Tigecycline Is Modified by the Flavin-Dependent Monooxygenase TetX†

Ian F. Moore, Donald W. Hughes, and Gerard D. Wright*

Antimicrobial Research Centre, Department of Biochemistry and Biomedical Sciences and the Department of Chemistry, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5, Canada

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ABSTRACT: The clinical use of tetracycline antibiotics has decreased due to the emergence of efflux and ribosomal protection-based resistance mechanisms. Currently in phase III clinical trials, the glycylcycline derivative tigecycline (GAR-936) containing a 9-*tert*-butylglycylamido group is part of a new generation of tetracycline antibiotics developed during the 1990s. Tigecycline displays a broad spectrum of antibacterial activity and circumvents the efflux and ribosomal protection resistance mechanisms. The TetX protein is a flavin-dependent monooxygenase that modifies first and second generation tetracyclines and requires NADPH, Mg^{2+}, and O_2 for activity. We report that tigecycline is a substrate for TetX and that bacterial strains containing the *tet*(*X*) gene are resistant to tigecycline. The resistance is due to the modification of tigecycline by TetX to form 11a-hydroxytigecycline, which we have shown has a weakened ability to inhibit protein translation compared with tigecycline. We have explored the basis of this decreased ability to block translation and found that hydroxylation occurs in the region of the molecule important for coordinating magnesium. 11a-Hydroxytigecycline forms a weaker complex with magnesium than tigecycline; the crystal structure of tetracycline in complex with the ribosome has shown that magnesium coordination is critical for binding tetracycline. Although *tet(X)* has not been isolated from any clinically resistant strains, our report demonstrates the first enzymatic resistance mechanism to tigecycline and provides an alert for the surveillance of resistant strains that may contain *tet(X)*.

Tetracycline antibiotics have been a mainstay of antibacterial therapy for over 5 decades. Despite their excellent oral activity and broad-spectrum antibacterial properties, their use has declined in the face of widespread resistance. Tetracyclines exert their bacteriostatic effect by binding to the 30S subunit of the ribosome and interfering with mRNA translation (*1*−*3*). The mechanism of inhibition of protein synthesis is through prevention of binding of the aminoacyl-tRNA to the acceptor site (A-site) (*4*, *5*). Crystal structures of tetracycline in complex with the ribosome reveal several binding sites on the 30S subunit (*6*, 7). In the highest occupancy site, site I, the hydrophilic side of tetracycline makes many hydrogen-bonding interactions with oxygen atoms of the ribosome's phosphate backbone. In addition to these interactions, a magnesium ion bound by oxygen atoms of carbons 11 and 12 of tetracycline also coordinates the oxygen atoms of the phosphate backbone within site I (Figure 1).

Bacterial resistance to tetracyclines manifests itself through expression of the *tet* genes of which more than 29 have been characterized (*1*). Two major mechanisms of resistance dominate: ribosomal protection (TetM, TetO, TetS), that release tetracyclines from the ribosome, and efflux (TetA, TetB, etc.), that actively transport tetracyclines across the cell membrane (*8*−*10*). Medicinal chemistry efforts in the early 1990s resulted in the discovery of the semi-synthetic glycylcycline derivatives of tetracyclines modified at position 9 of the tetracycle that maintain antibiotic activity and additionally were active against tetracycline-resistant bacteria carrying the *tet* genes for ribosomal protection and efflux (*11*). The minocycline derivative tigecycline (9-*tert*-butyl-

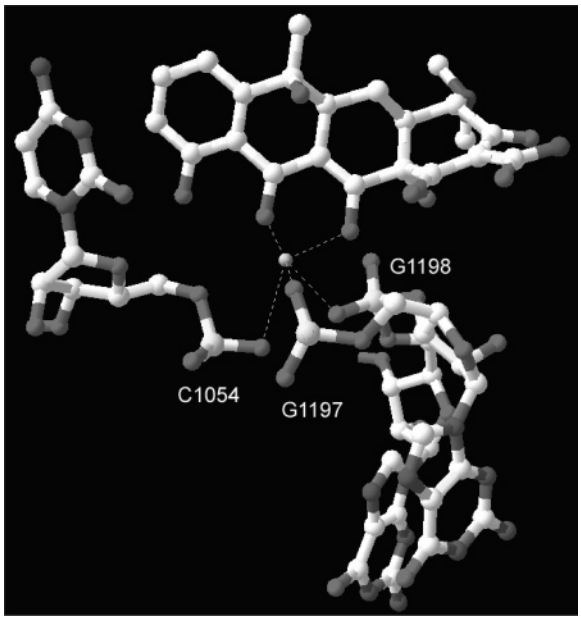

FIGURE 1: Tetracycline chelates Mg^{2+} through the oxygen atoms bound to carbons 11 and 12. Upon binding to the A-site of the ribosome, the 30S ribosomal RNA phosphates from cytosine 1054 and guanosines 1197 and 1198 provide the remaining coordination ligands. The image is from the *Thermus thermophilus* tetracycline structure (1HNW) (*6*).

