CALCIUM-DEPENDENT POTENTIATION OF THE PRO-INFLAMMATORY FUNCTIONS OF HUMAN NEUTROPHILS BY TIGECYCLINE *IN VITRO*.

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Running title: Effects of tigecycline on neutrophils.

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Objectives: Tigecycline is the prototype of the recently introduced, intravenously administered glycylcycline class of antibiotics, developed in response to the increasing problem of antibiotic resistance in Gram-positive bacteria, especially *Staphylococcus aureus,* as well as Gram-negative bacteria and anaerobes. However, relatively little is known about the immunomodulatory potential of tigecycline, specifically its interactions with human neutrophils. In the current study, we have investigated the effects of tigecycline at therapeutically-relevant concentrations and above (0.625 - 10 mg/L) on alterations in cytosolic Ca^{2+} concentrations, generation of antimicrobial reactive oxygen species (ROS) and release of granule proteases (elastase and the matrix metalloproteinases – MMPs-8 and -9) by human blood neutrophils activated with the chemoattractant, fMLP (1 μM).
Methods: Cytosolic Ca^{2+} concentrations were measured using fura-2/AM-based spectrofluorimetry and radiometric procedures, generation of ROS by oxygen consumption and myeloperoxidase-mediated auto-iodination, and protease release by ELISA procedures.
Results: Exposure of the cells to fMLP resulted in activation of the generation of ROS, as well as release of the granule proteases, all of which were significantly increased by preincubation of the cells with tigecycline in a dose-dependent manner. Tigecycline-mediated enhancement of these neutrophil functions was associated with elevations in the concentrations of cytosolic Ca^{2+}, which appeared to result from the Ca^{2+} ionophore activity of tigecycline.
Conclusion: Tigecycline, by functioning as a Ca^{2+}-ionophore, and independently of antimicrobial activity, potentiates the pro-inflammatory functions of human neutrophils *in vitro*.

Key words: Calcium ionophore; doxycycline; innate immunity; minocycline; oxidant-scavenger; tetracycline.

Introduction

Tigecycline is the prototype of the recently introduced, intravenously administered glycylcycline class of antibiotics, developed in response to the increasing problem of antibiotic resistance in Gram-positive bacteria, especially *Staphylococcus aureus*, as well as Gram-negative bacteria and anaerobes.[1-3] Tigecycline is similar to tetracyclines with respect to both molecular structure and mechanism of antimicrobial action, but possesses a broader spectrum of activity due to a minor structural modification, specifically a glycylcycline substitution at the C-9 position of the naphthalene ring.[1-3] Although it is concentrated by human neutrophils *in vitro*,[4] little is known about the effects of tigecycline on the pro-inflammatory/anti-inflammatory activities of these cells. Given their critical involvement in the eradication of extracellular pathogens, especially *Staphylococcus aureus*,[5,6] cooperative interactions between neutrophils and tigecycline are likely to be a determinant of the successful outcome of antimicrobial chemotherapy.[7] Alternatively, enhancement of the pro-inflammatory activities of these cells may exacerbate inflammation-mediated tissue damage and organ dysfunction through increased release of indiscriminate reactive oxygen species (ROS) and granule proteases.[7]

In the current study, we have investigated the effects of tigecycline on alterations in cytosolic Ca^{2+} concentrations, generation of antimicrobial ROS and release of granule proteases, following activation of neutrophils with the chemoattractant, fMLP. The generation of ROS was determined according to the magnitude of oxygen consumption by activated neutrophils, a measure of activity of the membrane-associated, electron-transporting, superoxide-generating enzyme-complex, NADPH oxidase. This was complemented by measurement of the activity of the myeloperoxidase (MPO) / hydrogen peroxide (H_2O_2) / halide system. This is a composite assay of the production of superoxide and H_2O_2, as well as the release of MPO from primary granules. MPO and H_2O_2 interact to form hypochlorous acid, an extremely potent antimicrobial ROS. Measurement of the primary granule protease, elastase, and the secondary / tertiary granule proteases, matrix metalloproteinases (MMPs)-8 and -9, was included because of their involvement in promoting neutrophil migration and killing of microbial pathogens, while increases in

cytosolic Ca^{2+} precede and are a prerequisite for activation of both generation of ROS and release of granule enzymes.[7]

The underlying hypothesis is that tigecycline modulates the pro-inflammatory activities of human neutrophils, possibly by altering cytosolic Ca^{2+} concentrations in these cells, due to the calcium ionophore activity of this class of antimicrobial agent.[8]

Materials and Methods

Antibiotics

Tigecycline was provided by Wyeth Pharmaceuticals (Wyeth, NJ, USA), while doxycycline hydrate, minocycline hydrochloride and tetracycline hydrochloride were purchased from the Sigma Chemical Co (St Louis, Mo, USA). All 4 agents were dissolved in distilled H_2O at a stock concentration of 5 g/L and diluted thereafter in indicator-free Hanks' Balanced Salt Solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$, Highveld Biological (Pty) Ltd, Johannesburg, South Africa). In most of the assays described below tigecycline was used at final concentrations of 0.625 – 10 mg/L, the exception being in assays of influx of extracellular Ca^{2+} at which concentrations of up to 40 mg/L were used. Following intravenous infusion of 100 mg tigecycline, peak serum levels of up to 27 mg/L have been documented immediately following infusion, with a mean value of 1.94 mg/L,[9] while a mean value of about 6 mg/L has been reported following a single infusion of 400 mg of the antibiotic to patients with multidrug-resistant *Klebsiella pneumoniae* or *Acinetobacter baumannii* urosepsis.[10] Leucocyte and tissue (colon, gallbladder, lung) concentrations are considerably higher than those of serum.[4, 9-11]

Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co.

