for synthetic modification toward the development of an effective Tc blocking agent. Such compounds, used alone or in combination with a standard tetracycline, show improved antibacterial activity.

The tetracyclines are a group of structurally-related antibiotics that have been used in the clinical treatment of bacterial infections since introduced in the late 1940s. The seven currently in use all bear the naphthacene nucleus with functional group modifications notable at carbons 5, 6, or 7 (Figure 1). The most commonly prescribed tetracyclines are tetracycline (Tc), doxycycline, and minocycline.[1] While simple structural changes have improved bioavailability, they have not enhanced antimicrobial activity, especially against tetracycline-resistant (Tc^r) bacteria.[2,3]

The ability of broad-spectrum Tc antibiotics to combat bacterial infection has been thwarted by the emergence of bacterial resistance.[1-3] More than a dozen genetically distinguishable Tc^r determinants have been described.[2-5] They include mechanisms of active efflux (classes A–E in enteric bacteria, classes K and L among Gram-positive bacteria) and ribosomal protection (classes M and O among Gram-positive and Gram-negative organisms).[3,8] Expression of the efflux determinant in enteric bacteria results in the production of an inner membrane 42–43 kD protein, designated the Tet protein,[9] which mediates active efflux of Tc and its analogues from Tc^r cells, thereby affecting bacterial cell survival. Once the Tet protein is expressed in a bacterial cell, it renders a tetracycline antibiotic virtually useless as a growth inhibitor at therapeutic concentrations.



Compound	R_5	R_{6a}	R_{6b}	R_7
Chlortetracycline	H	CH_3	OH	Cl
Oxytetracycline	OH	CH_3	OH	H
Tetracycline	H	CH_3	OH	H
Demeclocycline	H	H	OH	Cl
Methacycline	OH	$=CH_2$	-	H
Doxycycline	OH	CH_3	H	H
Minocycline	H	H	H	$N(CH_3)_2$

Figure 1. Structures of the clinically used tetracyclines and numbering system of the naphthacenecarboxamide ABCD ring system.

This study investigated molecular position variants in the Tc molecule in relation to the structural requirements for interference with Tet protein function and the potential to inhibit the active efflux of tetracycline in whole cells. The compounds tested were obtained from other laboratories, compound stores, and pharmaceutical companies or were synthesized according to methods reported in a previous publication.[10] These compounds were chosen specifically to define the essential and nonessential molecular requirements for blocking the Tc efflux system specified in resistant bacteria. This information would

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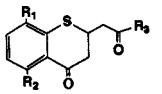
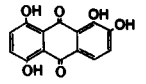
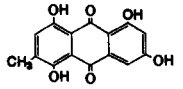
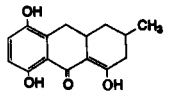
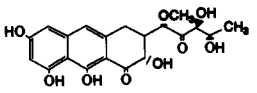
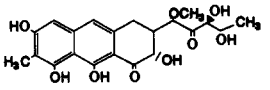
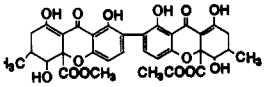
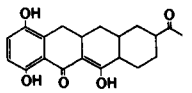
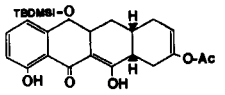
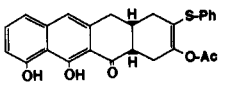
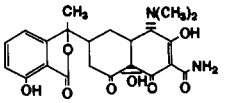
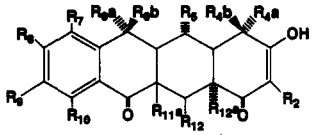
Table 1. Compounds and Tetracyclines Tested for Activity in Inhibiting Tetracycline Uptake in Everted Membrane Vesicles

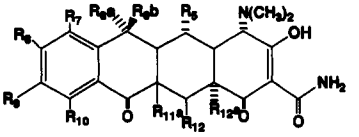
	compound	R_1	R_2	R_3	IC_{50}, μM[a]	t_R,[b] min	source[i]
	A. Partial Ring Structures						
	**1** (EMD 36928)	Cl	OH	OH	>150	NA[c]	A
	2 (EMD 36927)	Cl	OCH_3	OH	>150	14.19	A
	3 (EMD 46279)	Cl	OH	H	>75	NA[c]	A
	4 (EMD 36930)	H	OH	H	>75	16.83	A
	**5** (quinalizarin)				>150	NA[c]	B
	**6** (emodin)				>150	23.60	B
	**7**				>130	23.60	C
	**8** (olivin)				>100	NA[c]	B
	**9** (chromomycin B)				>100	NA[c]	B
	**10** (secalonic acid A)				>75	NA[c]	B
	**11**				>75	24.90	D
	**12** (TBDMSi = $Si(CH_3)_2C(CH_3)_3$, Ac = $COCH_3$)				>150	NA[c]	D
	**13** (Ph = phenyl, Ac = $COCH_3$)				>150	21.17[d]	D
	**14** (isotetracycline)				>75	NA[c]	E

