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Enhanced *in Vitro* Tigecycline Activity in the Presence of Chelating Agents

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Abstract

Lack of availability of novel antibiotic agents and the rise of resistance to existing therapies has led clinicians to utilize combination therapy to adequately treat bacterial infections. Here we examine how chelators may impact the *in vitro* activity of tigecycline (TIG) against *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*. Minimum inhibitory concentrations (MICs) were determined by broth dilution with and without various combinations of chelators (EDTA and other tetracyclines), and metal ions (i.e. calcium, magnesium). Trimethoprim (TMP) was used as non-chelating control. The addition of metal ions led to increases in MICs while addition of EDTA led to decrease in MICs. Chelating effects of EDTA were reversed by the addition of magnesium and most profoundly calcium. Similar effects of EDTA and calcium were observed for tetracycline (TET) and TMP. When other tetracyclines (TET, oxytetracycline (OXY) and chlortetracycline (CHL)) were used as chelators at concentrations below their MICs, TIG MICs decreased for *P. aeruginosa*, not for *E. coli*. Some decreases in TIG MICs were observed for *K. pneumoniae* when TET and CHL were added. The dose-dependent decrease in TIG MIC was observed for tetracycline and reversed by the addition of calcium. The presence of effects of EDTA and calcium on TMP MICs indicates that mechanisms outside of TIG chelation likely play a role in enhanced activity. Full characterization of an unexpected interaction such as TIG-TET with different microorganisms could provide valuable insights into the underlying mechanisms and design of physiologically viable chelators as candidates for future combinations regimens.

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Preliminary results were presented at the American College of Clinical Pharmacology (ACCP) 2014 Annual Meeting in Atlanta, GA[14], the American Society of Clinical Pharmacology and Therapeutics (ASCPT) 2015 in New Orleans, LA[15], and the 2nd International Caparica Conference in Antibiotic Resistance (IC2AR 2017) in Caparica, Portugal (P-32).

Keywords

antibacterial; drug combinations; calcium chelation; mechanistic investigation

1 Introduction

Lack of novel antibiotic approvals and increase in antibiotic resistance, clinicians repeatedly turn to the use of antibiotic combinations to expand antibiotic spectrums or enhance activity of existing agents through novel mechanisms. Often the interactions with another agent have been exploited to improve the efficacy of therapeutic agents. These interactions could be pharmacokinetic or pharmacodynamic in nature and should be characterized appropriately to determine the true potential clinical impacts. A pharmacokinetic interaction would affect the active drug concentration available to affect a target. Conversely, a pharmacodynamic interaction would impact the ability of the drug to kill or inhibit growth of bacteria. A pharmacodynamic interaction may also impact the nature of the interaction between the bacteria and the drug. Separate from this, pharmacodynamic effects of other drugs or chemicals may, independent of the drug of interest, impact the overall effect.

Tetracyclines are known to chelate metal ions but our group has recently investigated the interaction of metal ions with the extent of plasma protein binding of tigecycline (TIG) which exhibits atypical nonlinear plasma protein binding (i.e. decrease in free fraction as total concentrations increase followed by an increase in free fraction at highest concentrations) [1], a phenomenon also observed with other tetracyclines [2,3]. Thinking of what potential interaction TIG may have *in vitro* with chelators, a pharmacokinetic interaction may occur due to the competition of chelators for metal ions, allowing for more unchelated drug, which may lead to enhanced activity. On the other hand, the addition of chelators may also lead to a pharmacodynamic interaction in which the drug behaves differently against one or more target sites, exhibits different maximal antibacterial activity, or a different concentration-effect relationship as it pertains to the killing or inhibition of bacterial proliferation. Independent of these interaction aspects, a separate pharmacodynamic effect may also be exhibited by the addition of a chelator, for example, the chelation of metal ions may inhibit bacterial proliferation independent of drug effect.

Our investigation explores the interactions of chelating agents with the *in vitro* activity of TIG.

2 Materials and Methods

2.1 Chemicals, Bacteria, Equipment and Stock Solutions

Ethylenediaminetetraacetic acid (EDTA) and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). TIG was obtained from TSZ CHEM (Framingham, MA). TET, and trimethoprim (TMP) were obtained from Medisca Incorporated (Plattsburgh, New York). Oxytetracycline (OXY), chlortetracycline (CHL), and magnesium chloride were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Calcium chloride was obtained from Allied Chemical Corporation (Morristown, NJ) and Fisher Scientific (Pittsburgh, PA).