Neutrophils

Permission to draw blood from healthy, adult human volunteers was granted by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria and informed consent was obtained from all participants. Purified human neutrophils were prepared from heparinised blood (5 units of preservative-free heparin/mL of blood). Neutrophils were separated from

mononuclear leucocytes by centrifugation on Histopaque®-1077 cushions at 400*g* for 25 min at room temperature. The resultant erythrocyte/neutrophil layer was sedimented with 3% gelatine for 15 min at 37°C to remove most of the erythrocytes. Following centrifugation (280*g* at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity and viability (>90%), were resuspended to $1x10^7$ cells/mL in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and held on ice until used.

Neutrophil function assays

Where possible, assays were selected which were not prone to interference by the yellow colour of tigecycline.

Myeloperoxidase (MPO)-mediated iodination

Neutrophils ($1x10^7$) were pre-incubated without or with tigecycline (0.625 - 10 mg/L) for 10 min at 37°C in HBSS containing 1 µCi of iodine-125 (as $Na^{125}I$, 37 MBq, PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and 20 nmol/mL cold carrier NaI. Following pre-incubation, the cells were activated by addition of the synthetic chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 1 µM final) in combination with cytochalasin B (CB, 0.5 mg/L). The tubes, containing a final volume of 5 mL, were then incubated for a further period of 5 min at 37°C after which the reactions were terminated by the addition of an equal volume of ice-cold PBS and the tubes transferred to an ice-bath. The neutrophils were pelleted by centrifugation, washed 3 times with PBS, and the levels of radioactivity in the pellets measured using a PerkinElmer Wallac Wizard 2470 automated gamma counter. The results are expressed as nmol ^{125}I / 10^7 neutrophils.

In an additional series of experiments, the effects of doxcycline, minocycline, and tetracycline at concentrations of 5 and 10 mg/L on fMLP/CB-activated neutrophil auto-iodination were investigated.

Oxidant scavenging by tigecycline

A cell-free ROS-generating system based on MPO-mediated iodination of bovine serum albumin (BSA) was used to measure the ROS-scavenging potential of tigecycline. Briefly, reaction systems contained purified MPO (100 milliunits/mL) isolated from human neutrophils, glucose oxidase (1.55 units/mL, from bovine liver), HBSS containing 5 mM glucose, 20 nmol/mL cold carrier NaI, 2 µCi ^{125}I, 2 mg BSA, without and with tigecycline, at a fixed final concentration of 10 mg/L in a final volume of 1 mL. Reactions were initiated by the addition of glucose oxidase to generate hydrogen peroxide (H_2O_2) and the tubes incubated for 15 min at 37°C. The reactions were stopped and the BSA precipitated by the addition of 3 mL 20% trichloroacetic acid (TCA). The protein precipitates were washed 3 times with TCA followed by measurement of the extent of iodination of BSA. The results are expressed as nmol ^{125}I / 2 mg BSA.

Oxygen consumption

This was measured using a thermo-regulated 3-channel oxygen electrode (Model DW1, Hansatech Ltd, King's Lynn, Norfolk, UK). Neutrophils ($2x10^6$) were pre-incubated for 10 min at 37°C in HBSS without or with tigecycline, at a fixed concentration of 10 mg/L in most experiments and at 5 mg/L in a limited series, after which the cells were activated by the addition of fMLP/CB and the PO_2 of the cell-suspending medium monitored for 5 min. The results are expressed as nmol O_2 consumed over the first minute following addition of fMLP/CB when the reaction was linear.

In an additional series of experiments, the effects of the Ca^{2+}-chelating agent, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA, 10 mM) on tigecycline (10 mg/L)-mediated modulation of fMLP/CB-activated O_2 consumption by neutrophils were investigated. EGTA was added to the cells 1 min prior to fMLP/CB.

Release of granule proteases

The granule proteases elastase (primary granules) and matrix metalloproteinases-8 and -9 (MMP-8 and -9, secondary and secondary/tertiary granules respectively) were measured following activation

of neutrophils with either fMLP/CB (elastase) or fMLP only (MMPs-8 and-9). Neutrophils ($2x10^6$) were pre-incubated without or with tigecycline (0.625 - 10 mg/L) at 37°C followed by addition of the stimulant. After a further period of incubation for 15 min at 37°C, the reactions were stopped by the addition of an equal volume of ice-cold HBSS and the tubes placed on ice. The cells were pelleted by centrifugation and the supernatants assayed using ELISA procedures for elastase (Hycult Biotechnology, Uden, the Netherlands), MMP-8, and MMP-9 (Quantikine®, R&D Systems, Minneapolis, MN, USA) and the results expressed as ng/mL supernatant. The ranges for the elastase, MMP-8 and MMP-9 assays were 0.162 – 2.809, 0.156 – 10, and 0.312 – 10 ng/ml respectively.

Spectrofluorimetric measurement of cytosolic Ca^{2+}

Fura-2/AM was used as the fluorescent Ca^{2+}-sensitive indicator for these experiments.[12] Neutrophils ($1x10^7$/mL) were incubated with fura-2/AM for 30 min at 37°C, then pelleted by centrifugation and resuspended in HBSS. The fura-2-loaded cells were then pre-incubated for 5 min at 37°C without or with tigecycline at a fixed, final concentration of 2.5 mg/L (higher concentrations of tigecycline could not be used due to interference with the assay system). The cells were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable baseline was obtained (1 min), the cells were activated by the addition of fMLP (1 μM) and alterations in fluorescence intensity monitored over a 5 - 10 min period.