B. Tetracycline Ring Structures



compound	R_2	R_4a	R_4b	R_5	R_6a	R_6b	R_7	R_8	R_9	R_{10}	$R_{11}a$[j]	R_{12}	R_{12a}	IC_{50}, μM[a]	t_R,[b] min	source[i]
C2 modifications																
15 (tetracyclinonitrile)	CN	$N(CH_3)_2$	H	H	CH_3	OH	H	H	H	OH		OH	OH	9.2	11.75	E, F
16 (2-decarboxamido-2-Ac-Tc)	$COCH_3$	$N(CH_3)_2$	H	H	CH_3	OH	H	H	H	OH		OH	OH	15.4	NA[c]	E
C4 modifications																
17 (4-de(dimethylamino)-Tc)	$CONH_2$	H	H	H	CH_3	OH	H	H	H	OH		OH	OH	6.1	19.88[e]	F
18 (4-epi-Tc)	$CONH_2$	H	$N(CH_3)_2$	H	CH_3	OH	H	H	H	OH		OH	OH	12.3	5.68	F
19 (Tc-4*N*-methiodide)	$CONH_2$	$N(CH_3)_4I$	H	H	CH_3	OH	H	H	H	OH		OH	OH	5.0	7.81	G
C6 modifications																
20 (doxycycline)	$CONH_2$	$N(CH_3)_2$	H	OH	CH_3	H	H	H	H	OH		OH	OH	9.2	6.88	E
21 (5a,6-6-CH_3-anhydro-Tc)	$CONH_2$	$N(CH_3)_2$	H	H	CH_3	-	H	H	H	OH		OH	OH	20.0	24.49	E
22 (6-demethyl-6-deoxy-Tc)	$CONH_2$	$N(CH_3)_2$	H	H	H	H	H	H	H	OH		OH	OH	4.6	10.71	E
23 (6-methylene-Tc)	$CONH_2$	$N(CH_3)_2$	H	H	$=CH_2$	-	H	H	H	OH		OH	OH	9.2	9.38	E
24 (5-OH-6-methylene-Tc)	$CONH_2$	$N(CH_3)_2$	H	OH	$=CH_2$	-	H	H	H	OH		OH	OH	2.6	10.31	E
25 (thia-Tc)	$CONH_2$	$N(CH_3)_2$	H	H	6-S-	-	H	H	H	OH		OH	OH	4.6	NA[c]	A

Table 1 (Continued)



	R_5	R_5a	R_6b	R_7	R_8	R_6	R_{10}	$R_{11}a$[j]	R_{12}	$R_{12}a$	IC_{50}, μM[a]	t_R,[b] min	source[i]
C6–C13 and D-ring modifications													
26 (13-mercapto-5-OH-6-deoxy-Tc)	OH	CH_2SH	H	H	H	H	OH		OH	OH	4.6	17.72[f]	E
27 (13-(acetylthio)-5-OH-6-deoxy-Tc)	OH	CH_2SCOCH_3	H	H	H	H	OH		OH	OH	4.6	18.56	E
28 (7,13-epithio-6-deoxy-Tc)	H	CH_2–	H	S	H	H	OH		OH	OH	1.5	23.51	E
29 (13-(phenylsulfinyl)-5-OH-6-deoxy-Tc)	H	$CH_2SOC_6H_5$	H	H	H	H	OH		OH	OH	0.7	18.68	E
30 (13-(propylthio)-5-OH-6-deoxy-Tc)	OH	$CH_2S(CH_2)_2CH_3$	H	H	H	H	OH		OH	OH	0.7	20.18	J
31 (13-[(3-chloropropyl)thio]-5-OH-6-deoxy-Tc)	OH	$CH_2S(CH_2)_3Cl$	H	H	H	H	OH		OH	OH	0.6	19.67	J
32 (13-[(2-carboxyethyl)thio]-5-OH-6-deoxy-Tc)	H	$CH_2S(CH_2)_2CO_2H$	H	H	H	H	OH		OH	OH	43.2	14.68	J
33 (minocycline)	H	H	H	$N(CH_3)_2$	H	H	OH		OH	OH	2.3	10.19	F
34 (9-(dimethylamino)-6-demethyl-6-deoxy-Tc)	H	H	H	H	H	$N(CH_3)_2$	OH		OH	OH	15.4	8.10	E
35 (9-nitro-6-demethyl-6-deoxy-Tc)		H	H	H	H	NO_2	OH		OH	OH	12.3	NA[c]	E
36 (8-methoxy-6-demethyl-6-deoxy-chlor-Tc)	H	H	H	Cl	OCH_3	H	OH		OH	OH	19.5	NA[c]	H
37 (12a-deoxy-Tc)	H	CH_3	OH	H	H	H	OH		OH	H	>75	NA[c]	E
38 ($O^{10,12a}$-diacetyl-Tc)	H	CH_3	OH	H	H	H	O_2CCH_3		OH	O_2CCH_3	>75	23.58[g]	E
39 (O^{12a}-(acetyloxy)-Tc)	OH	CH_3	OH	H	H	H	OH		OH	O_2CCH_3	>75	NA[c]	E
40 (6-demethyl-6-deoxy-11a-fluoro-Tc)	H	H	H	H	H	H	OH	F	=O	OH	>75	NA[c]	E
41 (6-methylene-11a-chloro-Tc)	H	$=CH_2$	–	H	H	H	OH	Cl	=O	OH	>75	NA[c]	E
42 (4a,12a-anhydro-Tc)	H	CH_3	OH	H	H	H	OH		OH		>75	NA[c]	E
43 (5a,11a-dehydro-chlor-Tc)	H	CH_3	OH	Cl	H	H	OH		=O	OH	>75	7.23[h]	E
anthracyclines													
44 (adriamycin)											>75	20.23	I
45 (steffimycin B)											>75	25.37	G
46 (steffimycin C)											>75	NA[c]	G
47 (steffimycinone)											>75	22.03	G

