Tetracycline Analogs Affecting Binding to Tn*10*-Encoded Tet Repressor Trigger the Same Mechanism of Induction†

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Received November 9, 1995; Revised Manuscript Received February 29, 1996⊗

ABSTRACT: We examined the influence of substituents in tetracycline (tc) analogs modified at positions 2 and 4−9 and anhydrotetracycline (atc) on induction of the Tn*10*-encoded Tet repressor (TetR) by a quantitative *in vitro* induction assay. The equilibrium association constants of the modified tc to TetR were independently determined to distinguish effects on binding from those on induction. We found a correlation between the binding affinity and induction of TetR for most tc analogs. While a substitution at position 5 revealed only minor effects, changes at position 6 increased binding and induction efficiencies up to 20-fold. A chlorine at position 7 or 8 enhanced binding and induction about 4- and 9-fold, respectively. Substituents at position 9 decreased binding up to 5-fold. Epimerization of the dimethylamino function at position 4 in 4-epi-tc resulted in about 300-fold-reduced binding and 80-fold-reduced induction. Substitution of this grouping by hydrogen in 4-de(dimethylamino)-tc resulted in no binding and no induction. The respective atc analog failed to induce as well, although binding was still observed. The dimethylamino function may, thus, play a role in triggering the conformational change of TetR necessary for induction. Substitution of the 2-carboxamido by a nitrilo function did not influence binding and induction efficiencies. Atc showed about 30-fold increased binding and induction, being the most effective inducer tested in this study. The equilibrium association constants of most TetR−$[Mg\text{-}tc]^+$ and TetR−$([Mg\text{-}tc]^+)_2$ analog complexes with *tet* operator are decreased about 10^2- and 10^8-fold, respectively, as compared to those of free TetR. This suggests that these tc analogs share the same molecular mechanism of TetR induction.

In Gram-negative bacteria, resistance to tetracycline (tc)[1] is mostly mediated by a membrane-bound carrier protein (Yamaguchi et al., 1990), which leads to the active efflux of the antibiotic from the resistant cell (McMurray et al., 1980). Seven genetic determinants encoding efflux pumps, named A−E, G, and H, which share extensive sequence homology (Mendez et al., 1980; Marshall et al., 1986; Zhao & Aoki, 1992; Hansen et al., 1993), have been characterized up to now.

Regulation of expression of the Tn*10*-encoded determinant is mediated by Tet repressor (TetR) (Beck et al., 1982), which binds to two operator (*tetO*) sites (Hillen et al., 1983; Wray & Reznikoff, 1983). The inducer $[Mg\text{-}tc]^+$ binds to TetR and leads to a reduced affinity for *tetO*. The crystal structure of the TetR−$([Mg\text{-}tc]^+)_2$ complex (Hinrichs et al., 1994; Kisker et al., 1995) and functional characterizations of noninducible TetR mutants suggest that a substantial conformational change of TetR is triggered by tc binding (Müller et al., 1995).

$[Mg\text{-}tc]^+$ binding to TetR has been studied by nitrocellulose filter retention (Hillen et al., 1982) and fluorescence measurements (Takahashi et al., 1986, 1991). We have recently developed a quantitative *in vitro* assay for thermodynamic analysis of induction, from which the equilibrium binding constants of complex formation of TetR−$[Mg\text{-}tc]^+$ and TetR−$([Mg\text{-}tc]^+)_2$ with *tetO* can be derived (Lederer et al., 1995). Efficient induction of resistance gene expression is ensured by a binding constant of about 10^9 M^{-1} for the TetR−$[Mg\text{-}tc]^+$ complex (Takahashi et al., 1986, 1991), leading to a decrease of *tetO* affinity to the level of nonspecific DNA binding (Lederer et al., 1995). The high efficiency of induction and the ability of tc to efficiently penetrate most cells led to the use of TetR as a regulator of gene expression in eukaryotic cells (Dingermann et al., 1992; Faryar & Gatz, 1992; Wirtz & Clayton, 1995; Gossen & Bujard, 1992; Deuschle et al., 1995).

Tc is the parent compound for a widely used class of antibiotics. Many chemical analogs of tc have been synthesized and studied for their antibacterial effects (Rogalski, 1985). Some of them have markedly different affinities for TetR (Degenkolb et al., 1991) and are 100-fold more efficient inducers. Such derivatives were used for TetR-regulated expression in eukaryotic systems (Gossen et al., 1995). In this article, we provide a thermodynamic description of induction exerted by the tc analogs shown in Figure 1. The

† This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie. T.L. and W.H. were supported by grants from the Deutsche Forschungsgemeinschaft.

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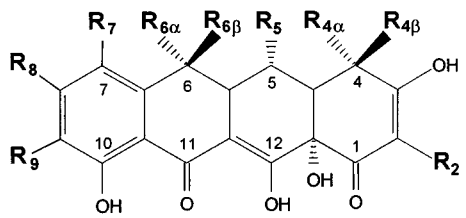
⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1996.

[1] Abbreviations: TetR, Tet repressor; tc, tetracycline; atc, anhydrotetracycline; bp, base pair; ddma, dimethylamino.

