Calcium-dependent potentiation of the pro-inflammatory functions of human neutrophils by tigecycline in vitro

Riana Cockeran1*, Ndiafhi D. Mutepe1, Annette J. Theron1, Gregory R. Tintinger2, Helen C. Steel1, Paraskevi I. Stivaktas1, Guy A. Richards3, Charles Feldman3 and Ronald Anderson1

1Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Faculty of Health Sciences, University of Pretoria and Tshwane Academic Division of the National Health Laboratory Service, Pretoria, South Africa; 2Department of Internal Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa; 3Division of Pulmonology, Department of Internal Medicine, Faculty of Health Sciences, Charlotte Maxeke Johannesburg Academic Hospital and the University of the Witwatersrand, Johannesburg, South Africa

*Corresponding author. Department of Immunology, PO Box 2034, Pretoria, South Africa. Tel: +27-12-319-2624; Fax: +27-12-323-0723; E-mail: riana.cockeran@up.ac.za

Received 30 June 2011; returned 17 August 2011; revised 20 September 2011; accepted 23 September 2011

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 investigated the effects of tigecycline at therapeutically relevant concentrations and greater (0.625–10 mg/L) on alterations in cytosolic Ca2+ concentrations, generation of antimicrobial reactive oxygen species (ROS) and release of granule proteases [elastase, matrix metalloproteinase-8 (MMP-8) and matrix metalloproteinase-9 (MMP-9)] by human blood neutrophils activated with the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; 1 μM).

Methods: Cytosolic Ca2+ 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 pre-incubation 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 Ca2+, which appeared to result from the Ca2+ ionophore activity of tigecycline.

Conclusions: Tigecycline, by functioning as a Ca2+ ionophore, and independent of antimicrobial activity, potentiates the pro-inflammatory functions of human neutrophils in vitro.

Keywords: 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 S. 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

© The Author 2011. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com
inflammation-mediated tissue damage and organ dysfunction through increased release of indiscriminate reactive oxygen species (ROS) and granule proteases.\textsuperscript{7}

In the current study we investigated the effects of tigecycline on alterations in cytosolic Ca\textsuperscript{2+} concentrations, generation of antimicrobial ROS and release of granule proteases following activation of neutrophils with the chemoattractant N-formyl-l-leucyl-l-phenylalanine (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\textsubscript{2}O\textsubscript{2})/halide system. This is a composite assay of the production of superoxide and H\textsubscript{2}O\textsubscript{2}, as well as the release of MPO from primary granules. MPO and H\textsubscript{2}O\textsubscript{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 metalloproteinase-8 (MMP-8) and matrix metalloproteinase-9 (MMP-9) was included because of their involvement in promoting neutrophil migration and killing of microbial pathogens, while increases in cytosolic Ca\textsuperscript{2+} precede and are a prerequisite for activation of both the generation of ROS and the release of granule enzymes.\textsuperscript{7}

The underlying hypothesis is that tigecycline modulates the pro-inflammatory activities of human neutrophils, possibly by altering cytosolic Ca\textsuperscript{2+} concentrations in these cells, due to the calcium ionophore activity of this class of antimicrobial agent.\textsuperscript{9}

\section*{Materials and methods}

\subsection*{Antibiotics}

Tigecycline was provided by Wyeth Pharmaceuticals (Madison, NJ, USA), while doxycycline hydrate, minocycline hydrochloride and tetracycline hydrochloride were purchased from the Sigma Chemical Co. (St Louis, MO, USA). All four agents were dissolved in distilled water at a stock concentration of 5 mg/L and diluted thereafter in indicator-free Hanks\textsuperscript{\textregistered} balanced salt solution (HBSS; pH 7.4, 1.25 mM CaCl\textsubscript{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\textsuperscript{2+}, in 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\textsuperscript{0} 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.\textsuperscript{10} Leucocyte and tissue (colon, gallbladder, lung) concentrations are considerably higher than those of serum.\textsuperscript{9–11}

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

\subsection*{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 heparinized blood (5 U of preservative-free heparin/mL of blood). Neutrophils were separated from mononuclear leucocytes by centrifugation on Histopaque\textsuperscript{\textregistered}-1077 cushions at 400 g for 25 min at room temperature. The resultant erythrocyte/neutrophil layer was sedimented with 3% gelatin 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 1 × 10\textsuperscript{7} cells/mL in PBS (0.15 M, pH 7.4) and held on ice until used.