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* To whom correspondence should be addressed. Tel: 905-525-9140 (ext. 22454). Fax: 905-522-9033. E-mail: wrightge@mcmaster.ca.

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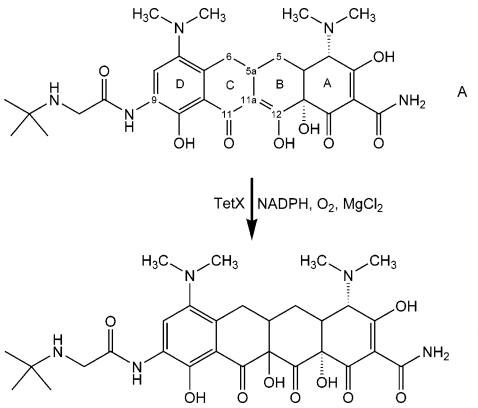

FIGURE 2: The structure of tigecycline (A) and the product of regiospecific hydroxylation catalyzed by TetX producing 11a-hydroxytigecycline (B).

glycylamido-minocycline (GAR-936, Figure 2A) is currently in phase III clinical trials (*12*).

Unlike the *tet* genes responsible for ribosomal protection and efflux, the *tet(X)* gene product is a tetracycline modification enzyme (*13*, *14*). TetX requires FAD,[1] NADPH, Mg^{2+}, and O_2 for activity (*15*). The *tet(X)* gene was originally discovered in the obligate anaerobe *Bacteroides fragilis*; however, only when the gene was transferred to aerobically growing *Escherichia coli*, was antibiotic resistance activity uncovered. We have recently reported the overexpression and purification of TetX and shown that it is a flavin-dependent monooxygenase that regiospecifically hydroxylates oxytetracycline at carbon 11a (*15*). The product 11a-hydroxyoxytetracycline is stable only under very acidic solutions; in solutions of pH greater than 1, it decomposes rapidly into products that are not easily identifiable. Because of the inherent instability of 11a-hydroxyoxytetracycline, it is not an effective antibiotic and a resistance phenotype is observed.

Tigecycline shows great promise as the prototype for the next generation of tetracycline derivatives as it maintains antibiotic spectrum and potency while overcoming resistance arising from the expression of ribosomal protection and efflux proteins (*16*). Although *tet(X)* has so far not been identified in clinically-derived bacterial strains resistant to tetracyclines, we report here that *tet(X)* provides resistance to tigecycline and hydroxylation of tigecycline by TetX to produce 11a-hydroxytigecycline decreasing its potency. This is the first report of enzyme-based resistance to this new class of antibiotic.

MATERIAL AND METHODS

Chemicals and Reagents. Tigecycline was generously provided by Wyeth Research. TetX was purified as previously described (*15*).

Spectrophotometric Assay of Tigecycline Hydroxylation. TetX (30 μg) was added to a tigecycline solution (40 μM) in 25 mM TAPS, pH 8.5, containing NADPH (80 μM) and $MgCl_2$ (10 mM). UV spectra were recorded every minute. Kinetic parameters were measured in a 96 well microtiter plate by following either the disappearance of tigecycline at 400 nm ($\epsilon_{400} = 13\,400\ M^{-1}\ cm^{-1}$) or NADPH ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) at 340 nm using a Molecular Devices SpectraMax Plus microtiter plate reader. When tigecycline disappearance was monitored, solutions contained NADPH (1 mM), $MgCl_2$ (10 mM), TetX (8 μg), and up to 1 mM tigecycline in 25 mM TAPS, pH 8.5. When the disappearance of NADPH was monitored, solutions contained NADPH (300 μM), $MgCl_2$ (10 mM), TetX (20 μg), and up to 250 μM tigecycline in 25 mM TAPS, pH 8.5. Steady-state kinetic parameters were determined by fitting initial rate (v_o) data to the standard Michaelis−Menten equation using Grafit 4 software (*17*):

$$v_o = k_{cat}[E_t][S]/([S] + K_m)$$

where $[E_t]$ is the total enzyme concentration.

LC−MS Identification of the Tigecycline−TetX Reaction Product. An aliquot of the assay solution was analyzed by reverse-phase liquid chromatography with mass spectrometry detection using an Applied Biosystems Q-Trap instrument. A Dionex Acclaim 120 C18 column was equilibrated with 95% water and 5% acetonitrile both containing 0.05% formic acid. A linear gradient was developed over 20 min to 3% water and 97% acetonitrile.