Radiometric assessment of Ca^{2+} influx

This procedure was used to measure the net influx of $^{45}Ca^{2+}$ into fMLP (1 μM)-activated neutrophils uncomplicated by either concomitant efflux of the cation or the colour of tigecycline. The cells were pre-incubated for 10 min at 37°C in Ca^{2+}-replete (1.25 mM) HBSS to ensure that intracellular Ca^{2+} stores were full in order to minimise spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to activation with fMLP) in the influx assay. The cells were then pelleted by centrifugation and resuspended to a concentration of $1x10^7$/mL in HBSS containing 12.5 μM cold

carrier $CaCl_2$. The Ca^{2+}-loaded neutrophils ($2x10^6$/mL) were then incubated for 10 min at 37°C in the absence or presence of tigecycline (5 and 10 mg/L) followed by simultaneous addition of fMLP (1 μM) and 2 mCi/L $^{45}Ca^{2+}$ (as $^{45}[Ca]Cl_2$, 185 MBq, PerkinElmer Life and Analytical Sciences), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The cells (10^7) in a final volume of 5 mL were then incubated for 5 min at 37°C after which chemoattractant-activated, store-operated uptake of Ca^{2+} is complete,[13] and the reactions stopped by the addition of 10 mL of ice-cold Ca^{2+}-replete HBSS to the tubes, which were transferred immediately to an ice bath. The cells were then pelleted by centrifugation, washed with 15 mL of ice-cold Ca^{2+}-replete HBSS and the cell pellets dissolved in 0.5 mL 0.1% Triton X-100 / 0.1 M NaOH and the radioactivity measured in a liquid scintillation spectrometer. The results are presented as the amount of cell-associated radioactivity (pmol $^{45}Ca^{2+}/10^7$ cells).

To measure the effects of tigecycline, (at a fixed, final concentration of 10 mg/L), on the efflux of Ca^{2+}, neutrophils (10^7/mL) were pre-incubated for 20 min at 37°C in HBSS containing $^{45}Ca^{2+}$ (5 mCi/L) as the only source of the cation to enable loading of neutrophil intracellular stores with $^{45}Ca^{2+}$. Following this loading step, the cells were pelleted by centrifugation, washed once with, and resuspended in Ca^{2+}-replete (1.25 mM) HBSS. The $^{45}Ca^{2+}$-loaded neutrophils ($2x10^6$/mL) in Ca^{2+}-replete HBSS were then pre-incubated for 10 min without and with tigecycline followed by addition of fMLP (1 μM) or an equal volume of HBSS to unstimulated systems and incubated for 1 min at 37°C after which efflux is complete.[13] Reactions were terminated and the cells processed as above and the amount of residual cell-associated $^{45}Ca^{2+}$ determined.

In an additional series of experiments, the effects of the following on the spontaneous uptake of $^{45}Ca^{2+}$ (*ie* in the absence of fMLP) were also investigated: i) exposure to tigecycline at concentrations of 2.5 - 40 mg/L; and ii) exposure to doxycycline, minocycline, tetracycline, or tigecycline at a fixed concentration of 40 mg/L.

Cell viability

Neutrophils ($2x10^6$/mL) were treated with doxycycline, minocycline, tetracycline, or tigecycline, at a fixed, final concentration of 40 mg/L for 20 min at 37°C, followed by a 10 min exposure to 45 mg/L propidium iodide (PI) at room temperature, and flow cytometric detection of uptake of PI as a marker of cell membrane damage.

Statistical analysis

With the exception of the fura-2 fluorescence experiments, some of which are presented as representative traces, the results of each series of experiments are presented as the mean values ± standard deviation (SD), with the number of replicates for each drug concentration and drug-free control system for each experiment, and the number of different donors used, shown in the figure legends. Levels of statistical significance were determined by comparing the absolute values for each drug-treated system with the corresponding values for the relevant drug-free control systems for each assay using the Wilcoxon matched pairs signed ranks test. Intra-day and inter-day coefficients of variance for the major assays are shown in the figure legends.

Results

MPO-mediated iodination

The effects of tigecycline on MPO-mediated neutrophil auto-iodination are shown in Figure 1A. Tigecycline caused dose-related stimulation of the generation of ROS by activated neutrophils, which reached a plateau at 5 - 10 mg/L and achieved statistical significance ($P<0.05$) at concentrations of 0.6 mg/L and higher. The effects of doxycycline, minocycline, and tetracycline at concentrations of 5 and 10 mg/L are shown in Figure 1B. The other tetracyclines, like tigecycline, also significantly increased the generation of ROS by fMLP/CB – activated neutrophils.

Oxidant scavenging

In the absence and presence of tigecycline (10 mg/L), the magnitude of iodination of BSA by the MPO / glucose oxidase / ^{125}I system was 0.546 ±

0.146 nmol and 0.176 ± 0.039 nmol ^{125}I / 2 mg BSA respectively (P< 0.05), while the corresponding value for the MPO / glucose oxidase-free control system was 0.064 ± 0.016 nmol ^{125}I / 2 mg BSA. These observations demonstrate that tigecycline scavenges ROS generated by the MPO / H_2O_2 / ^{125}I system.

Oxygen consumption

The effects of tigecycline (10 mg/L) on oxygen consumption by fMLP/CB – activated neutrophils are shown in Figure 2, which are the traces from a typical experiment. The magnitude of O_2 consumption by activated neutrophils was increased in the presence of tigecycline. The data for 19 different experiments using cells from 5 different donors for the control and tigecycline (10 mg/L)-treated systems were 36.7 ± 9.9 and 42.5 ± 11.5 nmol O_2 consumed / 1 min respectively (P<0.05).

Inclusion of EGTA significantly attenuated the stimulatory effects of tigecycline (10 mg/L) on O_2 consumption by neutrophils, the values for the control system and tigecycline-treated systems being 34.6 ± 4.8 and 40.7 ± 6.4 nmol O_2 consumed / 1 min, while the corresponding values in the presence of EGTA were 32.7 ± 4.4 and 31.4 ± 5.8 nmol O_2 consumed / 1 min respectively (P<0.05 for comparison of the tigecycline-treated systems without and with EGTA, data for 12 different experiments using cells from 5 different donors).

Granule proteases

The effects of tigecycline on the release of the granule proteases elastase, MMP-8, and MMP-9 are shown in Figure 3. Treatment of the cells with the antibiotic resulted in dose-related enhancement of release of all 3 proteases, especially MMP-9, achieving statistical significance (P<0.05) at concentrations of 0.625 and 5 mg/L for the MMPs and elastase respectively.