[a] Determined from the everted vesicle assay. [b] Single peak on HPLC at 280 nm, purity calculated from the area percent, approximately >95%. [c] Not available due to insufficient material or not assayable by this HPLC method. [d] Percent purity, 84.8%. [e] Percent purity, 93.5%. [f] Percent purity, 82.3%. [g] Percent purity, 91.8%. [h] Percent purity, 80.9%. [i] A = E. Merck, Darmstadt West Germany; B = Aldrich Chemical Co., Milwaukee, WI; C = see ref 23; D = see ref 24; E = Pfizer Central Research, Groton, CT; F = Lederle Laboratories, Pearl River, NY; G = Upjohn Pharmaceutical Co., Kalamazoo, MI; H = Schering Corp., Bloomfield, NJ; I = Ives Laboratories, New York, NY; J = synthesized according to previous methods in this laboratory.[10] [j] 11a–12 keto–enol tautomerism unless noted.

help define the minimal structural attributes needed to inhibit Tet protein function. They also provide data for future molecular modifications of Tc toward the development of effective Tet protein blocking agents as antibacterial drugs.

We examined all analogues using an established model of Tc efflux,[6] the everted membrane vesicle assay. The everted vesicle preparation serves as an efficient assay for molecules capable of blocking Tet protein mediated uptake of Tc and as a model for examining the structure–activity relationships of analogues, *vis à vis* the Tc efflux protein.

Everted membrane vesicles derived from *Escherichia coli* D1-209 possessing plasmid R222, which bears the class B Tc^r determinant on Tn*10*, were used. This determinant is found in many bacterial genera and is the most prevalent among Tc-resistant *E. coli*.[5] Energized everted inner membrane vesicles containing the Tet protein can accumulate radiolabeled tetracycline ([^{3}H]Tc) by Michaelis–Menten applicable kinetics with an approximate K_m range of 8–20 μM.[6,11] We found that when a compound is added to Li lactate energized vesicles together with [^{3}H]Tc, inhibition can be measured as a decrease in accumulation of labeled drug as compared to uptake without analogue.[10] Vesicles deenergized with the protonophore CCCP [(3-chlorophenyl)hydrazono]propanedinitrile served as the negative control; the positive control was uptake of [^{3}H]-Tc alone. Inhibitors of Tc transport exhibited a decrease in [^{3}H]Tc accumulation that was measured at the 2.5-min time point following energization. This endpoint was chosen because of its reproducibility among vesicle preparations and compounds, reflecting the intravesicular accumulation and extravesicular escape of [^{3}H]Tc.[10] This value was used to derive the IC_{50} value (concentration causing a 50% inhibition of [^{3}H]Tc uptake) as compared to control vesicles without the potential inhibitor.