Activity of TetX in the Presence of Catalase. The oxidation of tigecycline by TetX in the presence of catalase was monitored by UV spectroscopy. TetX (19 μg) was added to a cuvette containing tigecycline (75 μM) in 25 mM TAPS, pH 8.5, containing NADPH (1 mM), $MgCl_2$ (10 mM), and catalase (2400 units and 12 000 units; 1 unit will decompose 1.0 μmol H_2O_2 per minute at pH 7.0 and 25 °C). The reference cuvette contained all of the above components except tigecycline, and difference UV spectra were recorded every minute.

Antibiotic Activity of Enzymatically Modified Tigecycline. A NADPH regenerating system consisting of $NADP^+$ (1 mM), glucose-6-phosphate (40 mM), and glucose-6-phosphate dehydrogenase (5 units) in 25 mM TAPS, pH 8.5, and 1 mM $MgCl_2$ was incubated for 10 min at 37 °C. TetX (90 μg) was added followed by tigecycline (250 μM). A separate control reaction containing the above NADPH regenerating system and tigecycline was performed to monitor nonenzymatic oxidation of tigecycline. After addition of tigecycline and at 20 min intervals, aliquots (50 μL) were removed and trifluoroacetic acid (0.5 μL) added to stop the reaction. The extent of product formation was analyzed by HPLC, and a 10 μL aliquot of the reaction containing TetX was spotted on a sterile filter disk to assess antimicrobial activity. The sterile filter disks were placed on tryptic soy agar plates containing *Micrococcus luteus* diluted to an OD_{625} of 0.08−0.1, and the plates were incubated for 48 h at 37 °C. HPLC separation was performed on a Dionex Acclaim 120 C18 column equilibrated with 95% water and 5% acetonitrile both containing 0.05% trifluoroacetic acid. The reaction products were eluted with a linear gradient to 3% water and 97% acetonitrile over 10 min.

Purification of Enzymatically Modified Tigecycline. The product of TetX-catalyzed modification of tigecycline was isolated from two large-scale reactions (5 mL total volume) containing the same NADPH regenerating system, TetX (1.6

[1] Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate (reduced); FAD, flavin adenine dinucleotide; TAPS, *N*-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; LC−MS, liquid chromatography mass spectrometry, MIC, minimum inhibitory concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

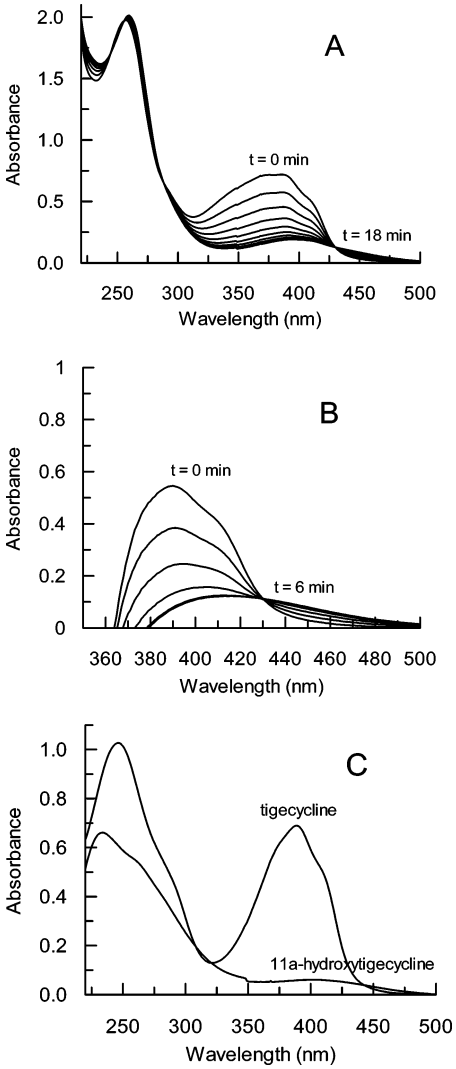

FIGURE 3: Hydroxylation of tigecycline results in a decrease in UV absorbance of the antibiotic. (A and B) The time course of the hydroxylation of tigecycline catalyzed by TetX in the presence of NADPH, Mg^{2+}, and O_2. Panel A follows the spectral changes in both NADPH and tigecycline upon oxidation. The reaction solution contains 80 μM NADPH, 40 μM tigecycline, 10 mM Mg^{2+}, and 30 μg of TetX. Panel B follows the spectral changes in the region of the tigecycline spectrum not overlapping with NADPH. The presence of the isosbestic point at 433 nm is indicative of a simple conversion of substrate into a single product. The UV absorbance of NADPH is subtracted by including an equal concentration in both the reference and sample cuvettes. The reaction solution contains 40 μM tigecycline, 1 mM NADPH, 10 mM Mg^{2+}, and 30 μg of TetX. Panel C shows an overlap of the UV spectra of equal concentrations of tigecycline and purified 11a-hydroxytigecycline.