S0006-2960(95)02683-3 CCC: $12.00

A



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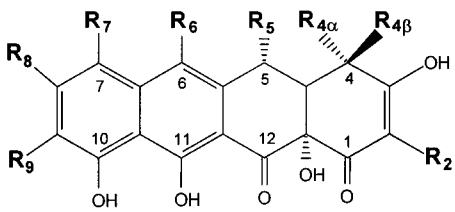


C

R_2	$R_{4\beta}$	$R_{4\alpha}$	R_5	R_6	$R_{6\beta}$	$R_{6\alpha}$	R_7	R_8	R_9	
$CONH_2$	H	$N(CH_3)_2$	H	-	OH	CH_3	H	H	H	tetracycline (tc)
$CONH_2$	H	$N(CH_3)_2$	H	CH_3	-	-	H	H	H	anhydro-tc (atc)
CN	H	$N(CH_3)_2$	H	-	OH	CH_3	H	H	H	2-nitrilo-tc
$CONH_2$	$N(CH_3)_2$	H	H	-	OH	CH_3	H	H	H	4-epi-tc
$CONH_2$	H	H	H	-	OH	CH_3	H	H	H	4-de(dimethylamino)-tc
$CONH_2$	H	H	H	CH_3	-	-	H	H	H	4-de(dimethylamino)-atc
$CONH_2$	H	$N(CH_3)_2$	OH	-	OH	CH_3	H	H	H	5-hydroxy-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	CH_3	H	H	H	H	6-deoxy-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	OH	H	H	H	H	6-demethyl-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	H	H	H	H	H	6-deoxy- 6-demethyl-tc
$CONH_2$	H	$N(CH_3)_2$	OH	-	$=CH_2$		H	H	H	6-methylene-5-hydroxy-tc
$CONH_2$	H	$N(CH_3)_2$	OH	-	H	CH_3	H	H	H	6-deoxy-5-hydroxy-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	OH	CH_3	Cl	H	H	7-chloro-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	H	H	H	H	NH_2	9-amino-6-deoxy-6-demethyl-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	H	H	H	Cl	NH_2	9-amino-8-chloro-6-deoxy-6-demethyl-tc
$CONH_2$	H	$N(CH_3)_2$	H	-	H	H	H	H	$NH\text{-}COCH_2\text{-}N(CH_3)_2$	9-(N,N dimethylglycylamido)-6-deoxy-6-demethyl-tc

FIGURE 1: Chemical structure of tc and analogs used in this study. (A) Structure of tc. (B) Structure of atc. (C) The substituents of the analogs are marked as R_2 to R_9 (with respect to the corresponding C atom), and the respective designation of the analog is given in the right part of the table. Substituents within the boxed cells indicate the differences to tc or atc, respectively.

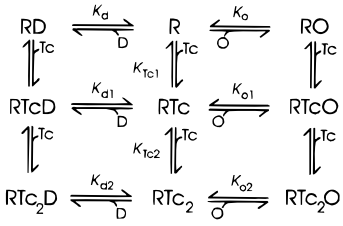
binding constants of each $[Mg\text{-}tc]^+$ analog to TetR were also determined to distinguish between their affinity for TetR and the ability to induce the conformational change of TetR leading to induction.

MATERIALS AND METHODS

Materials and General Methods. Tc was from Fluka (Buchs, Switzerland), atc from Janssen Chimica (Beerse, Belgium), and 5-hydroxy-tc from Sigma St. Louis, MO). All other tc analogs were synthesized by Lederle Laboratories (Pearl River, NY). Tc and analogs were purified prior to use by reverse-phase chromatography using a Nucleosil 100 7-C_{18} column (250 × 20 mm) obtained from Machery & Nagel (Düren, FRG) and a Pharmacia LKB HPLC solvent conditioner (2156), controller (2152), pump (2150), and variable wavelength monitor (2151). Tc analogs were monitored at 275 nm. Optimal separation was analytically determined. The preparation was done isocratically according to the optimized parameters using gradients from buffer A (H_2O + 0.1% trifluoroacetic acid) to buffer B (90:10 acetonitrile−H_2O + 0.1% trifluoroacetic acid). After purification, the samples were rechromatographed by means of a Nucleosil 120 5-C_{18} column (250 × 4 mm) and analyzed by integration of the elution profile, using Integration Pack, vers 3.90, provided from Kontron (Munich, FRG). The concentration of tc was determined spectroscopically, using $\epsilon_{355} = 13\,320\ M^{-1}\ cm^{-1}$ in 0.1 M HCl (Takahashi et al., 1986), and those of tc derivatives were determined by saturating fluorescence titration with known amounts of TetR. TetR was purified as described (Oehmichen et al., 1984). The concentration of TetR was determined by saturating fluorescence titration with known amounts of tc.

In Vitro Induction Assay. A 349 bp DNA fragment containing *tetO*$_1$ was prepared from pWH964 (Niederweis et al., 1992) and purified by CsCl gradient centrifugation. After digestion with *Eco*RI, the respective fragments were 5′ end-labeled using [γ-^{32}P]ATP as described (Maxam & Gilbert, 1980). Redigestion with *Bam*HI yielded a 203 bp *tetO*$_1$-containing DNA fragment. The restriction fragments were separated on a 5% PAA gel, and the 203 bp DNA was eluted, precipitated twice with ethanol, vacuum dried, and dissolved in H_2O.