All compounds were also examined for their ability to disrupt membrane energization or pH gradient formation in vesicles from Tc susceptible cells by an acridine orange fluorescence assay.[12] When vesicles are energized there is a decrease of fluorescence of acridine orange dye. If a compound acts as an antagonist by disruption of the energized vesicle, the proton gradient collapses, restoring fluorescence to its original level. In this manner all compounds were examined for functional antagonist activity; none interfered with pH gradient formation or disrupted the vesicle membranes.

Compounds with IC_{50} values lower than or comparable to the K_m of Tc ($\leq$20.0 μM, e.g., 13-(propylthio)-5-hydroxy-α-6-deoxy-Tc **30**, Table 1) were considered as structural analogues possessing molecular accommodation at the

binding site for Tc and the Tet protein with the potential to inhibit the efflux of Tc within the whole cell. Those compounds with large IC_{50} values (>20 μM) were considered not complementary to the active efflux binding site on the Tet protein and were not considered further *vis à vis* inhibitors of Tc^r cell growth (e.g. isotetracycline, **14**, Table 1). However, these analogues were valuable in showing which molecular substructures affected the interaction of Tc with the Tet protein.

From the findings, the most potent analogues were further tested alone and together with doxycycline for the effect *in vitro* (minimum inhibitory concentration, MIC) against Tc^r *E. coli*, *Enterococcus faecalis* and *Staphylococcus aureus*. The resulting MICs were examined for additivity or synergy.

Results

Structure–Activity Relationships of Tc Uptake Inhibition. The ability to block uptake of [^{3}H]Tc in everted vesicle preparations was examined for partial and complete tetracyclic ring analogues of Tc. Partial ring structure analogues **1–10**, Tc derivatives **11–43**, and anthracyclines **44–47** were tested and the IC_{50} values determined (Table 1). The activity of the compounds tested revealed certain trends in the structure–activity requirements for the inhibition of Tc uptake. Compounds **1–10** with partial ring systems, either bicyclic or tricyclic substructures, did not inhibit [^{3}H]Tc uptake. Large IC_{50} values were obtained with partial ring systems DC and DCB **1–7** and tricyclic and tetracyclic derivatives **8–10**.

Analogues of Tc containing only the DCB ring substructure with adjacent keto–phenol groups (**6**) or phenolic keto–enol tautomerizable groups (**7**), similar to the 10–11a functional groups within tetracycline, exhibited no inhibition of [^{3}H]Tc uptake. Anthracene compounds olivin (**8**), chromomycin B (**9**), and secalonic acid A (**10**), as well as an analogue containing the exact DCB substructure of 7-OH-Tc with a modified A ring (**11**), and tetracycline synthetic intermediates (**12, 13**), all exhibited large IC_{50} values. Similarly, a precise Tc analogue with an interrupted C ring, isotetracycline (**14**), also was inactive.

The C2 carboxamide-modified tetracycline analogues studied, tetracyclinonitrile (**15**) and 2-decarboxamido-2-acetyl-Tc (**16**), exhibited activity similar to parent Tc. These results suggest that modifications of the C2 carboxamide group do not drastically alter the Tc analogue interaction with Tet protein, at least in relation to functional group structural differences of the 2-carboxamide group, either by carbonyl or amide modification. Moreover, among partial ring systems and C2 analogues of Tc, an intact DCBA tetracycline nucleus must be present in order to affect the Tet protein efflux mechanism.

Studies of analogues at the C4 position, exemplified by compounds **17–19**, indicate that removal of the dimethylamino group, as in 4-de(dimethylamino)-Tc (**17**) or epimerization of the dimethylamino group, from the natural β isomer to the α position, 4-epi-Tc (**18**), showed activity similar to or better than that of Tc (6.1 and 12.3 μM, respectively). Tetracycline-4*N*-methiodide (**19**) with an IC_{50} of 5.0 μM also exhibited somewhat better inhibitory activity than Tc.

Several C6-substituted analogues maintained or exhibited increased activity in inhibiting Tc uptake. Removal of the 6-α-OH group leading to 5-hydroxy-α-6-deoxy-Tc (**20**, doxycycline) resulted in an IC_{50} of 9.2 μM, whereas the dehydrated 5-deoxy analogue 5a,6,6-methyl-anhydro-Tc (**21**) showed less activity at 20 μM. Sancycline (6-demethyl-6-deoxy-Tc, **22**), 6-methylene-Tc (**23**), and 5-hydroxy-6-methylene-Tc (**24**, methacycline) exhibited inhibitory constants of 4.6, 9.2, and 2.6 μM, respectively. A sancycline analogue containing a sulfur atom substitution at position 6, thiatetracycline (**25**), exhibited an IC_{50} of 4.6 μM.