\subsection*{Neutrophil function assays}

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

\subsection*{MPO-mediated iodination}

Neutrophils (1 × 10\textsuperscript{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\textsubscript{125}I, 37 MBq; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and 20 nmol/mL cold carrier NaI. Following preincubation, the cells were activated by the addition of the synthetic chemoattractant 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 three 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 125I/10\textsuperscript{7} neutrophils.

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

\subsection*{Oxidant scavenging by tigecycline}

A cell-free ROS-generating system based on MPO-mediated iodination of BSA was used to measure the ROS-scavenging potential of tigecycline. Briefly, reaction systems contained purified MPO (100 mU/mL) isolated from human neutrophils, glucose oxidase (1.55 U/mL, from bovine liver), HBSS containing 5 mM glucose, 20 nmol/mL cold carrier NaI, 2 μCi 125I, 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 H\textsubscript{2}O\textsubscript{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 of 20% trichloroacetic acid (TCA). The protein precipitates were washed three times with TCA followed by measurement of the extent of iodination of BSA. The results are expressed as nmol 125I/2 mg BSA.

\subsection*{Oxygen consumption}

This was measured using a thermoregulated 3-channel oxygen electrode (Model DW1, Hansatech Ltd, King’s Lynn, Norfolk, UK). Neutrophils (2 × 10\textsuperscript{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 partial pressure of oxygen (pO\textsubscript{2}) of the cell-suspending medium monitored for 5 min. The results are expressed as nmol O\textsubscript{2} consumed over the first minute following the addition of fMLP/CB when the reaction was linear.

In an additional series of experiments, the effects of the Ca\textsuperscript{2+}-chelating agent, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; 10 mM) on tigecycline (10 mg/L)-mediated
modulation of fMLP/CB-activated O2 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 MMP-8 and MMP-9 (secondary and secondary/tertiary granules, respectively) were measured following activation of neutrophils with either fMLP/CB (elastase) or fMLP only (MMP-8 and MMP-9). Neutrophils (2 × 10⁶) were pre-incubated without or with tigecycline (0.625–10 mg/L) at 37°C followed by the 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) and 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 0.4–25, 0.156–10 and 0.312–10 ng/mL, respectively.

Spectrofluorimetric measurement of cytosolic Ca2+

Fura-2/AM was used as the fluorescent Ca2+-sensitive indicator for these experiments. Neutrophils (1 × 10⁶/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 that were maintained at 37°C in a Hitachi 650 105 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 Ca2+ influx

This procedure was used to measure the net influx of ⁴⁵Ca⁴⁺ 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 Ca²⁺-replete (1.25 mM) HBSS to ensure that intracellular Ca²⁺ stores were full in order to minimize spontaneous uptake of ⁴⁵Ca⁴⁺ (unrelated to activation with fMLP) in the influx assay. The cells were then pelleted by centrifugation and resuspended to a concentration of 1 × 10⁷/mL in HBSS containing 12.5 μM cold carrier CaCl₂. The Ca²⁺-loaded neutrophils (2 × 10⁶/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 2mCi/L ⁴⁵CaCl₂ (as ⁴⁵Ca[Cl]₂; 185 MBq; PerkinElmer Life and Analytical Sciences) or ⁴⁵Ca²⁺ only to control unstimulated systems. The cells (10⁷) in a final volume of 5 mL were then incubated for 5 min at 37°C, after which chemotacticant-activated, store-operated uptake of Ca²⁺ is complete, and the reactions stopped by the addition of 10 mL of ice-cold Ca²⁺-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 Ca²⁺-replete HBSS and the cell pellets dissolved in 0.5 mL of 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 ⁴⁵Ca²⁺/10⁷ cells).