Fura–2/AM fluorescence

The results shown in Figure 4 are typical traces from 2 different experiments using cells from 2 different donors (7 in the series) showing alterations in cytosolic Ca^{2+} (fura-2 fluorescence) in neutrophils activated with fMLP in the absence and presence of tigecycline (2.5 mg/L). Addition of fMLP to

neutrophils was accompanied by the abrupt increase in fluorescence intensity due to phospholipase C / inositol triphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores, which was unaffected by tigecycline. This was followed by a rapid decrease in fluorescence intensity due to efflux and resequestration of Ca^{2+}, which levelled off at about 2 min after the addition of fMLP coincident with store-operated Ca^{2+} influx.[13] In the presence of tigecycline, the rate of decline in fluorescence intensity was slower, compatible with higher post-peak cytosolic Ca^{2+} concentrations.

$^{45}Ca^{2+}$ efflux and influx

Activation of neutrophils with fMLP resulted in efflux of about 60% of neutrophil-associated $^{45}Ca^{2+}$, which was unaffected by tigecycline (not shown). With respect to fMLP-activated, store-operated influx of $^{45}Ca^{2+}$, the magnitudes of uptake of the cation measured 5 min after the addition of the chemoattractant were 33 ± 11.2, 43 ± 12.7, and 43 ± 13.7 pmol $^{45}Ca^{2+}$ for the control system and for systems treated with 5 and 10 mg/L tigecycline respectively, relative to a value of 5.2 ± 4.5 pmol $^{45}Ca^{2+}$ for the unstimulated (no fMLP) control system (P<0.05 for comparison of the tigecycline-treated system with the fMLP-activated control system).

The effects of tigecycline (2.5 – 40 mg/L) on the spontaneous (no fMLP) uptake of $^{45}Ca^{2+}$ by neutrophils, as well as the comparative effects of this agent and doxycycline, minocycline, and tetracycline at fixed concentrations of 40 mg/L, are shown in Figure 5. Treatment of neutrophils with tigecycline resulted in a dose-related increase in the net influx of $^{45}Ca^{2+}$, which was also evident with doxycycline and tetracycline, with minocycline being significantly (P<0.05) more potent than the other agents.

Viability

Exposure of the neutrophils to doxycycline, minocycline, tetracycline or tigecycline at a final concentration of 40 mg/L for 20 min at 37°C had no effects on cell viability according to propidium iodide exclusion, the respective values being 96.1 ± 0.1, 96.0 ± 0.3, 96.0 ± 0.3 and 97.0 ± 0.2% viability relative to a control value of 97.0 ± 0.1% (data from 4 determinations).

Discussion

In the current study, exposure of neutrophils to tigecycline prior to activation with fMLP/CB resulted in dose-related enhancement of the generation of ROS, according to increased activity of the MPO / H_2O_2 / halide system. These pro-oxidative interactions of tigecycline with neutrophils appeared to result from increased consumption of O_2 by fMLP/CB-activated cells, compatible with enhanced activity of NADPH oxidase and generation of superoxide and H_2O_2. The levelling-off of the tigecycline-mediated increase in activity of the MPO / H_2O_2 / halide system observed at concentrations of 5 – 10 mg/L of the antibiotic is probably attributable to the counteracting, hypohalous acid-scavenging properties of tigecycline as described in the current study, as well as in previous studies for other members of the tetracycline group of antibiotics,[14-16] demonstrating that these agents possess both pro-oxidative and anti-oxidative properties.

In the therapeutic setting, the pro-oxidative interactions of tigecycline with neutrophils, if predominant, may be either beneficial or harmful. In the case of the former, the increased generation of microbicidal ROS by tigecycline-treated neutrophils is likely to contribute to host defence against bacterial pathogens, already weakened by the direct antibiotic action of tigecycline.[17,18] On the other hand, however, increased production of indiscriminate ROS poses the potential hazard of oxidant-mediated damage to bystander host cells and tissues.[19]

Treatment of neutrophils with tigecycline also resulted in dose-related enhancement of release of proteolytic enzymes from primary (elastase), secondary (MMP-8 and MMP-9), and tertiary (MMP-9) granules, with release of MMP-9 being most affected by exposure of the cells to tigecycline. As is the case with the pro-oxidative properties of tigecycline, pro-proteolytic activity may be protective or harmful. Beneficial effects on host defences include the direct antimicrobial activity of elastase, targeting both Gram-negative and –positive bacterial pathogens,[20] while this protease, together with MMP-8 and MMP-9, facilitates the migration of neutrophils across vascular endothelium and extracellular matrices.[21-23] However, excessive release of these proteases has been implicated in the pathogenesis of inflammation-mediated

brain and lung injury in bacterial meningitis and pneumonia, including hospital-acquired pneumonia.[24-29]

From a mechanistic perspective, the pro-oxidative and pro-inflammatory interactions of tigecycline with neutrophils were associated with increases in cytosolic Ca^{2+}, a second messenger critically involved in activation of both NADPH oxidase and degranulation, particularly mobilisation of secondary and tertiary granules.[30,31] Using fura-2/AM-based spectrofluorimetry, activation of tigecycline-treated neutrophils with fMLP resulted in a sustained increase in post-peak cytosolic Ca^{2+} concentrations, with no detectable effects on either pre-activation basal levels of the cation, or on the abruptly-occurring peak response coincident with its release from intracellular stores. The apparent lack of effect on basal Ca^{2+} may simply reflect the fact that the maximum concentration of tigecycline which could be used in the spectrofluorimetric assay was 2.5 mg/L. The effects of tigecycline at higher concentrations on Ca^{2+} influx, uncomplicated by the yellow colour of the antibiotic, were investigated by radiometric detection of cell-associated cation. Using this procedure, tigecycline-mediated augmentation of Ca^{2+} uptake was observed not only with fMLP-activated neutrophils, but also with unstimulated cells, compatible with a mechanism related to the Ca^{2+} ionophore activity of tigecycline, as opposed to enhancement of store-operated influx of the cation.