Compounds containing a sulfur atom attached to the exocyclic carbon at position 13, as in 13-mercapto-5-hydroxy-α-6-deoxy-Tc (**26**) and 13-(acetylthio)-5-hydroxy-α-6-deoxy-Tc (**27**), both exhibited IC_{50} values of 4.6 μM while 7,13-epithio-α-6-deoxy-Tc (**28**) and 13-(phenylsulfinyl)-5-hydroxy-α-6-deoxy-Tc (**29**), showed IC_{50} values of 1.5 and 0.7 μM. Position 13 homologues 13-(propylthio)-5-hydroxy-α-6-deoxy-Tc (**30**), 13-[(3-chloropropyl)thio]-5-hydroxy-α-6-deoxy-Tc (**31**) exhibited IC_{50} values of 0.7 and 0.6 μM, respectively, while the more polar derivative 13-[(2-carboxyethyl)thio]-5-hydroxy-α-6-deoxy-Tc (**32**) exhibited an IC_{50} of 43.2 μM.

Modified Tcs at positions 7, 8, and 9 on the aromatic D ring are presented as compounds **33–36**. Minocycline (**33**) with an IC_{50} of 2.3 μM also ranked high in overall potency among the compounds tested. The structural isomer of minocycline, 9-(dimethylamino)-6-demethyl-6-deoxy-Tc (**34**), and the 9-nitro analogue, 9-nitro-6-demethyl-6-deoxy-Tc (**35**), both exhibited decreased activity (15.4 and 12.3 μM, respectively). Substitutions at position 8, as in the 8-methoxy derivative 8-methoxy-6-deoxy-6-demethyl-chlorTc (**36**), resulted in activity similar to Tc (19.5 μM).

Modified analogues at positions C10-12a all showed a significant decrease in inhibitory activity. Removal of the 12a-hydroxy group of Tc, as in 12a-deoxy-Tc (**37**), or blockage of the C10 and/or 12a hydroxyl groups by acetyl moieties, as exemplified by $O^{10,12a}$-diacetyl-Tc (**38**) and O^{12a}-(acetyloxy)-Tc (**39**), resulted in a loss of inhibitory activity. Halogenated 11a-position derivatives, 6-demethyl-6-deoxy-11a-fluoro-Tc (**40**), and 6-methylene-11a-chloro-Tc (**41**) similarly exhibited low inhibitory activity. Formation of double bonds within the A or B rings of the naphthacene ring system, as in 4a,12-anhydro-Tc (**42**) or C and D rings, as in 5a,11a-dehydro-chlorTc (**43**) also resulted in a loss of activity.

Members of the anthracycline class of antineoplastic agents adriamycin (**44**), steffimycins B and C (**45, 46**), and steffimycinone (**47**) all were inactive as inhibitors of [^{3}H]-Tc uptake. These agents were chosen because of their containing a naphthacene ring system and also that certain mammalian cancer cell lines have demonstrated resistance to these compounds, by a mechanism linked to active efflux.[13,14]

Antimicrobial Activity in Vitro. The MICs of the 13-alkyl derivatives 13-(propylthio)-5-hydroxy-α-6-deoxy-Tc (**30**), 13-[(3-chloropropyl)thio]-5-hydroxy-α-6-deoxy-Tc (**31**), and 13-[(2-carboxyethyl)thio]-5-hydroxy-α-6-deoxy-Tc (**32**) were determined against Tc-susceptible and Tc-resistant (Tc^r, efflux-type determinants) *E. coli* (Tc^r determinant class A and B), *S. aureus* (Tc^r determinant class K), and *E. faecalis* (Tc^r determinant class L). All compounds were compared to a clinically used antibiotic, doxycycline. Two of the three derivatives (**30, 31**) showed activity similar to doxycycline against the Tc-susceptible *S. aureus* and *E. faecalis*. However, all three showed poor

Table 2. Minimum Inhibitory Concentration of Doxycycline and 13-(Propylthio)-5-OH-Tc Analogues against Tetracycline-Susceptible Bacterial Strains

	MIC, μg/mL[a]			
organism (strain)	doxycycline	13-(propylthio) (30)	13-[(3-chloropropyl)thio] (31)	13-[(2-carboxyethyl)thio] (32)
E. coli (ML308-225)	0.5	40	10	>40
E. coli (D31m4)	0.5	10	2.5	20
S. aureus (RN450)	0.25	0.6	0.6	2.5
E. faecalis (ATCC9790)	0.25	1.25	0.6	10

[a] Amount of compound which inhibited growth after 18 h of incubation at 37 °C.