mg), and tigecycline (4.5 mg). Product formation was monitored by HPLC, and upon completion, the reaction was stopped by the addition of HCl to 0.1 M. The reaction mixture was applied onto a Waters Sep-Pak Plus C18 column, and the column was washed with water. The product was eluted with 1% acetonitrile in water followed by 5% acetonitrile in water. All fractions containing the product of enzymatically modified tigecycline were combined and lyophilized. Prior to NMR analysis, the combined product fractions were purified by preparative scale HPLC on a Grace Vydac C18 preparative column using the same conditions as the analytical separation. The purified reaction product was dissolved in 0.1 M DCl/D_2O, and ^1H and ^{13}C NMR spectra were recorded on a Brucker AV 700 instrument. Proton spectra were obtained at 700.23 MHz, and ^{13}C spectra were recorded at 176.09 MHz.

Minimal Inhibitory Concentration (MIC) Determinations. MICs were performed in cation-adjusted Mueller−Hinton broth following NCCLS guidelines, and the results were recorded after incubation for 24 h at 37 °C. The MICs of tigecycline and 11a-hydroxytigecycline were tested against *E. coli* W3110 containing the *tet(X)* gene in a pUC19 background and *E. coli* W3110 containing pUC19 vector only.

The effect of the level of expression of TetX on MIC was tested against *E. coli* BL21(DE3) containing *tet(X)* in a pET28 vector and *E. coli* BL21(DE3) containing pET28 vector only. TetX expression was induced with IPTG at 0.5 and 0.05 mM.

In Vitro Translation Assay. To assess the impact of modification of tigecycline on ribosome function, in vitro translation assays were performed. Reactions were carried out using the ActivePro In Vitro Translation Kit from Ambion. The protein PanK (37 kDa) was translated from a pET28a construct with *panK* cloned into the Nde I and Hind III restriction sites. Tigecycline and purified 11a-hydroxytigecycline were incubated with the contents of the kit for 5 min prior to the initiation of translation by the addition of 2 μg plasmid DNA. [^{35}S]-Methionine was included in the reaction mixture to label PanK (6 Met). Translation reactions were allowed to continue for 2 h, then stopped by cooling on ice. Proteins were precipitated by the addition of acetone and collected by centrifugation. The collected pellet was resuspended in SDS loading buffer and denatured at 100 °C. The samples were separated on an 11% SDS−polyacrylamide gel, then dried and quantified using a phosphorimager.

Measurement of the Dissociation Constant of Chelation Complexes of Magnesium and Tigecycline and 11a-Hydroxytigecycline. The dissociation constants of chelation complexes with magnesium were determined in the presence of tigecycline (20 μM) and 11a-hydroxytigecycline (20 μM) with varying concentrations of $MgCl_2$ (50 μM to 20 mM) in 50 mM MOPS, pH 7.5. As described previously, absorbance at 380 nm was used to determine the dissociation constant of the tigecycline complex (*18*), and 430 nm was used for determination of the 11a-hydroxytigecycline chelation complex. Dissociation constants were determined by nonlinear regression fitting of the binding isotherms to the equation, $[\text{Bound}] = \text{Capacity}[\text{Mg}]_{\text{free}}/(K_d + [\text{Mg}]_{\text{free}})$.

RESULTS

TetX Inactivates Tigecycline. The inactivation of tigecycline by TetX was established by UV−vis spectroscopy, HPLC, and a biological assay. Tetracyclines contain two UV absorbance maxima, one at 260 nm and one at 363 nm. The maximum at 363 nm is indicative of the aryl β-diketone chromophore of rings B, C, and D. The strong absorbance of NADPH at 340 nm overlaps most of the tigecycline UV spectrum except for a region above 360 nm (Figure 3A,B). Figure 3A shows the spectral changes in both NADPH and tigecycline upon reaction with TetX. Subtracting the absorbance of NADPH reveals a portion of the tigecycline spectrum, and as the reaction with TetX proceeds, the maximum at 400 nm decreases with time indicating that tigecycline is being modified by TetX in the presence of

NADPH (Figure 3B). The steady-state kinetic parameters for tigecycline modification were determined to be K_m, 44 ± 12 μM; k_{cat}, 0.36 ± 0.03 s^{-1}; and k_{cat}/K_m, 8.3 × 10^3 M^{-1} s^{-1} by following the disappearance of either NADPH at 340 nm or tigecycline at 400 nm. These values are similar to those observed for TetX-mediated oxidation of other tetracyclines (*15*).