This latter contention is supported by the following additional observations: i) three other tetracyclines also promoted uptake of Ca^{2+} by neutrophils, with doxycycline and tetracycline being equivalent to tigecycline, while minocycline, the most lipophilic, was 2-3 fold more potent than the other agents; ii) doxycycline, minocycline and tetracycline, like tigecycline, also caused increased activity of the MPO / H_2O_2 / halide system of fMLP/CB-activated neutrophils (the apparent lack of correlation with Ca^{2+} ionophore activity may reflect differences in the ROS-scavenging properties of these agents); iii) tigecycline-mediated enhancement of O_2 consumption by activated neutrophils was attenuated by inclusion of the Ca^{2+}-chelating agent, EGTA, in the cell-suspending medium; and iv) treatment of neutrophils with U-73122, an agent which inhibits phospholipase C and abolishes store-operated influx of Ca^{2+},[32] did not affect tigecycline-mediated uptake of the cation (data not shown).

The findings of the current study may seem somewhat surprising given that tetracyclines, particularly doxycycline and minocycline, have been reported to possess anti-inflammatory properties including inhibition of the expression and/or activity of MMPs,[16,33-38] inducible nitric oxide,[39-41] and NADPH oxidase.[40] However, tetracyclines also possess well-recognised pro-inflammatory/irritant properties, due at least in part to Ca^{2+} ionophore activity.[8] Pro-inflammatory activity contributes to tetracycline / doxycycline-mediated pleural fibrosis with obliteration of the pleural space (pleurodesis) and appears to result from activation of p38 MAP kinase and extracellular signal regulated kinases 1/2, both Ca^{2+}-dependent events, leading to synthesis of pro-inflammatory cytokines/chemokines by various cell types.[42-46] Furthermore, in a very recent study, exposure of retinal pigment epithelial cells to minocycline *in vitro* resulted in increased MMP-9 gene expression, underscoring the complexity of the effects of tetracyclines on the synthesis of MMPs.[45]

With respect to tigecycline, the clinical significance of pro-inflammatory activity if any, remains to be established. Nonetheless, the relatively high frequency of adverse events and increased mortality, especially in the setting of ventilator-associated pneumonia,[47-49] in comparison with other antimicrobial agents used in the treatment of severe infection caused by antibiotic-resistant pathogens, is noteworthy.

In conclusion, tigecycline, apparently as a consequence of its Ca^{2+} ionophore activity, has been found to augment the pro-inflammatory activities of isolated human neutrophils, which may, to some extent, be counteracted by the oxidant-scavenging properties of this antibiotic. Although the pro-inflammatory interactions of tigecycline with neutrophils may contribute to the eradication of microbial pathogens, they also pose the potential hazard of increased inflammation-mediated tissue damage in severe bacterial infection.

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Guy Richards has served on the speakers' bureau for Pfizer and has developed a guideline for the appropriate use of Tigecycline independently of the company.

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Legends to Figures

Figure 1: Effects of A) tigecycline (TG, 0.6 – 10 mg/L, and B) doxycycline (Doxy), tetracycline (Tetra) and minocycline (Mino) (5 and 10 mg/L, B) on the generation of ROS by the neutrophil myeloperoxidase / H_2O_2 / halide system measured according to the extent of neutrophil auto-iodination following activation of the cells with fMLP/CB (f/CB). The results of 6 or 5 experiments, with 3 - 4 replicates in each system, using cells from 6 or 5 different individuals in series A and B respectively, are presented as the mean values as nmol ^{125}I / 10^7 cells ± SD. The intra-day and inter-day variabilities of the fMLP/CB control system were 2.4% and 17.5 % respectively.
* $P < 0.05$

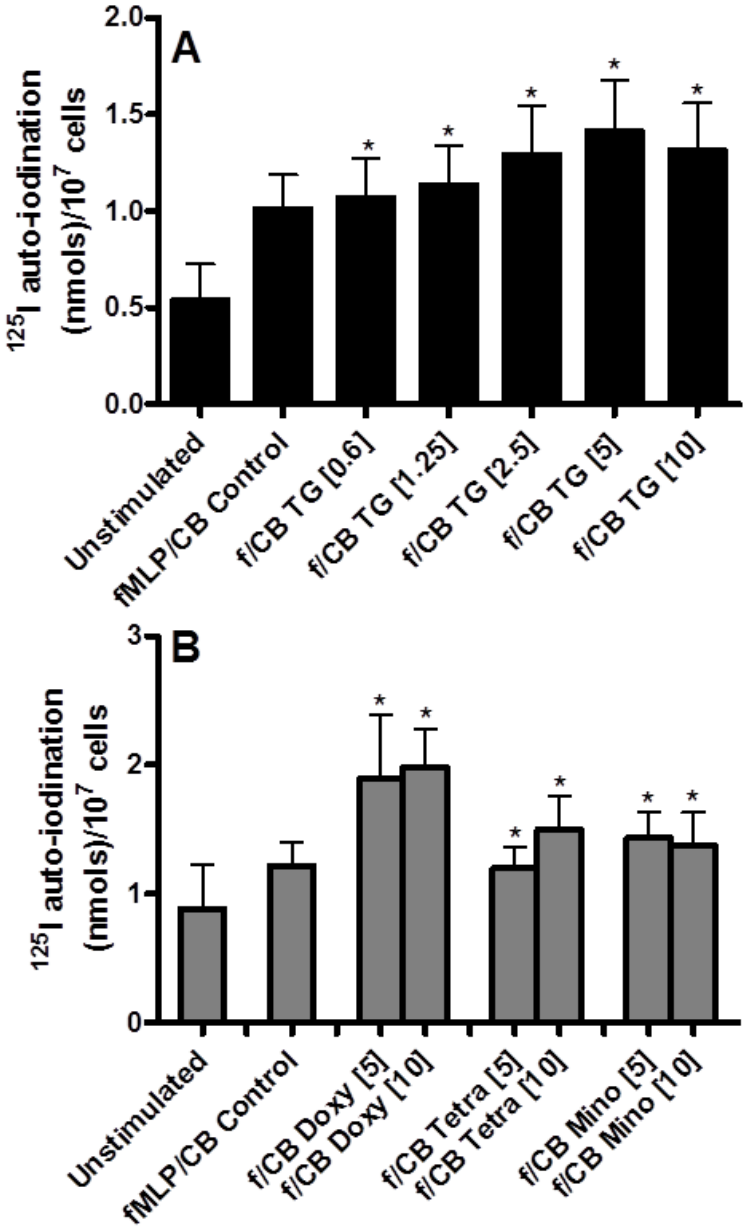
Figure 2: A typical trace using cells from a single donor (5 different experiments using cells from 5 different donors in the series) showing the effects of tigecycline (TG) at 5 and 10 mg/L on the magnitude of oxygen consumption by neutrophils activated with fMLP/CB. The intra-day and inter-day variabilities of the fMLP/CB control system were 8.4% and 35.0% respectively.