Scheme 1



One picomole of fragment containing 20 000 cpm was incubated in 40 μL of 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 2.5 mM NaCl, 10 μg of nonspecific DNA (pWH802; Unger et al., 1984), 1×10^{-7} M TetR, and different amounts of tc analogs at ambient temperature for 30 min. Prolonged incubation times did not alter the results, indicating that full equilibrium was reached after 30 min. Methylation, cleavage of the methylated DNA, and gel electrophoresis of the products were done as described (Heuer & Hillen, 1988). Autoradiography was done using Hyperfilm-MP (Amersham, Braunschweig, FRG) for 24 h with an intensifying screen. The autoradiographs were densitometrically scanned with an Ultroscan XL laser densitometer (Pharmacia LKB, Freiburg, FRG). The protection of the *tetO*-guanine(2) band (Heuer & Hillen, 1988) was used to score the extent of operator binding in dependence of the inducer concentration. For quantification, the intensity of the guanine(2) in *tetO* was normalized with respect to the intensities of two guanine residues which are not affected by TetR binding. The induction was defined to be 0% in the absence of tc and 100% in the absence of TetR. A detailed description is given elsewhere (Lederer et al., 1995).

Analysis of Induction Data (Inducer-Dependent Dissociation of TetR from tetO). The induction data were analyzed using a scheme of coupled equilibria (Scheme 1) which considers all reactions, including the binding of tc to RO, RTcO, RD, and RTcD, respectively. Details are described elsewhere (Lederer et al., 1995).

In Scheme 1, R, O, and D represent the repressor dimer, the operator, and nonspecific DNA, respectively. The concentration of all species was calculated by the half-interval resolution method for a given set of binding parameters and total protein and DNA concentrations (Takahashi et al., 1989). The theoretically determined amount of free *tetO* as a function of inducer concentration was then compared with the experimental result by a least squares analysis to estimate the binding constants K_{o1} and K_{o2}. To facilitate the analysis, the parameter f1, relating K_o, K_{o1}, and K_{o2}, was defined as follows; $K_{o1} = \ln[f1 \times \exp(K_o) + (1 - f1) \times \exp(K_{o2})]$. f1 varies between 0 and 1. If each subunit of TetR dimer interacts with DNA in an independent manner, f1 should be 0.5, because the contribution of binding energy of one subunit without tc is $\exp(K_o)/2$ and that of the subunit with tc is $\exp(K_{o2})/2$. If the binding of one tc per dimer completely abolishes *tetO* binding, f1 should be 0 and, thus, $K_{o1} = K_{o2}$. When the binding of two tc per dimer is required for complete induction, f1 should equal 1 and $K_{o1} = K_o$ [for details, see Lederer et al. (1995)].

Determination of Association Equilibrium Constants. TetR−$[Mg\text{-}tc]^+$ binding constants were obtained from fluorescence titration at limiting Mg^{2+} concentrations as described (Takahashi et al., 1991). We adjusted free Mg^{2+} concentrations ranging from 10^{-11} to 10^{-7} M using a buffer containing EDTA as a metal chelator (Perrin & Dempsey, 1974). The buffer contained 20 mM EDTA in 50 mM Tris-HCl (pH 8.3), 150 mM NaCl, and 1 mM dithiothreitol. Mg^{2+} concentrations between 10^{-7} and 10^{-2} M were adjusted by dilution of the added Mg^{2+} stock solution using a buffer without EDTA. In all cases, the concentration of TetR and tc analogs was equimolar and varied in the range of 0.4−2.3 μM. The data analysis required the determination of the equilibrium constant K_M for the binding of Mg^{2+} to the respective tc derivative. A total of 2000 μL of 100 mM Tris-HCl (pH 8.3), 150 mM NaCl, 20 mM EDTA, and 1 mM dithiothreitol containing 0.4−2.3 μM tc analog was titrated with Mg^{2+} stock solutions (5, 50, and 500 mM). To avoid dilution of tc by addition of Mg^{2+}, we used Mg^{2+} solutions containing the same concentration of tc as present in the sample cuvette. The increase of tc fluorescence emission with increasing Mg^{2+} concentrations was measured and analyzed by Scatchard plotting (Scatchard, 1949).

Tc analogs with an amino function at position 9 did not provide significant fluorescence, and thus, the equilibrium association constants K_M and K_A were determined by absorption measurement (K_M) or competition experiments with tc (K_A).

RESULTS

Association Equilibrium Constants of tc with Mg^{2+}. The association constants of the tc analogs with Mg^{2+} (K_M) are summarized in the first column of Table 1. Analogs containing a 9-amino function showed no significant fluorescence. For these, the change of absorption upon Mg^{2+} binding was employed to determine K_M. We used wavelengths of 375 nm for 9-amino-6-deoxy-6-demethyl-tc and 380 nm for the respective 8-chloro derivative. K_M of $[Mg\text{-}tc]^+$ complex formation was also determined by absorption as a control. It yielded the same result as the fluorescence measurement (data not shown). We were not able to determine K_M for 4-de(dimethylamino)-atc, either by fluorescence or by absorption. Therefore, the K_M of atc was used for all further calculations. Various substitutions at positions 2 and 4−7 affected the Mg^{2+} affinity to the respective analog only slightly, as the K_M did not vary by more than 2-fold. In contrast, atc showed a 3−4-fold decreased K_M. Introduction of a 9-amino- or a 9-dimethylglycylamido grouping led to an about 3-fold increase of K_M. An additional 8-chloro substituent in the 9-amino derivative resulted in a further 2−3-fold increase of Mg^{2+} affinity, being the highest K_M determined in this study.