The ability of TetX to modify tigecycline in the presence of the enzyme catalase was also followed by UV−vis spectroscopy. Addition of up to 12 000 units of catalase had no effect on the rate of tigecycline oxidation.

The biological activity of the TetX−tigecycline reaction product was assessed by a disk assay with a tetracycline-susceptible test organism *Micrococcus luteus*. Figure 4A shows the time-dependent loss of tigecycline antibiotic activity upon incubation with TetX in the presence of NADPH and magnesium. The loss of antibiotic activity was simultaneously monitored by reverse-phase HPLC and found to be the result of the formation of a single product peak (Figure 4B). No product peak was observed upon incubation of tigecycline and the NADPH regenerating system in the absence of TetX after 20 h. Unlike the product of reaction of TetX and oxytetracycline, which begins to degrade to a second product before complete transformation into 11a-hydroxyoxytetracycline(*15*), only one product peak was observed in the reaction between TetX and tigecycline. This product peak was observed by HPLC to be stable over time at pH 8.5.

TetX Hydroxylates Tigecycline at Carbon 11a. The mass of the TetX−tigecycline reaction product was analyzed by LC−MS. The product peak had an *m*/*z* value of 602.4 corresponding to the addition of one oxygen atom to tigecycline (*m*/*z* 586.3).

Tables 1, 2, and 3 list the ^1H chemical shifts, coupling constants, and ^{13}C NMR shifts of the purified TetX−tigecycline reaction product and tigecycline determined under identical conditions. The same numbers of proton resonances are seen in the spectrum of tigecycline and the TetX−tigecycline reaction product, and no changes in proton coupling are observed. Changes in chemical shift are observed for the two protons attached to carbon 6 that shift to higher frequency by an average of 0.4 ppm.

Significant changes in chemical shift are observed in the ^{13}C NMR spectrum of the TetX−tigecycline reaction product. Carbon 5a shifts to higher frequency by 8.7 ppm, carbon 11a shifts to lower frequency by 26.8 ppm, and carbon 12 shifts to higher frequency by 30.6 ppm. These changes in chemical shift are consistent with hydroxylation at carbon 11a. Upon hydroxylation of the enol localized between carbons 11a and 12, carbon 11a undergoes a hybridization change from sp^2 to sp^3 consistent with a shift to lower frequency of 26.8 ppm (Figure 2B). Carbon 12, which is converted from the enol tautomer to a carbonyl center in the product, shifts to higher frequency by 31.2 ppm. Carbon 5a, which now has an oxygen atom adjacent, shifts to higher frequency by 8.7 ppm. These results are consistent with those observed for the TetX−oxytetracycline reaction product (*15*); however, in the case of 11a-hydroxyoxytetracycline in acid solution, an intramolecular ketal is formed between the oxygen atom at carbon 6 and carbon 12 and no higher frequency shift is observed for carbon 12. Tigecycline is unsubstituted at carbon 6 and therefore does not undergo an analogous intramolecular hemi-ketal reaction upon formation of 11a-hydroxytigecycline.