Table 3. Minimum Inhibitory Concentration and Growth Inhibitory Activity of Doxycycline or the 13-(Propylthio)-5-OH-Tc Analogues Alone and in Combination against Tetracycline-Resistant Bacterial Strains

		MIC, μg/mL[a] Tc[r] determinant				
compound	IC_{50}	A[c]	B[c]	B*[d]	K[e]	L[f]
A. Alone						
13-(propylthio) (30)	0.7	>50	>50	12.5	12.5	1.56
13-[(3-chloropropyl)thio] (31)	0.6	50	>50	6.25	6.25	1.56
13-[(2-carboxyethyl)thio] (32)	43.2	>50	>50	>50	12.5	3.12
doxycycline	9.2	50	>50	12.5	12.5	6.25
B. In Combination (Doxycycline/Analogue)[b]						
13-(propylthio) (30)		6/3(S)	NE	6/1.5(A)	6/3(A)	1.5/0.8(A)
13-[(3-chloropropyl)thio] (31)		3/3(S)	NE	3/1.5(S)	3/1.5(S)	1.5/0.4(S)
13-[(2-carboxyethyl)thio] (32)		25/50(A)	NE	5/25(A)	3/6(A)	1.5/1.5(A)

[a] Amount of compound which inhibited growth after 18 h of incubation at 37 °C. [b] (S) = synergy, (A) = additive, NE = no effect. [c] In ML308-225 (Tet A on plasmid pIP15; Tet B on plasmid R222). [d] In *E. coli* D31m4 (Tet B on plasmid pHCM1). [e] In *S. aureus* RN4250 (Tet K on plasmid pT181). [f] In *E. faecalis* ATCC9790 (Tet L on plasmid MV158).

antibacterial activity against *E. coli* ML308-225 (Table 2). Cell wall impermeability contributed to the inactivity, as observed by the greatly increased activity of compounds **30** and **31** against the cell wall mutant *E. coli* D31m4. This bacterium possesses an outer membrane lipopolysaccharide defect.[15] Such mutations make the cell more permeable to hydrophobic compounds.[16]

All three compounds (**30–32**) and doxycycline were ineffective as growth inhibitors against the Tc[r] Gram-negative organism *E. coli* D1-299 (ML308-225 bearing the class A determinant on plasmid pIP15) and D1-209 (ML308-225 bearing the class B determinant on plasmid R222) (>50 μg/mL) (Table 3). Against the outer membrane mutant D31m4 bearing the class B determinant on pHCM1,[17] the compounds showed increased activity, with the more lipophilic 13-(3-chloropropyl) derivative (**31**) demonstrating an increase in potency over the more polar carboxyl homologue (MICs of 6.25 vs >50 μg/mL, respectively).

Against *S. aureus* RN 4250 (pT181, Tet K), the compounds were similar in activity, demonstrating MICs of 6.25–12.5 μg/mL. The 13-(3-chloropropyl) derivative (**31**), however, was slightly more potent. Against *E. faecalis* ATCC 9790 (pMV158, Tet L), compounds **30** and **31** exhibited better antibacterial activity (1.56 μg/mL) while the carboxyl homologue (**32**) and doxycycline showed less activity (3.12 and 6.25 μg/mL, respectively).

Checkerboard titration of the 13-(propylthio) derivatives with doxycycline indicated that the more lipophilic derivatives **30** and **31** acted in synergy against *E. coli* D1-299 (bearing Tet A), while the carboxylic acid derivative **32** was much less effective. None of the compounds examined were active against *E. coli* D1-209 (bearing Tet B); however, the lipophilic derivatives were more effective against the Tet B determinant in the outer membrane mutant D31m4.

Against Tc[r] *S. aureus* (class K) and *E. faecalis* (class L), compounds **30** and **31** showed far greater combined activity than **32**, and compound **31** showed synergy.

Discussion

These studies used analogues of Tc to examine the necessary molecular requirements for blocking Tet protein mediated uptake of [^{3}H]Tc into everted inner membrane vesicles from Tc[r] *E. coli*. The effect of structural modifications of Tc on [^{3}H]Tc uptake can initially describe molecular features that must be present in order to provide maximum inhibition of efflux. The intact tetracycline ABCD naphthacene structure is essential for activity as are the presence of phenolic keto–enol tautomerizable groups on the lower periphery of the ring system at positions 10, 11, and 12. The loss of activity from Tc analogues modified on the lower ring system suggests key features of the Tc–Tet protein interaction. Most important is that the molecular substructures of the tetracyclic ring system required for inhibition of everted vesicle uptake are limited. The results obtained from blocked hydroxy group derivatives and halogenated analogues along the lower periphery of tetracycline (position 10–12a) suggest that the probable substrate for Tc efflux is a tetracyclic structure that allows formation of keto–enol functional groups. Blocking these positions results in the impedement of formation of keto–enol tautomerization along this periphery. The formation of keto–enol groups correlates well with activity and the formation of Mg^{2+} or Ca^{2+} chelates, since oxygen-containing functional groups at positions 10–12a of Tc have been shown to be high-affinity chelators of divalent metal ions.[18,19] That the efflux system functions as a cation:Tc antiport system has been shown experimentally.[11]

The 4-(dimethylamino) functional group in the β-configuration is preferred, although the α isomer can retain notable activity. Furthermore, removal of the dimethylamino group does not markedly affect activity, *vis à vis* Tc.