Association Equilibrium Constants of $[Mg\text{-}tc]^+$ with TetR. The binding constants of the TetR−tc complexes were determined to characterize their molecular interactions. Each repressor dimer can bind two tc molecules without detectable cooperativity as described (Takahashi et al., 1986, 1991). We found the best agreement between experimental and fitted data for cooperativity values (α) between 1 and 10. However, the quality of the fits was not very sensitive to small changes of α, indicating that this assay does not detect potential cooperativity. Therefore, we determined the intrinsic association constants K_A with $\alpha = 1$ for all derivatives (Table 1, column 2). The binding constants of various $[Mg\text{-}tc]^+$ to TetR varied by about 4 orders of magnitude between the weakest (4-epi-tc) and the strongest (atc) binders.

Table 1: Association Equilibrium Constants of Tc Derivatives with Mg^{2+} (K_M) and TetR (K_A) and the Respective Efficiencies of Induction of TetR[a]

derivative	$K_M \times 10^3$ (M^{-1})	$K_A \times 10^9$ (M^{-1})	concentration at half induction $\times 10^{-6}$ (M)
tc	2.40 ± 0.40	3.30 ± 1.300	2.20 ± 0.11
atc	0.68 ± 0.02	100.00 ± 20.000	0.09 ± 0.01
2-nitrilo-tc	2.90 ± 0.50	3.80 ± 3.300	0.65 ± 0.03
4-epi-tc	2.90 ± 0.30	0.01 ± 0.002	170.00 ± 7.50
4-de(dimethylamino)-tc	4.50 ± 0.60	no binding	no induction
4-de(dimethylamino)-atc	nd	0.03[b]	no induction
5-hydroxy-tc	4.20 ± 0.30	2.60 ± 0.900	3.00 ± 0.21
6-deoxy-tc	3.30 ± 0.90	1.80 ± 0.800	1.80 ± 0.11
6-demethyl-tc	3.30 ± 0.20	0.17 ± 0.080	8.20 ± 0.56
6-deoxy-6-demethyl-tc	3.50 ± 1.00	8.00 ± 4.000	0.34 ± 0.02
6-methylene-5-hydroxy-tc	2.60 ± 0.30	24.00 ± 3.000	0.17 ± 0.01
6-deoxy-5-hydroxy-tc	2.70 ± 0.30	23.00 ± 5.000	0.14 ± 0.01
7-chloro-tc	1.30 ± 0.20	10.00 ± 5.000	0.24 ± 0.02
9-amino-6-deoxy-6-demethyl-tc	8.60 ± 0.80	1.20 ± 0.700	nd
9-amino-8-chloro-6-deoxy-6-demethyl-tc	22.50 ± 2.00	8.10 ± 3.700	nd
9-(*N*,*N*-dimethyl-glycyl-amido)-6-deoxy-6-demethyl-tc	7.50 ± 1.00	0.64 ± 0.110	nd

[a] The induction efficiency is given as the concentration of inducer necessary for half-maximal induction. [b] As K_M could not be determined for 4-de(dimethylamino)-atc, K_A was calculated using K_M of atc.

A tc derivative with a 2-nitrilo replacing the 2-carboxamido function showed the same K_A as tc. Thus, this alteration has no effect on TetR binding.

The strongest effects on TetR binding were found for alterations at position 4. Epimerization of the dimethylamino function at this position resulted in an about 300-fold decrease of K_A, while substitution of this function by hydrogen in 4-de(dimethylamino)-tc reduced binding below the sensitivity of the assay. In contrast, binding of the respective atc analog, 4-de(dimethylamino)-atc, was detectable. K_A of this analog was decreased about 3000-fold. We note that we used the K_M of atc for calculation of K_A because we did not succeed in determining K_M for this derivative. This result demonstrates that the dimethylamino grouping at position 4α is necessary for efficient binding of tc to TetR.

The introduction of a 5-hydroxyl group in 5-hydroxy-tc slightly decreased K_A about 2-fold, indicating that this substituent may not contact TetR.

A methyl or a hydroxyl function at position 6*β* showed an about 10- or 15-fold-reduced binding constant, respectively. In contrast, derivatives with a methyl group at position 6α as well as the planar 6-methylene function showed an about 5-fold-increased binding constant. This result shows that substituents at these positions may be involved in TetR−tc interaction.

A chloro function at position 7 or 8 had a positive effect on the affinity to TetR. At position 7, it led to an about 4-fold-increased binding constant, whereas binding was stimulated about 8-fold in the 8-chloro derivative.

Analogs with an amino function at position 9 did not show significant fluorescence. We therefore determined their binding constants by competition experiments with tc. Tc and the respective derivative were mixed in varying ratios, and the fluorescence of the TetR−$([Mg\text{-tc}]^+)_2$ complex was measured in comparison to the fluorescence of the complex without added derivative. The 9-amino- and the 9-dimethylglycylamido grouping had a negative effect on TetR binding as they decreased K_A about 7- and 13-fold, respectively. Interestingly, in absence of Mg^{2+}, the binding constant to TetR was about 2 orders of magnitude higher for the 9-amino derivatives (K_A of about 10^7 M^{-1}) than that for tc (K_A of about 10^5 M^{-1}; Takahashi et al., 1986).