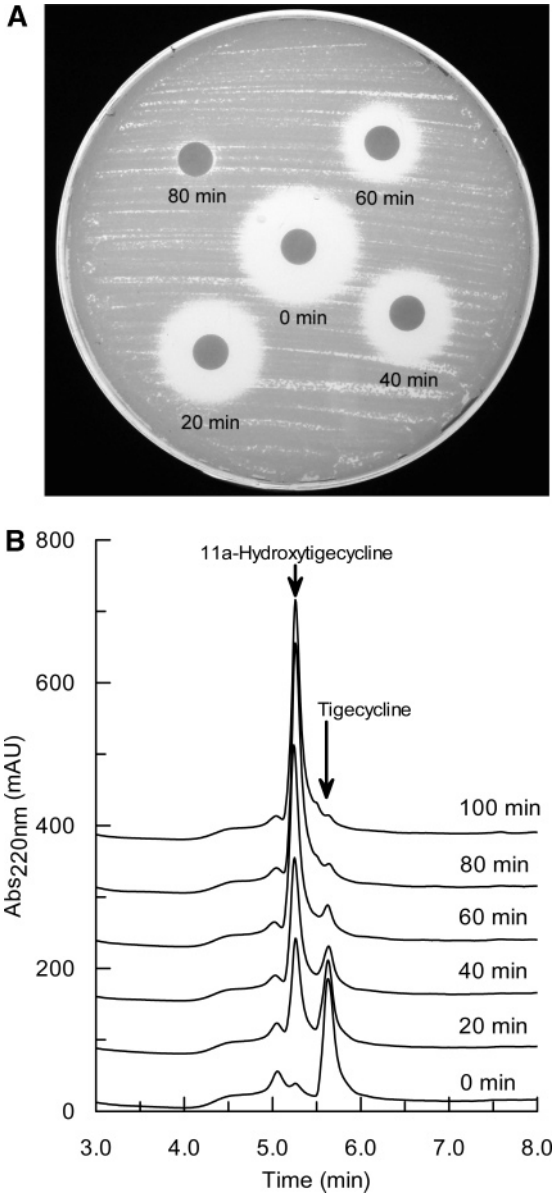

FIGURE 4: (A) TetX inactivates tigecycline. Antibiotic activity is abolished upon incubation of tigecycline with TetX, NADPH, Mg^{2+}, and O_2. The time course of loss of antibiotic activity is shown using the tigecycline-susceptible bacterium *M. luteus* and evidenced by the shrinking of the zone of growth inhibition around disks containing the reaction products. (B) Progress of the inactivation of tigecycline by TetX. Tigecycline is converted into a single product (11a-hydroxytigecycline) by TetX in the presence of NADPH, Mg^{2+}, and O_2. 11a-Hydroxytigecycline was observed to be stable over time at pH 8.5. Aliquots for analysis were removed immediately after the addition of tigecycline to the reaction mixture and every 20 min thereafter. HPLC separation was performed on a C18 column equilibrated with 95% water and 5% acetonitrile both containing 0.05% trifluoroacetic acid. The reaction products were eluted with a linear gradient to 3% water and 97% acetonitrile over 10 min. An aliquot of each time point was spotted on the sterile disks shown in panel A.

Antibiotic Properties of 11a-Hydroxytigecycline. The MIC of tigecycline against an *E. coli* strain containing *tet(X)* was 2 μg/mL, and the MIC of *E. coli* W3110 containing an empty pUC19 vector was 0.5 μg/mL. The MIC of 11a-hydroxytigecycline is 64 μg/mL against both strains. The difference

Table 1: ^{1}H Chemical Shifts of Tigecycline and 11a-Hydroxytigecycline in 0.1 M DCl/D_2O Obtained at 700.23 MHz

	chemical shifts (ppm)	
proton	tigecycline	11a-hydroxytigecycline
4	4.071	3.773
4-N(CH_3)$_2$	3.017, 2.948	3.042, 2.871
4a	3.007	3.246
5a	2.262	2.162
5′ [a]	1.713	1.653
5[a]	3.132	2.930
6[a]	3.064	3.413
6′ [a]	2.554	3.021
7-N(CH_3)$_2$	3.272	3.269
8	8.419	8.528
NH***CH*$_2$CO	4.139	4.104
C(CH_3)$_3$	1.408	1.381

[a] The C-5 and C-6 methylene protons are nonequivalent, and the nonprimed and primed numbers represent pseudoequatorial and pseudoaxial structures.

Table 2: ^{1}H-^{1}H Coupling Constants of Tigecycline and 11a-Hydroxytigecycline in 0.1 M DCl/D_2O

	coupling constant (Hz)	
proton	tigecycline	11a-hydroxytigecycline
	Proton (^{3}J)	
4, 4a	1.6	2.2
4a, 5	2.6	4.3
4a, 5′	13.5	13.2
5, 5a	4.9	4.2
5′, 5a	11.2	13.3
5a, 6	4.6	4.8
5a, 6′	13.6	2.4
	Proton (^{2}J)	
5, 5′	−13.8	−14.9
6, 6′	−15.4	−17.0

in MIC for tigecycline observed in the presence of the *tet-(X)* gene is much smaller than the change in MIC observed for the other tetracyclines. The effect of the level of TetX expression on MIC was tested against *E. coli* strains harboring *tet(X)* in an inducible T7 RNA polymerase expression system. The MIC of tigecycline against *E. coli* BL21(DE3) containing *tet(X)* in pET28 was 0.125 μg/mL. This MIC increased to 0.5 μg/mL upon induction of TetX expression with either 0.05 or 0.5 mM IPTG. The MIC of tigecycline against *E. coli* BL21(DE3) containing and empty pET28 vector was 0.125 μg/mL with or without IPTG.

In addition to MIC, the ability of 11a-hydroxytigecycline to block protein synthesis in an in vitro protein translation assay was investigated. Tigecycline and 11a-hydroxytigecycline were added to in vitro translation reactions in equal concentrations, and the production of a radiolabeled protein (PanK) was quantified (Figure 5). As expected, the addition of tigecycline in the in vitro assay system resulted in dramatic attenuation of protein production (97% inhibition compared to a reaction mixture in the absence of antibiotic). On the other hand, addition of the same amount of 11a-hydroxytigecycline resulted in less than 20% inhibition or approximately 18−30 times more protein transcribed in the presence of 11a-hydroxytigecycline than tigecycline.