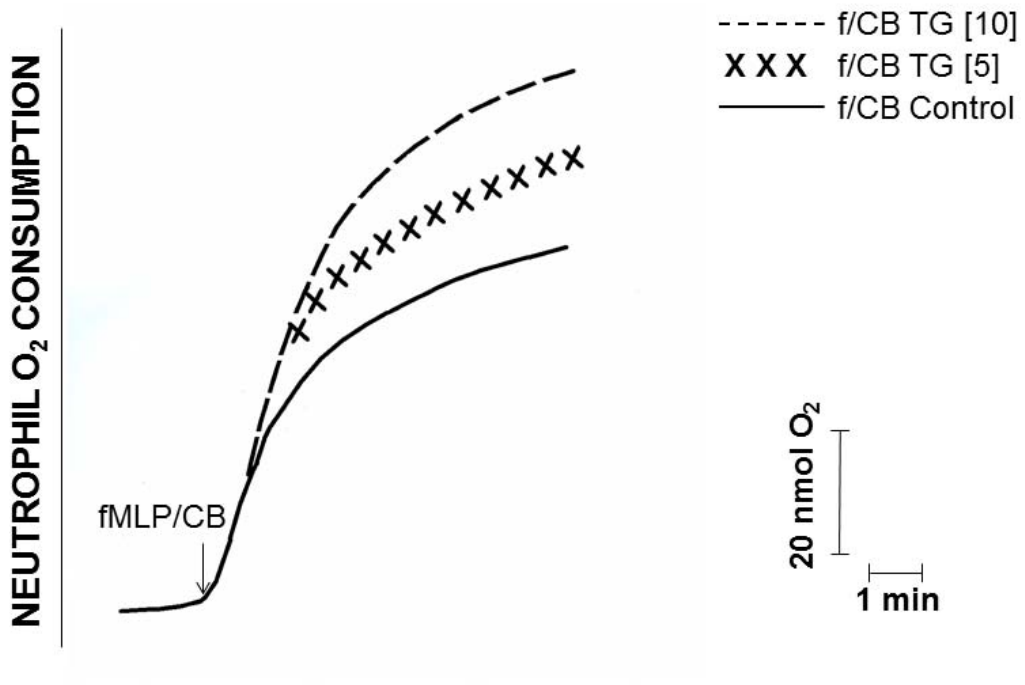
Figure 3: Effects of tigecycline (TG, 0.6 – 10 mg/L) on the release of elastase (A), matrix metalloproteinase-8 (MMP-8, B) and matrix metalloproteinase-9 (MMP-9, C) from neutrophils activated with fMLP/CB (elastase) or fMLP only (MMP-8 and MMP-9). The results of 12 - 13 experiments using cells from 6 - 7 different donors are expressed as the mean concentration (ng/mL) in the cell-free supernatants ± SD. The intra-day variations of the fMLP control system were 9.5%, 2.3% and 2.9%, while the inter-day variabilities were 47.9%, 24.6%, and 36.3% for elastase, MMP-8 and MMP-9 respectively.
*P<0.05