Atc showed the highest affinity to TetR found in this study with an about 30-fold-increased binding constant.

Quantification of Induction by tc Analogs. A methylation protection assay in which the extent of methylation protection of a guanine in *tetO* is determined in dependence of the inducer concentration (Lederer et al., 1995) was used for quantification of *in vitro* induction. We determined the equilibrium association constants of TetR−$[Mg\text{-tc}]^+$ to *tetO* (K_{o1}) and TetR−$([Mg\text{-tc}]^+)_2$ to *tetO* (K_{o2}) by using a scheme of coupled equilibria to fit the experimental data (see Materials and Methods; Scheme 1). The constants obtained for the tc derivatives are summarized in Table 2. The binding constant of TetR to *tetO* (K_o) was set to 1×10^{15} M^{-1} for all calculations (Lederer et al., 1995). The f1 value relates the binding constants K_o, K_{o1}, and K_{o2} to each other. As K_o is constant in the absence of inducer, f1 can be regarded as a measure of the contributions of the first and second bound tc molecules to induction (for details, see Materials and Methods). In addition, the apparent induction efficiencies as represented by the concentration of inducer necessary for half-maximal induction are listed in Table 1 (column 3).

2-Nitrilo-tc showed a slightly enhanced induction efficiency compared to that of tc, as is shown in Figure 2. Half-induction occurred at about 3-fold lower concentrations of the inducer. Whereas K_{o2} for tc and 2-nitrilo-tc was at the level of nonspecific binding, K_{o1} for TetR−$[Mg\text{-2-nitrilo-tc}]^+$ was about 4 times lower than that for TetR−$[Mg\text{-tc}]^+$. This is also reflected by the slightly decreased f1 value.

Tc derivatives affecting the 4-dimethylamino grouping led to the largest decrease of induction efficiency (Figure 3), resembling their effect on binding (compare above). Induction was completely abolished when the dimethylamino grouping was substituted by hydrogen. Whereas 4-de(dimethylamino)-atc, in contrast to 4-de(dimethylamino)-tc, showed some residual binding to TetR, no induction was detected for both derivatives. Epimerization of the 4α-dimethylamino grouping to 4*β* led to an about 80-fold reduction in the apparent induction efficiency. The induction data of this analog were only poorly fitted by our reaction model. A freshly purified sample of 4-epi-tc contains about

Table 2: Association Equilibrium Constants of TetR−[Mg-tc]$^+$ (K_{o1}) and TetR−([Mg-tc]$^+$)$_2$ (K_{o2}) with *tetO*[a]

derivative	K_{o1} (M^{-1})	K_{o2} (M^{-1})	f1
tc	$2.9 \pm 0.9 \times 10^{12}$	2×10^5 to 8×10^5	0.73 ± 0.02
atc	$3.7 \pm 0.8 \times 10^{12}$	4×10^5 to 5×10^7	0.70 ± 0.03
2-nitrilo-tc	$7.1 \pm 1.4 \times 10^{11}$	5×10^5 to 7×10^7	0.60 ± 0.04
4-epi-tc[b]	3.0×10^{12}	2.0×10^5	0.74
4-de(dimethylamino)-tc		no induction	
4-de(dimethylamino)-atc		no induction	
5-hydroxy-tc	$3.1 \pm 0.9 \times 10^{12}$	4×10^5 to 3×10^7	0.69 ± 0.03
6-deoxy-tc	$1.1 \pm 0.4 \times 10^{12}$	3×10^5 to 4×10^7	0.66 ± 0.04
6-demethyl-tc	$3.5 \pm 2.1 \times 10^{11}$	5×10^5 to 2×10^7	0.57 ± 0.07
6-deoxy-6-demethyl-tc	$8.3 \pm 1.8 \times 10^{11}$	2×10^5 to 5×10^7	0.63 ± 0.04
6-methylene-5-hydroxy-tc	$1.5 \pm 0.3 \times 10^{12}$	4×10^5 to 4×10^7	0.65 ± 0.03
6-deoxy-5-hydroxy-tc	$1.3 \pm 0.4 \times 10^{12}$	3×10^5 to 3×10^7	0.66 ± 0.03
7-chloro-tc	$7.9 \pm 3.1 \times 10^{11}$	4×10^5 to 2×10^6	0.66 ± 0.01
9-amino-6-deoxy-6-demethyl-tc	nd	nd	nd
9-amino-8-chloro-6-deoxy-6-demethyl-tc	nd	nd	nd
9-(*N*,*N*-dimethyl glycylamido)-6-deoxy-6-demethyl-tc	nd	nd	nd

[a] f1 can be regarded as a measure for the contribution of each tc molecule to induction (see Materials and Methods). [b] The parameters were calculated assuming a contamination by tc of 2% as verified by HPLC analysis.