Dissociation Constants of Chelation Complexes of Tigecycline and 11a-Hydroxytigecycline with Magnesium. The crystal structure of tetracycline in complex with the ribosome (Figure 1) illustrates the importance of metal chelation by

Table 3: ^{13}C Chemical Shifts of Tigecycline and 11a-Hydroxytigecycline in 0.1 M DCl/D_2O Obtained at 176.09 MHz

	chemical shifts (ppm)	
carbon	tigecycline	11a-hydroxytigecycline
1	192.47	194.70
2	95.36	96.87
2-$CONH_2$	171.76	171.50
3	186.23	186.58
4	68.93	69.34
4-N(CH_3)$_2$	42.07	43.53, 42.40
4a	33.72	37.68
5	32.16	27.00
5a	30.82	39.47
6	28.38	24.00
6a	130.87	129.01
7	129.52	130.84
7-N(CH_3)$_2$	46.09	46.21
8	119.55	121.53
9	124.85	125.41
10	153.35	154.30
10a	115.95	113.04
11	192.92	187.29
11a	107.95	81.12
12	170.60	201.22
12a	73.60	79.50
9-NHCO	165.16	165.21
NH***CH*$_2$CO	42.65	42.66
(CH_3)$_3$***C***	57.23	57.24
***(CH*$_3$*)*$_3$C	24.34	24.34

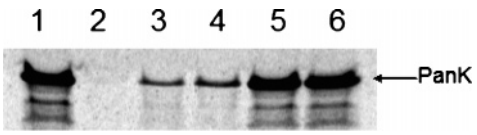

FIGURE 5: Impact of the antibiotic tigecycline and inactivation product 11a-hydroxytigecycline on in vitro protein synthesis. Phosphorimage of the amount of [^{35}S]-methionine-labeled PanK synthesized after 2 h in the presence of tigecycline and 11a-hydroxytigecycline using a commercial in vitro translation kit. Lane 1 contains only plasmid DNA; lane 2 contains no plasmid DNA; lanes 3 and 4 contain tigecycline at 4 and 2 μg/mL, respectively; and lanes 5 and 6 contain 11a-hydroxytigecycline at 4 and 2 μg/mL, respectively.

the tetracycline class of antibiotics. The formation of a metal tetracycline chelation complex can be detected by a bathochromic shift in the UV spectrum. The UV maximum of tigecycline has been shown to shift from 350 to 380 nm in the presence of excess $CoCl_2$, and a dissociation constant of 23 μM was determined (*18*). In the presence of excess $MgCl_2$, a bathochromic shift to 380 nm is also observed. 11a-Hydroxytigecycline has a UV maximum centered at 398 nm that shifts to 403 nm in the presence of 10 mM $MgCl_2$. The largest change in absorbance between a solution of 11a-hydroxytigecycline containing 10 mM magnesium and one containing no magnesium was seen at 430 nm. This wavelength was used to measure the dissociation constant of the magnesium chelation complex. Figure 6 shows the binding isotherms of complex formation between magnesium and tigecycline and 11a-hydroxytigecycline. The dissociation constant determined for the magnesium−tigecycline complex was 1.1 mM and for the magnesium−11a-hydroxytigecycline complex was 3.6 mM.

DISCUSSION

Decades of tetracycline study have revealed that modifications on the hydrophilic side of the molecule (carbons 1, 2a,

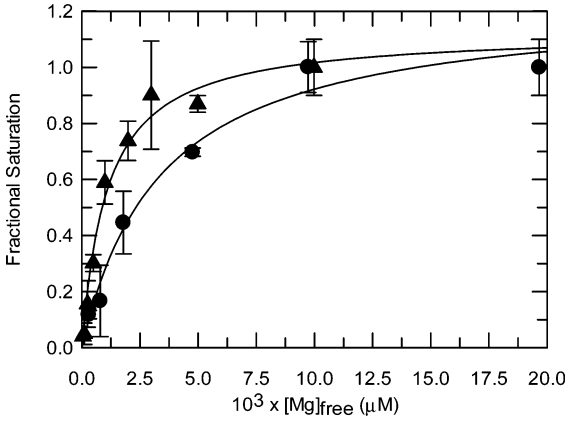

FIGURE 6: Binding isotherms of chelation complex formation between magnesium and tigecycline (▲) and magnesium and 11a-hydroxytigecycline (●) determined by UV spectroscopy. The dissociation constant of the magnesium−tigecycline complex is 1.08 ± 0.02 mM, and for the magnesium−11a-hydroxytigecycline complex, it is 3.6 ± 0.7 mM.