Bacterial dispersions in normal saline were prepared using the A-JUST Turbidimeter (Abbott Laboratories) along with McFarland Turbidity Standards (Remel Microbiology Products, Lenexa, KS). Cation-adjusted Mueller Hinton Broth was obtained from Becton, Dickinson and Company (Sparks, MD). *Pseudomonas aeruginosa* ATCC® 27853 and a tetracycline-resistant methicillin-resistant *Staphylococcus aureus* isolate were provided by UF Health Microbiology Laboratory (Gainesville, FL), and clinical isolates *Escherichia coli* ARC3600 (NDM-1, CMY-6, OXA-1) and *Klebsiella pneumoniae* ARC3802 (NDM-1, SHV-2a, SHV-11, CTX-M-15, TEM-1) were provided by JMI Laboratories (North Liberty, IA).

2.2 Minimum Inhibitory Concentration (MIC) Determination

MICs were determined by serial macrodilution method (total volume = 1 mL) in culture media (prepared fresh weekly and stored at 4°C). Various chemical components in the following experiments were incubated with the bacteria (1.5×10^6 CFU/mL) of interest. The 24-well plates were incubated at 37°C for 16–24 h prior to visual MIC determination where the lowest concentration with no visible growth was the MIC. Negative controls without bacteria were included to confirm absence of bacterial contamination in broth and stock solutions. Positive controls with bacteria were also included with and without inactive components. Experiments were performed in duplicate.

2.2.1 Effect of Metal Ion Chelation on *in Vitro* MICs of TIG and TET—The impacts of metal ion chelation on *in vitro* activity was determined by the addition of Mg^{2+} or Ca^{2+} and/or EDTA to MIC assays for TIG and TET against various bacteria. TIG MIC assays were performed for *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. MICs were determined with and without divalent metal ions, 4.2 mM Mg^{2+} and 10.4 mM Ca^{2+} , and/or 1.5 mg/mL EDTA. *P. aeruginosa* and *E. coli* TIG MICs were also assessed in the presence of 0.65, 1.3, 2.6, and 5.2 mM Ca^{2+} . *P. aeruginosa* TIG MICs were performed in the presence of additional EDTA concentrations (0.094, 0.19, 0.38, and 0.75 mg/mL) and additional Ca^{2+} /EDTA combinations (0.65 and 2.6 mM Ca^{2+} , with 1.5 mg/mL EDTA). TET MICs assays were performed for *P. aeruginosa* with and without the additions of varying levels of Ca^{2+} (0.65, 1.3, 2.6, 5.2, and 10.4 mM) and/or EDTA (0.094, 0.19, 0.38, 0.75, 1.5 mg/mL). TMP was selected as a negative control drug to test against *P. aeruginosa*, as it is not subject to metal ion chelation. TMP MICs were determined in the presence and absence of 1.5 mg/mL EDTA and/or varying levels of Ca^{2+} (0.65, 1.3, 2.6, 5.2, and 10.4 mM).

2.2.2 TIG Interactions with Tetracyclines

2.2.2.1 OXY, CHL and TET: TIG MICs were determined in the presence of three other tetracyclines (CHL, OXY and TET) at concentrations below their determined MICs against *P. aeruginosa*, *E. coli* and *K. pneumoniae* were tested in combination with serial two-fold dilutions of TIG to determine if tetracyclines, which also act as chelators, exhibit similar effects to EDTA.

2.2.2.2 Impact of TET on TIG MICs: The relationship between TIG and TET against *P. aeruginosa*, *E. coli*, and MRSA (clinical TET-resistant isolate) were examined further. Varying levels of TET (4, 8, and 12 mg/L) below the MIC (16–32 mg/L) were added to TIG

MIC assays. When TET led to a decrease in TIG MIC, the effects of adding 10.4 mM Ca^{2+} in combination with TET to TIG MIC assays were also assessed.

3 Results

3.1 Effect of Calcium Chelation on *in Vitro* MICs of TIG and TET

The effects on TIG MIC for all three bacterial pathogens are summarized in Table 1. While increased MICs are observed for all bacteria when Ca^{2+} or Mg^{2+} are added, the increase is most drastic with Ca^{2+} addition. The addition of EDTA alone led to a decrease in MICs for all bacteria. While this effect could be reversed by the addition of divalent metal ions, the reversal was most potent when adding Ca^{2+} . The MICs with varying Ca^{2+} levels for *P. aeruginosa* and *E. coli* are described in Table 2, and results for MICs in combination with varying EDTA with or without Ca^{2+} for *P. aeruginosa* are shown in Table 3. Dose-related increases in MIC with increasing levels of Ca^{2+} added were observed for both *E. coli* and *P. aeruginosa*. Dose-related decreases in MIC with increasing levels of EDTA added were observed for *P. aeruginosa*. A dose-dependent MIC increase, reversing the effects of EDTA by adding increasing levels of Ca^{2+} , was also observed for *P. aeruginosa*.