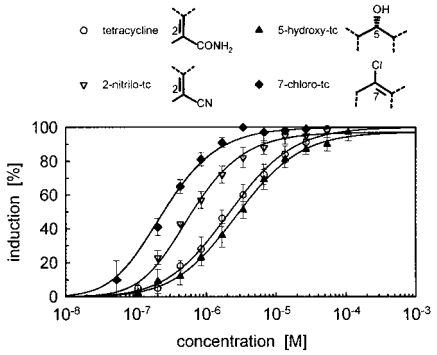


FIGURE 2: Influence of chemical substitutions at positions 2, 5, and 7 of tc on *in vitro* induction efficiencies. The induction efficiency is given in dependence of the inducer concentration. The symbols represent the experimental data, with standard deviations of repeated experiments marked as vertical error bars. The fitted theoretical induction curve is shown by the solid line. On top, part of the structure of the used derivatives is shown.

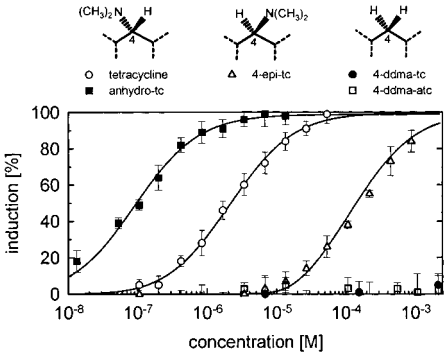


FIGURE 3: Influence of chemical substitutions at position 4 of tc and the structurally different atc on *in vitro* induction efficiencies. The induction efficiency is given in dependence of the inducer concentration. The symbols represent the experimental data, with standard deviations of repeated experiments marked as vertical error bars. The fitted theoretical induction curve is shown by the solid line. On top, part of the structure of the used derivatives is shown.

2% of tc due to spontaneous epimerization. Assuming this grade of contamination, we obtained good fits for $K_{o1} = 3 \times 10^{12}$ M^{-1} and $K_{o2} = 2 \times 10^5$ M^{-1}, identical results as for tc (Table 2). This is also reflected by the same f1 values. This result underlines the essential role of the 4α-dimethylamino moiety for induction and resembles the same finding described above for binding.

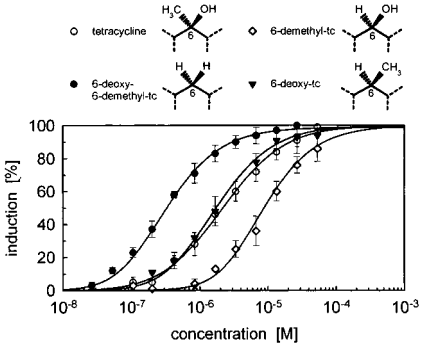


FIGURE 4: Influence of chemical substitutions at position 6 of tc on *in vitro* induction efficiencies. The induction efficiency is given in dependence of the inducer concentration. The symbols represent the experimental data, with standard deviations of repeated experiments marked as vertical error bars. The fitted theoretical induction curve is shown by the solid line. On top, part of the structure of the used derivatives is shown.

The introduction of a 5-hydroxyl grouping had no effect on the induction efficiency (Figure 2). Both the concentration at half-induction and the induction parameters K_{o1}, K_{o2}, and f1 were the same as for tc.

Substitution of the 6β-hydroxyl grouping by hydrogen increased the induction efficiency about 20−25-fold (Figure 4; compare 6-demethyl-tc with 6-deoxy-6-demethyl-tc or 5-hydroxy-tc with 6-deoxy-5-hydroxy-tc in Table 1), whereas substitution by a methyl increased induction only by 4−5-fold (Figure 4; compare 6-deoxy-tc with 6-demethyl-tc). This suggests that the negative effect of the hydroxyl is related to its hydrophilic character and to steric hindrance. The 6α-methyl had a positive effect on induction. Substitution by hydrogen led to a 3−4-fold-decreased induction efficiency (Figure 4; compare tc with 6-demethyl-tc or 6-deoxy-5-hydroxy-tc with 6-deoxy-6-demethyl-tc in Table 1). The 6-methylene grouping assumes a planar configuration, and the respective tc analog showed about the same induction efficiency as the ones with only a methyl grouping at this position (compare 6-methylene-5-hydroxy-tc with 6-deoxy-5-hydroxy-tc). K_{o1} and K_{o2} obtained with tc derivatives at position 6, except for 6-demethyl-tc, turned out to be the same as for tc (see Table 2).

A 7-chloro substitution led to an about 10-fold-increased induction efficiency compared to that of tc (Figure 2), whereas the binding constants K_{o1} and K_{o2} were only slightly

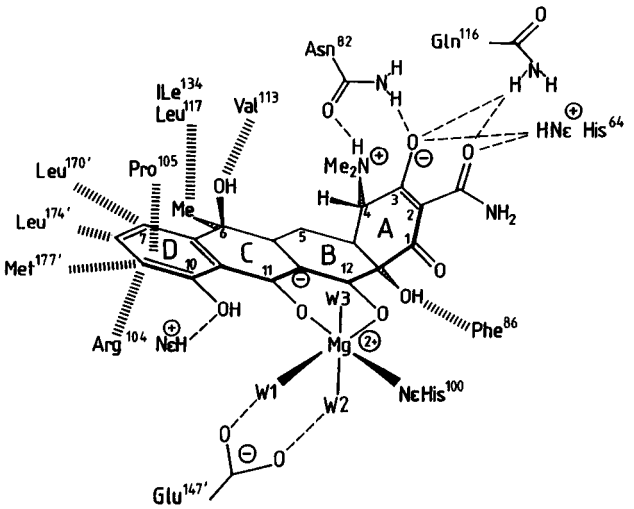


FIGURE 5: Chemical structure of tc and schematic description of the interactions between tc and TetR. Dashed lines are hydrogen bonds; hatched lines are hydrophobic interactions. W1, W2, and W3 are water ligands in the octahedral coordination shell of Mg^{2+}, and Me is methyl. Charges are marked + and −. From Hinrichs et al. (1994).

influenced. We were not able to determine induction efficiencies for derivatives at positions 8 and 9, because the results showed very high standard deviations in repeat experiments.