12, 11a, 11, 10a, and 10) abolish antibiotic activity, while modifications of the opposite side are well-tolerated. It is not surprising, therefore, that TetX modifies tetracyclines on the hydrophilic side of the molecule to result in resistance. The kinetic parameters of TetX activity do not vary widely with substrate, which might suggest that oxidation is non-enzymatic; the function of TetX might be to generate an active oxygen species such as hydrogen peroxide that then reacts with the tetracycle. We found that the presence of catalase did not affect the rate of tigecycline oxidation suggesting that a freely diffusing hydrogen peroxide is not the active oxygen species and that oxidation occurs in the TetX active site. The lack of apparent substrate discrimination likely indicates that the enzyme-binding pocket is specific for the hydrophilic site of the tetracycle, which is invariable for all the tetracyclines. The importance of the hydrophilic side of the tetracycle was revealed with the crystal structure of tetracycline in complex with the ribosome that showed critical interactions between the oxygen atoms of carbons 11 and 12 and guanosine bases 1197 and 1198 mediated by a magnesium ion (Figure 1) (*6*, *7*). Tigecycline and tetracycline have been shown to bind to identical or overlapping sites on the ribosome, and it has been surmised that they likely share the same mode of action (*19*).

We have demonstrated that TetX regiospecifically hydroxylates the semi-synthetic antibiotic tigecycline at position 11a and that this modification is correlated with loss of antibiotic activity. In the present study, we were unable to determine the stereochemistry at position 11a. The lack of a proton at carbon 11a forced us to look at NOE interactions in other parts of the molecule but did not allow unambiguous assignment. Assignment is likely to come by X-ray determination of crystals of 11a-hydroxytigecycline. The product 11a-hydroxytigecycline is also a much weaker inhibitor of protein translation than the parent compound.

Hydroxylation at carbon 11a significantly changes the physical properties of tigecycline. Tetracyclines are known metal chelators and the conversion of tigecycline to 11a-hydroxytigecycline dramatically affects its magnesium binding properties. The β-diketone moiety encompassing carbons 11, 11a, and 12 is an important site for metal binding (*20*). In tetracycline, this group has a pK_a of 7.5, and the major species in solution at neutral pH are the O12 anion and the O12 neutral form. Ionization at this oxygen provides for tighter magnesium binding. Upon transformation to 11a-hydroxytigecycline, this hydroxyl group is converted to a ketone and this pK_a (negative charge) is lost, and weaker binding to magnesium is observed. The dissociation constant for magnesium complex formation increases from 1.1 to 3.6 mM as a result of hydroxylation. Weaker binding to magnesium will lower the affinity of 11a-hydroxytigecycline for the ribosome, as observed in the decreased ability of 11a-hydroxytigecycline to block protein synthesis. In addition to this effect, removal of the resonance interaction between carbons 11, 11a, and 12 due to hydroxylation will affect the global conformation of the molecule. The tetracycline crystal structure reveals these three atoms to be nearly coplanar; in the 11a-hydroxytetracycline molecule, this planarity will be lost.

The impact of this modification is also revealed in the MIC of 64 μg/mL for 11a-hydroxytigecycline against *E. coli* compared to 0.5 μg/mL for tigecycline. Paradoxically, the presence of *tet(X)* in *E. coli* results in an MIC of only 2 μg/mL for tigecycline. Therefore, at present, even if tigecycline is a substrate for TetX and the product of enzymatic reaction is severely impaired in antibiotic activity, tigecycline may still be clinically active in organisms harboring the *tet(X)* gene. In addition, tigecycline has a 5-fold higher affinity for the ribosome than tetracycline and is 10 times more potent in inhibiting protein synthesis in a cell-free assay (*21*). Therefore, one would expect the antibiotic properties of tigecycline to be less sensitive to this type of modification than tetracycline.

There have not been any reports of TetX in clinical isolates conferring tetracycline resistance even though the *tet(X)* gene was isolated from *Bacteroides* transposons, increasing the chances of horizontal transfer. Therefore, under present conditions, TetX is not likely to influence the effectiveness of tigecycline. Should a mutant of TetX emerge with improved k_{cat} and this determinant be transferred to more clinically relevant organisms, the opportunity for clinical failure of this new antibiotic will present itself. Our study therefore is highly relevant demonstrating for the first time a resistance mechanism toward tigecycline and providing an alert for surveillance of clinical strains with *tet(X)* genes that may emerge with increased use of this antibiotic.

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