Results with analogues containing molecular differences at positions 2, 4, 5, 6, 7, 8, and 9 indicate that a degree of synthetic chemical modification is possible without the

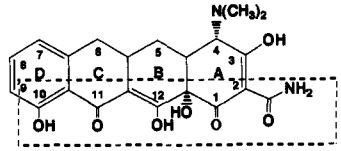


Figure 2. Essential region of tetracycline required for inhibition of Tet protein mediated uptake of Tc in everted vesicles from *E. coli* D1-209. The ABCD naphthacene ring structure and the phenolic diketone substructure 10–12a (boxed area) are required for inhibitory activity.

loss of efflux inhibitory activity. Position 13-alkyl derivatives, e.g., 7,13-epithio-Tc (**28**), 13-(phenylsulfinyl)-Tc (**29**), 13-(propylthio)-5-hydroxy-α-6-deoxy-Tc (**30**), and 13-[(3-chloropropyl)thio]-5-hydroxy-α-6-deoxy-Tc (**31**), were among the most potent inhibitors of efflux of the compounds studied (Table 1).

From these results an initial model for the minimal Tc structure needed for uptake inhibition emerges as a representation of these molecular attributes (Figure 2). The essential region (boxed area) represents the sites most sensitive to molecular modification, whereas positions C4–C9 alone or together show the most promise for the production of a specific uptake inhibitor. Interestingly, the molecular prerequisites for tetracycline action at the ribosome show similar qualitative structural characteristics.[20]

The 13-(propylthio)-5-OH-Tc derivatives were tested for activity against Gram-negative and Gram-positive bacteria. They were relatively inactive as single growth inhibitory agents against wild-type susceptible *E. coli* strains. The inactivity was related in part to the poor ability to enter Gram-negative cells. This point was evident in the increased activity of the more lipophilic derivatives (**30**, **31**) against the outer cell wall mutant of *E. coli* D31m4. The 13-propyl derivatives (**30**, **31**) showed good antibacterial activity against susceptible *S. aureus* and *E. faecalis*, although they were slightly less active than doxycycline (Table 2).

Against Tc^r derivatives of *E. coli* ML308-225, none of the analogues, nor doxycycline, was effective (MIC > 50 μg/mL). However, against the outer membrane mutant D31m4, bearing the Tet B determinant, compounds **30**, **31**, and doxycycline showed similar activity. The carboxylic acid derivative (**32**) remained inactive.

Against Tc^r *S. aureus* (class K) and *E. faecalis* (class L), the analogues showed antibacterial activity similar to or better than doxycycline. Analogues **30** and **31** showed better activity as antibacterial agents than **32**. The activity against *E. faecalis* bearing Tet L was similar to that of the *E. faecalis* susceptible strain.

When used in combination with doxycycline, the more lipophilic analogues (**30** and **31**) reduced the doxycycline or analogue average MIC by one to two dilutions. With compound **31**, both compounds were reduced by two or more dilutions and consistently showed synergy. This was particularly noted with compounds **30** and **31** against ML308-225 bearing the class A determinant.

The synergistic growth inhibitory activity of the more potent 13-(propylthio) efflux inhibitors when used in combination with doxycycline against Tc^r *E. coli* containing efflux systems paralleled the observations for efflux inhibition. The more lipophilic derivatives (**30**, **31**) are more potent inhibitors of efflux and are potent synergists against class A resistant bacteria. The decreased activity of these same compounds against class B containing organisms may be due to the increased resistance mediated by Tet B proteins[6] and the relative impermeability of the *E. coli* cell wall, since synergy was noted with compound **31** against the class B determinant in *E. coli* D31m4.

Similarly, inhibition was also revealed against gram positive Tc^r *E. faecalis* and *S. aureus*, where the more lipophilic analogues alone not only showed greater potency than doxycycline, but also demonstrated synergy with doxycycline. The low concentrations of doxycycline needed to reach an MIC (≤2-fold dilution) in combination with similar decreases in the concentration of an uptake inhibitor demonstrate a reversion of tetracycline resistance to a physiological state of tetracycline susceptibility.