The best inducer we found in this study was atc with a 25-fold-increased induction efficiency (Figure 3). Atc has a naphthol moiety instead of a single aromatic D-ring in tc. In contrast, we found no significant influence on the induction parameters K_{o1}, K_{o2}, and f1, suggesting that the changed structure does not influence the mechanism of induction.

DISCUSSION

Association Equilibrium Constants of tc with Mg^{2+}. Complex formation of tc with divalent cations can involve different groupings of tc depending on pH (Silva et al., 1972) and solvent (Schnarr et al., 1979). Tc is mostly deprotonated at C(12)−OH and C(3)−OH in aqueous solution at pH 8.3 and ligands Mg^{2+} probably via the 1,3-diketoenolate moiety at positions 11 and 12 (Figure 5; Schnarr et al., 1979). This hypothesis is consistent with the coordination of Mg^{2+} in the crystal structure of the TetR(D)−$([Mg\text{-}tc]^+)_2$ complex (Hinrichs et al., 1994; Kisker et al., 1995) and our finding that modifications at positions 2 and 4−7 of tc exhibit only little effects on Mg^{2+} binding. The results obtained here for the TetR(B) protein are interpreted on the basis of the high-resolution crystal structure of the TetR(D)−$([Mg\text{-}tc]^+)_2$ complex, because they share 63% identical residues and their crystal structures have the same peptide folding. Compared to the TetR(D) complex, the TetR(B)−$([Mg\text{-}tc]^+)_2$ structure is less well-resolved (Hinrichs et al., 1994; Kisker et al., 1995). Larger effects were observed for modifications at positions 8 and 9. An 8-chloro as well as a 9-amino- or a 9-dimethylglycylamido grouping resulted in 3-fold-increased binding of Mg^{2+}. This cannot be explained by electronic effects on the 11,12 oxygens and may, thus, be due to structural modifications. The aromatization of the C-ring in atc may be responsible for the decreased Mg^{2+} binding because the carbonyl at position 12 is no longer able to enolize. This is in agreement with the involvement of the 11,12 oxygens in Mg^{2+} binding.

Induction and Binding Capacity of tc Analogs. The relation between binding to and induction of TetR by various tc analogs can be analyzed to gain information about the induction mechanism. The 2-nitrilo substituent showed the same binding affinity and induction as a carboxamido grouping at this position in tc. Therefore, both substituents may not be important for tc binding to TetR. Nevertheless, a hydrogen bond between the carboxamido group and the Nε of His^{64} was observed in the TetR(D)−$([Mg\text{-}tc]^+)_2$ crystal structure (Figure 5; Hinrichs et al., 1994; Kisker et al., 1995). Therefore, the effect of the hydrogen bond may be of little energetic importance and/or may be overcompensated by other interactions in the 2-nitrilo derivative. In contrast to these results, 2-nitrilo-tc failed to induce TetR *in vivo*. This suggests interference of the 2-nitrilo grouping with uptake or diffusion of this analog into bacterial cells (D. Rothstein, personal communication).

Substitution of the 4-dimethylamino grouping by hydrogen revealed the strongest effects on binding and induction. This indicates a specific contact between this grouping and the TetR protein, which is supported by the crystal structure, where a hydrogen bond connects the 4-dimethylamino moiety and the Asn^{82} side chain (Figure 5; Hinrichs et al., 1994; Kisker et al., 1995). Epimerization of the 4-dimethylamino substituent makes this bond less likely due to the increased distance. In addition, steric interference with other side chains of TetR may interfere with binding and, thus, explain the decreased binding and induction of this analog. An Asn^{82} to Ser mutation in TetR leads to an about 100-fold-decreased K_A with tc and underlines the role of this amino acid for the TetR−tc interaction (Müller et al., 1995). 4-De(dimethylamino)-tc and the respective atc analog did not induce the TetR−*tetO* complex. The explanation for the tc analog is trivial, because it does not bind TetR under these conditions, whereas the atc derivative does show residual binding but no induction. We simulated the induction reaction according to the thermodynamic scheme for the atc derivative. Although K_A is decreased by nearly 3 orders of magnitude compared to that of atc, we should detect induction under our experimental conditions. Thus, K_{o1} and K_{o2} must be different from those of atc. This provides a hint for a specific role of the 4-dimethylamino group in the induction mechanism.