Results for TET MIC experiments are displayed in Table 3. TET exhibits a similar dose-dependent increase in MIC with increasing Ca^{2+} concentrations and decrease in MIC with increasing EDTA concentrations. The MIC lowering effect of EDTA can also be reversed with the addition of Ca^{2+} .

Results of TMP MICs are shown in Table 3. The addition of EDTA led to a drop in the MIC, while the addition of increasing levels of Ca^{2+} reversed the effects of EDTA, similar to those trends seen for TIG and TET.

3.2 Interactions with Tetracyclines

3.2.1 Impact of OXY, CHL, and TET on TIG MICs—MIC were first determined for OXY, CHL and TET against *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. For *P. aeruginosa* MICs for OXY, CHL, and TET were determined to be 8, 16, and 16–32 mg/L, respectively. For *K. pneumoniae* MICs were determined to be 2–4, 4, and 2–4 mg/L for OXY, CHL, and TET, respectively, while for *E. coli*, MICs for all three tetracyclines were greater than 32 mg/L. When TIG MIC was examined in combination with concentrations of each tetracycline below its MIC, no significant changes in MICs were observed for *K. pneumoniae* or *E. coli*, with the exception of a drop in the MIC from 1–2 (TIG alone) to 0.25–0.5 mg/L when TET 1 mg/L or CHL 2 mg/L was added against *K. pneumoniae*. Conversely decreases in the TIG MIC were observed for all added tetracyclines against *P. aeruginosa* with decreases from 8–16 mg/L (TIG alone) to 2–4, 4, and 2–8 mg/L in the presence of 4 mg/L OXY, 8 mg/L CHL, and 8 mg/L TET, respectively.

3.2.2 Impact of TET on TIG MICs—Table 4 shows the resulting changes in TIG MICs in the presence of TET with or without Ca^{2+} . There was a TET dose-related decrease in TIG MIC for *P. aeruginosa* but not for *E. coli* or MRSA. The addition of Ca^{2+} to the TIG MICs in the presence of TET led to an increase in the TIG MIC, reversing the effect of TET.

4 Discussion

These results show how a metal ion chelator, such as EDTA, leads to a decrease in TIG MICs, which can be reversed by the addition of divalent metal ions, with Ca^{2+} having a particularly potent effect for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. For *E. coli* and *P. aeruginosa* the dose-dependent effects of calcium on TIG MIC were demonstrated. The EDTA-dose dependent decrease in TIG MIC and Ca^{2+} -dependent reversal of this decrease were characterized for *P. aeruginosa*. While there are no physiological levels of EDTA, total serum calcium is typically 2.2–2.6 mM, which was covered by the range of calcium used (only 0.5–0.62 mM Ca^{2+} is present in the media). Similar effects were observed for a similar set of experiments for TET against *P. aeruginosa*, another calcium chelator.

Since EDTA is not a chelator that can be administered clinically, other tetracyclines, also with metal ion chelating properties, were tested, including TET, OXY and CHL. Interestingly, significant decreases in the MIC of TIG in the presence of other tetracyclines were mainly observed for *P. aeruginosa*. The effects of TET with TIG were investigated further. An effect was observed for *P. aeruginosa* but not for *E. coli* or MRSA. Dose-dependent effects of TET on TIG *P. aeruginosa* MIC were evaluated at concentrations below the TET MIC (16–32 mg/L) and the reversal of these effects were demonstrated by adding Ca^{2+} . These results exhibit the same trends as that seen with EDTA.

One hypothesis is that TIG and TET may compete for calcium, which may grant enhanced effects in combination. As to why these effects of TET may not be observed for other bacteria tested, there are many possibilities, some of which are discussed:

- 1) One possibility is that TIG may be approaching its maximum effect without the addition of a calcium chelator. As TIG already has activity against *E. coli* and *K. pneumoniae*, the window for which an increase in microbiological activity could occur may be smaller than for *P. aeruginosa*.
- 2) The other bacteria tested may not be as dependent on calcium for bacterial proliferation. In this case, the addition of a chelator may not have a profound effect, except with a more complete calcium chelation, like the one provided by the addition of EDTA.
- 3) The effects on MIC caused by a chelating agent may not be attributable only to the competitive chelation of TIG. For example, competition for efflux pumps (both TIG and TET are subject to efflux pumps[4]), and/or the ribosomal target site may exist.