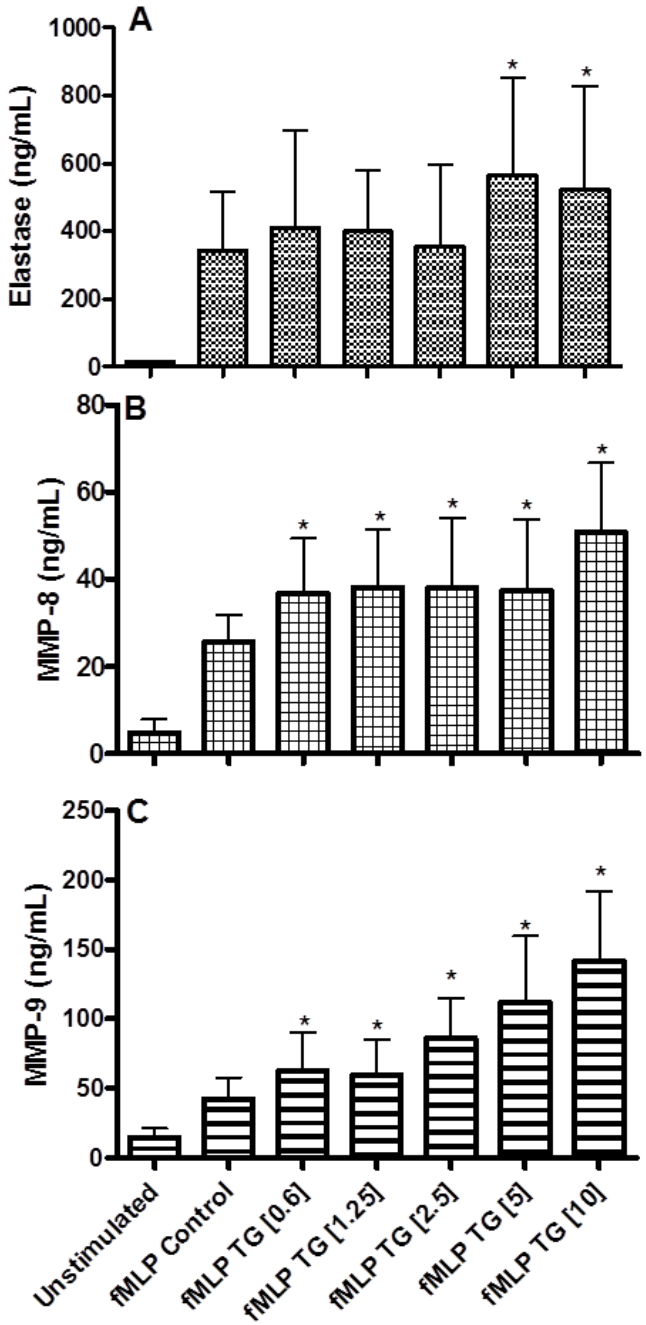
Figure 4: Typical traces from 2 experiments (7 in the series) using cells from 2 different donors showing the effects of tigecycline at a fixed, final concentration of 2.5 mg/L on alterations in neutrophil cytosolic Ca^{2+} concentrations following activation of the cells with fMLP added as indicated

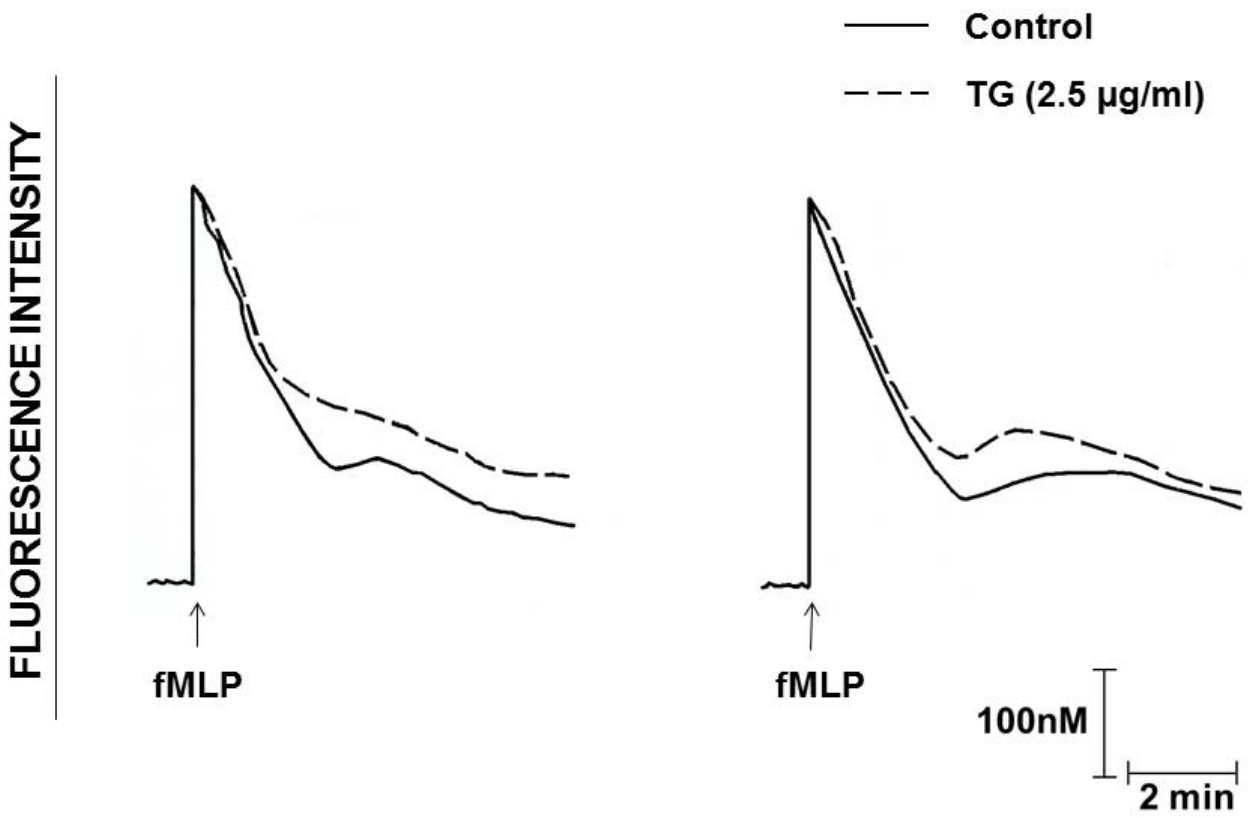
by the arrow after a stable baseline was obtained (1 min). The intra-day and inter-day variabilities for the fMLP control system were 4.1% and 14.0% respectively.