We found two major effects at position 6 of tc. (i) Substituents at position 6β generally decreased the affinity to TetR. (ii) Substitutions with increasing hydrophobicity increased the affinity. This leads to the conclusion that the protein environment in this region is hydrophobic and in closer contact to the 6β- than to the 6α-substituent. This conclusion is supported by the TetR(D)−$([Mg\text{-}tc]^+)_2$ crystal structure, which reveals three hydrophobic amino acids (Val^{113}, Leu^{117}, and Ile^{134} in the class D repressor) surrounding the 6α- and 6β-substituent of tc (Figure 5). The sequence alterations of Tn*10*-encoded TetR are Val^{113} to Leu and Ile^{134} to Leu, retaining the hydrophobicity of this region. The apparent induction by 6-demethyl-tc was only 3−4-fold-decreased compared to that of tc, which is in contrast to the about 20-fold-decreased binding constant K_A. In contradiction to this, K_A for 6-deoxy-6-demethyl-tc was decreased about 3−4-fold, compared to that of 6-deoxy-5-hydroxy-tc which also differs in the methyl group at position 6α.

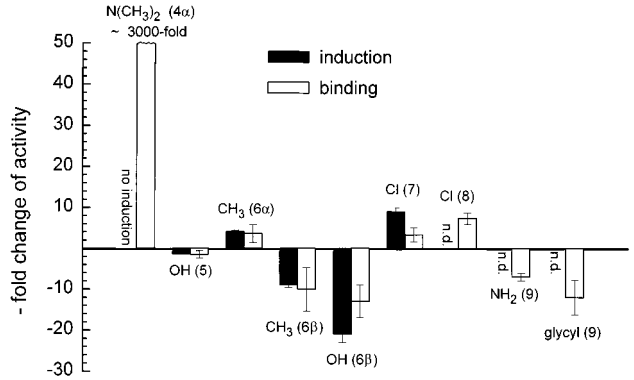


FIGURE 6: Influence of specific substituents on *in vitro* induction (filled bars) and binding (open bars) of TetR. Each substituent is marked over the respective bars. Bars facing to the top indicate a positive and those to the bottom a negative influence. The factors represent the influence of the respective moiety, compared to hydrogen at this position.

Small substituents at position 7, as in 7-chloro-tc, increase the affinity to TetR (Degenkolb et al., 1991). The same effect was shown here for induction. The positive effect of the chlorine in the D-ring of tc on binding was even more pronounced at position 8.

Both substituents at position 9, the amino- and dimethylglycylamido moieties, revealed a negative effect on binding. We assume that this is caused by steric interference, especially for the large dimethylglycylamido grouping. All 9-modified tc analogs yielded significant induction, but we were not able to quantify it. Efficient induction has also been shown for the 9-(*N*,*N*-dimethylglycylamido) derivative *in vivo* (Someya et al., 1995). The high metal-independent binding of these derivatives to TetR suggests a problem arising from nonspecific binding. That may contribute to the poor reproducibility of *in vitro* induction.

Atc was the most effective binder and inducer studied here. The 30-fold-increased K_A is partially caused by the loss of the 6α-hydroxyl, which has a negative effect on binding and induction (Figure 6). Nevertheless, the electronic change at the 11,12 oxygens and/or the planarization of the C-ring may also contribute to the change of K_A. The binding constants K_{o1} and K_{o2} did not differ significantly from those of the TetR−tc complex, indicating that binding parallels induction.

The different induction efficiencies found here *in vitro* seem to be quite similar in bacterial (Bertrand et al., 1984) and eukaryotic expression systems. It has been shown recently that atc and 6-deoxy-5-hydroxy-tc, which we found to be the best inducers, also appear to be the most effective inducers in HeLa cells (Gossen & Bujard, 1995). In these studies, 5-hydroxy-tc revealed about the same induction efficiency as tc, which is also consistant with our finding. In contrast, there are deviations between binding and induction of TetR *in vitro* and antibiotic potential of the respective derivatives *in vivo*, suggesting a mechanistic difference between binding to TetR and binding to the ribosome. Atc, an efficient inducer of TetR, exhibits destructive effects on the cytoplasmatic membrane and has only small effects on protein biosynthesis (Brunello & Chopra, 1992).

An overview of the influence of tc substituents on binding and induction is shown in Figure 6. The factors given in this figure were derived from comparisons of tc analogs having either the respective moiety or hydrogen at the respective position. Thus, they define the influence of each single modification. The nearly quantitative correlation between the efficiencies of binding and induction indicates the mechanistic coupling between both processes. Substituents affecting hydrogen bonds as well as hydrophobic interactions exert a strong influence on both binding and induction of the respective derivative. Nevertheless, the hydrogen bond between the dimethylamino at position 4 and Asn^{82} contributes most strongly to the binding free energy change (−21 kJ/mol), whereas hydrophobic interactions show a more gradual influence (about ±5 kJ/mol). This is in agreement with results of a mutational analysis of the respective positions in TetR, where similar results were obtained (Müller et al., 1995). Except for 4-de(dimethylamino)-atc, no derivatives were studied which could bind TetR, but which failed to induce it. In contrast, many TetR mutants have been characterized which bind tc but are not induced (Müller et al., 1995). Therefore, we propose that binding of the drug initiates the induction process but has no or little influence on its mechanism. This is also consistent with the result that the *tetO* binding constants K_{o1} and K_{o2} are nearly identical for all tc derivatives. It, thus, appears that they trigger the same induction mechanism in TetR.

ACKNOWLEDGMENT

We thank Drs. C. Berens and B. Hecht for critically reading the manuscript.

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BI952683E