We speculate that the uptake inhibitor has an increased affinity for the Tet protein in Tc^r *E. coli* bearing the determinant Tet B, displacing Tc as the substrate, thus blocking the efflux of doxycycline. We further postulate that once efflux inhibitors enter the bacterial cell and inhibit the Tet protein, therapeutic concentrations of doxycycline can enter and accumulate within the cell, leading to ribosomal dysfunction and growth inhibition. The unexpected finding of activity of these same analogues against *S. aureus* and *E. faecalis* bearing other efflux mechanisms of resistance[2] extends the utility of this assay. The data also suggest that the other efflux proteins involved in Tc resistance may share structural features in common with Tet protein, *vis à vis* Tc binding.

In summary, these studies provide new insights and information about the qualitative structure–activity relationships between Tc substrates and the Tet protein efflux antiport system. The observed potency of the 13-(alkylthio)-Tc derivatives led to an in depth structure–activity study of this position as it affected inhibition of Tc uptake.[10] The results obtained in this latter study helped derive a molecular model for a hydrophobic pocket in the Tet protein which accommodated the Tcs subjected to efflux.[10]

Our present findings extend our knowledge about the molecular requirements on the Tc molecule for inhibition of Tc uptake. They clearly indicate that modifications at some other positions are possible without interfering with analogue interaction with the Tet protein and help define a minimum structure for an efflux blocker. Furthermore, potent inhibitors of uptake also act synergistically with clinically used Tcs against Tc^r whole cells. The further understanding of the molecular attributes needed for inhibition of Tc efflux and growth inhibition characteristics will help in the development of compounds effective against tetracycline-resistant bacteria. In the future, efflux inhibitors either alone or in conjunction with Tcs may provide the clinician with further compounds useful against infections caused by Tc-resistant and -susceptible bacteria. The recent report of a new group of semisynthetic Tcs active against Tc^r and Tc-susceptible bacteria adds additional promise to the future of tetracycline antibiotics.[25]

Experimental Section

Compounds. All compounds were obtained with the consent of each respective laboratory and are listed by each individual source as well as by the retention time and percent purity, as determined by HPLC (Table 1). Analytical HPLC was performed on each compound to determine purity using a Waters Bondapack C18 reverse-phase column and a gradient system of two Waters 501 HPLC pumps at a 1.6 mL/min flow rate. The gradient was controlled by Waters Maxima Chromatography software fol-

lowing a linear gradient from 30% to 70% MeOH over 20 min. UV absorbance was monitored at 280 nm with a Waters Model 441 absorbance detector. Solvent system A: 0.02 M Na_2HPO_4 + 0.001 M Na_2EDTA adjusted to pH 4.5 with H_3PO_4. Solvent system B: 100% MeOH.[21] The synthetic methods, isolation procedures, and chemical characterization used for the 13-(propylthio) derivatives (compounds **30–32**) were as previously reported.[10]

Everted Vesicle Assay. Everted inner membrane vesicles were prepared from *E. coli* D1-209 containing plasmid R222, according to the method of McMurry,[6] which has been described in detail in a previous publication.[10] Analogue inhibition of Tc uptake was measured as a decrease in accumulated [^{3}H]Tc in everted membrane vesicles at 2.5 min from which the IC_{50} value was calculated. The potential disruption of the energized vesicles was assayed by a fluorescence assay using acridine orange uptake in energized vesicles from isogenic Tc-susceptible *E. coli*.[12,22]

Minimum Inhibitory Concentration (MIC). The *in vitro* antibacterial properties of the compounds listed were assayed by conventional procedures. The organisms were grown overnight in Luria broth at 37 °C (containing 1 μg/mL tetracycline for resistant strains), inoculated into Luria broth, and allowed to grow to an optical density (A_{530}) dependent upon the strain (D1-299 Tet A, 0.5; D1-209 Tet B, 0.5; RN4250 Tet K, 0.4; ATCC9790 Tet L, M, both 0.2). Two-fold dilutions of the test compound stock solution (10 mg/mL) were prepared in Luria broth to obtain the test concentration ranging from 200 to 0.030 μg/mL. Compounds were dissolved in H_2O or a 1:1 H_2O/MeOH solution. Each tube containing 1 mL of Luria broth was inoculated with 10 μL of culture per mL (approximately 10^6 cfu/mL) and incubated at 37 °C for 18–20 h. The minimum inhibitory concentration (MIC, μg/mL) was the lowest concentration of the test compounds that yielded no visible growth within the tube.

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