To measure the effects of tigecycline (at a fixed final concentration of 10 mg/L) on the efflux of Ca²⁺, neutrophils (10⁷/mL) were pre-incubated for 20 min at 37°C in HBSS containing ⁴⁵Ca⁴⁺ (5 mCi/L) as the only source of the cation to enable loading of neutrophil intracellular stores with ⁴⁵Ca²⁺. Following this loading step, the cells were pelleted by centrifugation, washed once with, and resuspended in, Ca²⁺-replete (1.25 mM) HBSS. The ⁴⁵Ca²⁺-loaded neutrophils (2 × 10⁶/mL) in Ca²⁺-replete HBSS were then pre-incubated for 10 min without with tigecycline followed by the 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 was complete. Reactions were terminated and the cells processed as above and the amount of residual cell-associated ⁴⁵Ca²⁺ determined.

In an additional series of experiments, the effects of the following on the spontaneous uptake of ⁴⁵Ca²⁺ (i.e. 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 (2 × 10⁶/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 means ± 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 rank 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 1(a). 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 ≥0.6 mg/L. The effects of doxycycline, minocycline and tetracycline at concentrations of 5 and 10 mg/L are shown in Figure 1(b). 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/¹²⁵I system was 0.546±0.146 nmol and 0.176±0.039 nmol ¹²⁵I/2 mg BSA (P < 0.05), respectively, while the corresponding value for the MPO/glucose oxidase-free control system was 0.064±0.016 nmol ¹²⁵I/2 mg BSA. These observations demonstrate that tigecycline scavenges ROS generated by the MPO/H₂O₂/¹²⁵I 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 five different donors for the control and tigecycline (10 mg/L)-treated systems were $36.7 \pm 9.9$ and $42.5 \pm 11.5$ 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 $34.6 \pm 4.8$ and $40.7 \pm 6.4$ nmol O$_2$ consumed/1 min, while the corresponding values in the presence of EGTA were $32.7 \pm 4.4$ and $31.4 \pm 5.8$ 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 five 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 three proteases, especially MMP-9, achieving statistical significance ($P<0.05$) at concentrations of 0.6 and 5 mg/L for the MMPs and elastase, respectively.

**Fura-2/AM fluorescence**

The results shown in Figure 4 are typical traces from two different experiments using cells from two different donors (seven in the series) showing alterations in cytosolic Ca$^{2+}$ (fura-2 fluorescence) in neutrophils activated with fMLP/CB (f/CB). The 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. 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 45Ca2+ 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 45Ca2+ 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 45Ca2+ 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 45Ca2+, 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 effect on cell viability according to PI 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 four 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/H2O2/halide system. These pro-oxidative interactions of tigecycline with neutrophils appeared to result from increased consumption of O2 by fMLP/CB-activated cells, compatible with enhanced activity of NADPH oxidase and generation of superoxide and H2O2. The levelling-off of the tigecycline-mediated increase in activity of the MPO/H2O2/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 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...
Effects of tigecycline on neutrophils

Figure 5. Effects of tigecycline (TGC; 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, four in the series). The results of both series of experiments are expressed as the mean values (pmol $^{45}$Ca$^{2+}$/10$^7$ cells)±S.D. The intra-day and inter-day variabilities for the control system were 2.2% and 14.6%, respectively. *P<0.05 when compared with the drug-free control system. †P<0.05 when compared with 40 mg/L minocycline.

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 mobilization 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 that 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- to 3-fold more potent than the other agents; (ii) doxycycline, minocycline and tetracycline, like tigecycline, also caused increased activity of the MPO/H$_2$O$_2$/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 that inhibits phospholipase C and abolishes store-operated influx of Ca$^{2+}$, 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-recognized 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.

**Funding**

This study was supported by the South African Medical Research Council as well as by a research grant (grant number 35733) from Wyeth Pharmaceuticals.

**Transparency declarations**

G. A. R. has served on the speakers’ bureau for Pfizer and has developed a guideline for the appropriate use of tigecycline independently of the company. C. F. has acted on the advisory board, received honoraria for lectures and received support for congress travel from Pfizer-Wyeth. The current study was partially supported by a research grant awarded to R. A. by Wyeth Pharmaceuticals. All other authors: none to declare. Wyeth Pharmaceuticals did not contribute to the design of experiments or to preparation of the manuscript.

**References**

Effects of tigecycline on neutrophils


33 Sadowski T, Steinmeyer J. Effects of tetracyclines on the production of matrix metalloproteinases and plasminogen activators as well as their natural inhibitors, tissue inhibitor of metalloproteinases-1 and plasminogen activator inhibitor-1. Inflamm Res 2003; 50: 175–82.