The second point was partially confirmed by the series of TMP experiments, where a drug with some activity against *P. aeruginosa*, not known to be subject to chelation, was selected. Similar trends observed for TMP, TIG, and TET when calcium was added and removed supports that a pharmacodynamic effect independent of a drug being subject to calcium chelation exists. These effects were more drastic for TIG as compared to TMP, which may be attributed to a greater maximum effect of TIG, or a concurrent interaction (i.e. a pharmacokinetic interaction) not present with TMP. Conversely, it is possible that the relative shift in MIC may be less for TMP as its starting MIC is much higher.

Metals such as Ca^{2+} and Mg^{2+} are important in biological processes. The impacts of EDTA on bacterial inhibition and enhancing antimicrobial activity have been previously observed[5–7]. The effects on Ca^{2+} and other cations on biofilm formation and attachment for *Pseudomonas spp.* have been extensively studied[8–10]. Mg^{2+} binding by extracellular DNA and Ca^{2+} binding alginate (a biofilm component) have been associated with induced resistance genes in *P. aeruginosa*[11,12]. Interestingly for *P. aeruginosa* Ca^{2+} seems to have a more substantial impact on binding to alginate than Mg^{2+} [13]. Perhaps the planktonic effects of chelation by EDTA also occur in the case of TET and other Ca^{2+} chelators.

These results merit further investigations into the impact of metal ion chelators on antibacterial activity specifically as it pertains to TIG and *P. aeruginosa*. The combination of TIG with other tetracyclines is unique and surprising in that drug combinations often consist of two drugs of different mechanisms and of different therapeutic classes. Full characterization of the TIG-TET interaction linked with clinical pharmacokinetic models could provide valuable insights into the design of physiologically viable chelators as candidates for future combination regimens and underlying mechanisms could be exploited to develop novel antibacterial agents.

5 Conclusion

This work has shown the potential for enhanced *in vitro* activity of TIG in the presence of metal ion chelation. This enhancement was reversible by the addition of metal ions, in particular Ca^{2+} for which the dose-dependent reversal was observed. A similar phenomenon was also observed for TMP for *P. aeruginosa*, supporting a separate pharmacodynamic mechanism. Using other tetracyclines in place of EDTA, TIG's activity was increased primarily against *P. aeruginosa*. These experiments support further exploration of the underlying mechanisms and the use of chelators, such as tetracyclines, in combination with antimicrobials to improve the efficacy.

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Table 1

Tigecycline MIC results with and without divalent metal ions and/or EDTA.

Added Component	TIG MIC (mg/L)		
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>K. pneumoniae</i>
None	8–16	0.5	0.25–0.5
4.2 mM Mg ²⁺	>32	2–4	1–2
10.4 mM Ca ²⁺	>64	>32	>32
1.5 mg/mL EDTA	0.0313–0.0625	<0.015	0.125
1.5 mg/mL EDTA + 4.2 mM Mg ²⁺	0.5	2–4	1–2
1.5 mg/mL EDTA + 10.4 mM Ca ²⁺	>=64	4	4–8

Table 2TIG MIC results for varying levels of Ca²⁺.

mM Ca ²⁺ Added	TIG MIC (mg/L)	
	<i>P. aeruginosa</i>	<i>E. coli</i>
0.65	16	0.5
1.3	16–32	2
2.6	≥64	2
5.2	>64	>32

Table 3

TIG, TET, and TMP MIC results for varying levels of EDTA with or without Ca²⁺.

mg/mL EDTA Added	mM Ca ²⁺ Added	TIG MIC (mg/L)	TET MIC (mg/L)	TMP MIC (mg/L)
		<i>P. aeruginosa</i>		
0	0	8–16	16–32	1024
0	0.65	16	32	ND
0	1.3	16–32	32	ND
0	2.6	>=64	32–64	ND
0	5.2	>64	>128	ND
0	10.4	>64	>128	1024–2048
0.094	0	4–8	8–16	ND
0.19	0	2–4	4	ND
0.38	0	0.125–0.25	0.5	ND
0.75	0	0.0625–0.125	0.5	ND
1.5	0	0.0313–0.0625	0.25	64–128
1.5	0.65	<0.0313–0.0625	0.25–0.5	64–128
1.5	1.3	ND	ND	128–256
1.5	2.6	0.125	0.5	256
1.5	5.2	ND	ND	1024
1.5	10.4	>=64	32–>128	>=2048

ND: not done

Table 4

TIG MIC results for varying sub-MIC levels of TET with or without Ca²⁺.

mg/L TET Added	mM Ca ²⁺ Added	TIG MIC (mg/L)		
		<i>P. aeruginosa</i>	<i>E. coli</i>	MRSA
0	0	8–16	0.5	0.5
4	0	4	0.5	ND
8	0	2–8	0.5	ND
12	0	0.5–2	0.5	1
4	10.4	>32	ND	ND
8	10.4	>32	ND	ND
12	10.4	32	ND	ND

ND: not done