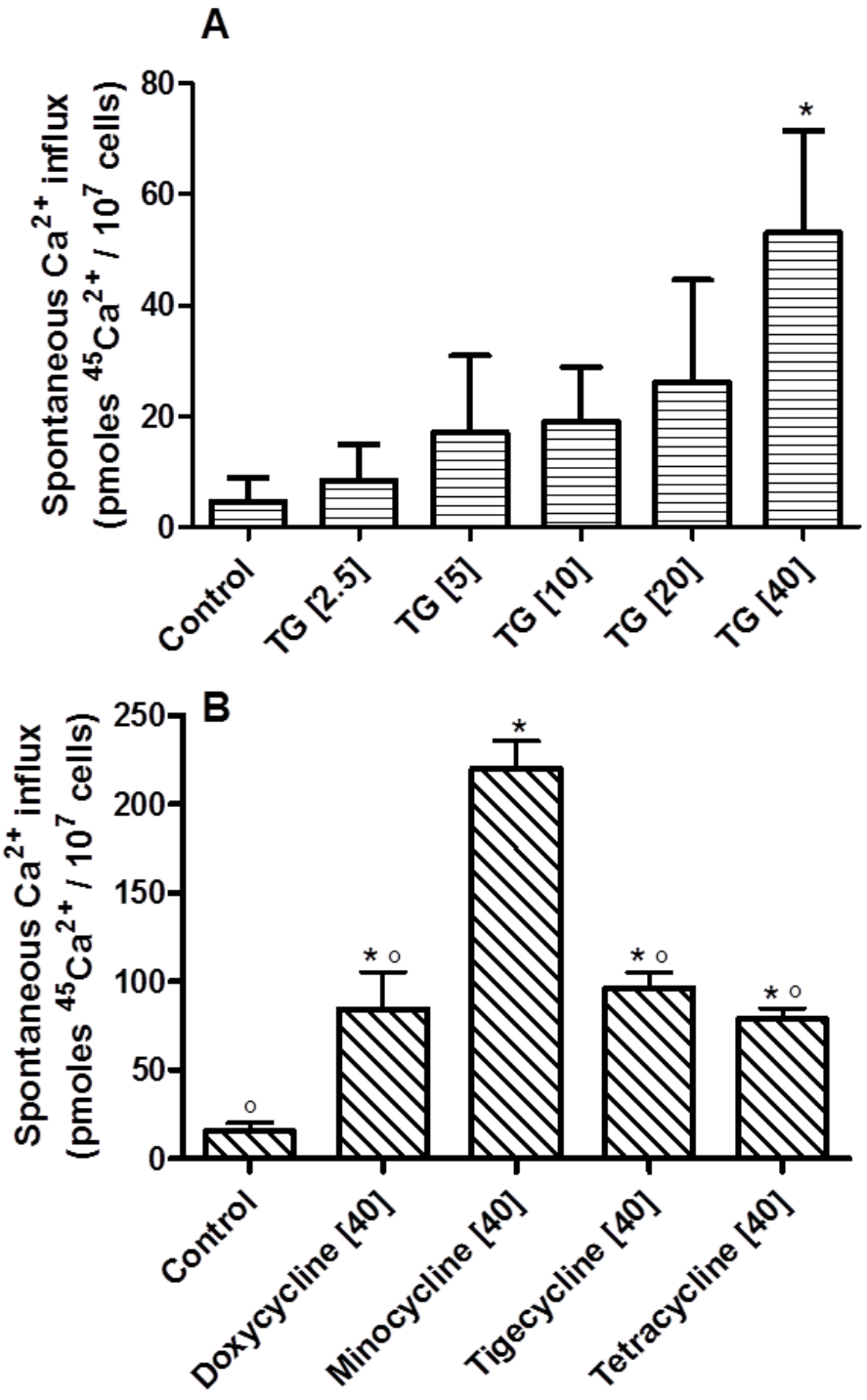
Figure 5: Effects of tigecycline (TG, 2.5 – 40 mg/L) on the spontaneous (no added stimulant such as fMLP) uptake of $^{45}Ca^{2+}$ by neutrophils following 10 min of exposure of the cells to the antibiotic (A, n=8) and assessment of the comparative effects of doxycycline, minocycline, tetracycline and tigecycline all at a fixed final concentration of 40 mg/L on the uptake of $^{45}Ca^{2+}$ by neutrophils (B, representative experiment, 4 in the series). The results of both series of experiments are expressed as the mean values (pmol $^{45}Ca^{2+}$/ 10^7 cells) ± SD. The intra-day and inter-day variabilities for the control system were 2.2% and 14.6% respectively.
*P<0.05 when compared to the drug-free control system, °P<0.05 when compared to minocycline 40 mg/mL.


A
^{125}I auto-iodination (nmols)/10^7 cells
2.0
1.5
1.0
0.5
0.0
*
Unstimulated
fMLP/CB Control
f/CB TG [0.6]
f/CB TG [1.25]
f/CB TG [2.5]
f/CB TG [5]
f/CB TG [10]
B
^{125}I auto-iodination (nmols)/10^7 cells
3
2
1
0
*
Unstimulated
fMLP/CB Control
f/CB Doxy [5]
f/CB Doxy [10]
f/CB Tetra [5]
f/CB Tetra [10]
f/CB Mino [5]
f/CB Mino [10]


NEUTROPHIL O_2 CONSUMPTION
fMLP/CB
f/CB TG [10]
X X X f/CB TG [5]
f/CB Control
20 nmol O_2
1 min


A
Elastase (ng/mL)
1000
800
600
400
200
0
B
MMP-8 (ng/mL)
80
60
40
20
0
C
MMP-9 (ng/mL)
250
200
150
100
50
0
Unstimulated
fMLP Control
fMLP TG [0.6]
fMLP TG [1.25]
fMLP TG [2.5]
fMLP TG [5]
fMLP TG [10]


Control
TG (2.5 µg/ml)
FLUORESCENCE INTENSITY
fMLP
fMLP
100nM
2 min


A
Spontaneous Ca^{2+} influx
(pmoles $^{45}Ca^{2+}$ / 10^7 cells)
80
60
40
20
0
*
Control
TG [2.5]
TG [5]
TG [10]
TG [20]
TG [40]
B
Spontaneous Ca^{2+} influx
(pmoles $^{45}Ca^{2+}$ / 10^7 cells)
250
200
150
100
50
0
*
* °
* °
* °
°
Control
Doxycycline [40]
Minocycline [40]
Tigecycline [40]
Tetracycline [40]