Immunomodulatory effects of tigecycline in Balb/c mice

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Tigecycline is a glyclcycline antibiotic approved by the FDA for the treatment of complicated infections. Despite its effectiveness, the FDA announced a warning of increasing mortality associated with its use. There is, however, no clear explanation for this side effect. Previous reports found a possible effect of tigecycline on leukocyte proliferation and pro-inflammatory cytokine release. We therefore investigated the effect of tigecycline on the immune components and response in Balb/c mice in vivo and in vitro. It was found that tigecycline enhanced lymphocyte proliferation and significantly increased cellular infiltration within the footpad, as based on DTH testing, but reduced the hemagglutination titer. In splenocyte cultures, tigecycline suppressed splenocyte proliferation with IC50 3–5 μmol L−1, significantly increased IL-2 secretion and reduced IL-17 secretion in a dose dependent mode. In conclusion, tigecycline is safe at therapeutic and sub-therapeutic doses, but it could still have an immunomodulatory effect at higher doses. Use of higher doses of tigecycline requires further investigation.

Keywords: tigecycline, immunomodulation, Balb/c mice, biochemical effect

Assessment of the investigational drug toxicity to the immune system is an important aspect in safety evaluation of pharmaceuticals, since it is very important to identify and evaluate the potential effects that produce immunotoxicity during drug development (1). Determining the mechanism of drugs’ immunotoxicity is an important issue to understand the clinical relevance of the adverse effects observed. Some drugs were found to induce toxicity by affecting the immune cells and lymphoid organ cellularity. The US office of technology and FDA defined immunotoxicants as substances that negatively affect the quality or the quantity of the immune response including immunosuppression, immunostimulation, allergy and autoimmunity (2).

Tigecycline (TGC) is a first-in-class glyclcycline; it is a 9-tert-butylglycylamido derivative of minocycline with bacteriostatic and bactericidal activity on Gram-positive, Gram-negative and atypical mycobacteria, but not on Pseudomonas aeruginosa and Proteus spp. (3). The Food and Drug Administration (FDA) approved TGC in 2005 for complicated infections.
Infections of skin structures, intra-abdominal and community acquired pneumonia, yet clinicians are using it as off-label for bacteremia, diabetic foot infections and hospital acquired pneumonia (4, 5). Moreover, TGC is used as an adjunctive therapy alone or in combinations for bacterial infections in hematological malignancies.

Due to the emergence of tetracycline resistance, TGC was rapidly approved for use in complicated infections. It was shown to be safe in phase II and III clinical trials with nausea and vomiting as the main side effects. After that, several case reports from clinical centers reported hematologic alteration and bone marrow suppression (6, 7). In September 2010, the FDA announced a black box warning for TGC due to increased risk adjusted mortality of 3 to 4 % compared to other regimens, and recommended its use only when other alternatives were not applicable (8).

Due to the wide application of TGC, investigation of a drug’s toxicity to the immune system is a standard part of general toxicity studies. Previous studies showed harmful effects of TGC on the immune system. It was found to penetrate neutrophils and stimulate their proliferation and cytotoxicity by acting as a calcium ionophore, thus augmenting the innate response (9). However, it could have a suppressive effect on antibodies produced by lymphocytes (10).

Cellular and humoral toxicity of TGC may vary according to the type of the present antigen. In \textit{in vivo} and \textit{ex vivo} murine models, T-helper (Th)-1 cytokines, but not Th-2 cytokines, were suppressed (11, 12). Inhibition of T-cell proliferation was seen when TGC was co-cultured with \textit{S. aureus} antigen (13) but not with endotoxin lipopolysaccharide (LPS) (14). Despite its inhibitory activity on Th-1, it did not affect production of Th-2 dependent antibodies in B cells (11). Conflicting results of previous researches highlight the need of investigating how tigecycline affects the immune system and its components.

Determining how TGC exerts its immunotoxicity is an important issue to understand the clinical relevance of the observed adverse effects. There are no previous studies that evaluated direct effects of TGC on B-lymphocytes and antibody production. The current study is aimed at evaluating the metabolic and immunomodulatory activities of tigecycline \textit{in vitro} and \textit{in vivo} using Balb/c model mice.

\section*{EXPERIMENTAL}

\subsection*{Animals}

Twenty eight pathogen-free female Balb/c mice (8–10 weeks old, 19–28 g body mass) were purchased from the animal house of the Applied Science University (Amman, Jordan) and housed in polystyrene cages in an air controlled room. All animals were maintained at a laboratory diet and tap water \textit{ad libitum} and were acclimatized for one week before the experiment (15). The experiments were conducted according to the principles given in the guide for the care and use of laboratory animals (16). Ethical approval for conducting animal studies was obtained from the Ethical and Graduate Studies Council of the Faculty of Pharmacy at the University of Jordan (Amman, Jordan).

\subsection*{Materials and reagents}

Tigecyl\textsuperscript{®} 500 mg vials were purchased from Hikma Pharmaceuticals (Jordan). Tetrazolium salt, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) powder,
concanavalin A (Con A), 2-mercaptoethanol (2-ME), bovine serum albumin (BSA) and RPMI-1640 were purchased from Sigma-Aldrich (Germany). Fetal bovine serum (FBS), penicillin and streptomycin, non-essential amino acids (NEAA) and sodium pyruvate solution were purchased from Euroclone, USA.

**Drug preparation, dose and exposure schedules**

A Tigecyl® 500 mg vial was dissolved in normal saline (NS) to a concentration of 0.5 mg mL\(^{-1}\) and kept at \(-80^\circ\)C until use. Human therapeutic dosing regimen of tigecycline is 100 mg \(i.v.\) as a loading dose, then 50 mg \(i.v.\) twice daily for 14–21 days (17). Mice were divided into four groups (I-IV) of seven animals each. Group I (control group) received NS by \(i.p.\) injection and groups II–IV were treated with TGC at doses of 178.6, 714.3 and 1428 µg kg\(^{-1}\) bm corresponding to 0.25-, 1- and 2-times the human therapeutic dose. Each group received a loading dose of 0.5X, 2X and 4X, respectively, on the first day of dosing, then was maintained on 0.25X, 1X and 2X, resp., twice daily for 20 days by \(i.p.\) injection.

**Body and metabolic organs masses**

Experimental animals were weighed at the beginning of the experiment and just before the excision. Changes in body mass were recorded. At the end of the experiment, livers and kidneys were extracted and weighed. The mean mass of each group was compared to the mean mass of the control group.

**Determination of white blood cell (WBC) indices**

Before excision, blood (0.2 mL) was collected from the retro-orbital plexus under light anesthesia from each mouse in sterile heparin tubes (Minicollect\(^{®}\), China) using heparinized capillaries, so that the blood to anticoagulant ratio was 1:0.075, \(V/V\). WBC count was determined by diluting 25 µL of EDTA blood with 475 µL of 10 % acetic acid. Cells were counted using a hemocytometer (18). For differential leukocyte count, a blood film was stained with Giemsa and Wright for each treated animal and counted under a light microscope (Nikon, China) at 100X.

**Biochemical markers of acute liver and kidney injuries**

Blood was collected in sterile plain tubes and left at room temperature for 2 h before serum was separated. Serum was stored at \(-20^\circ\)C for later use to measure alanine transaminase (ALT), aspartate transaminase (AST), serum creatinine (Scr) and urea using Alfa Wassermann diagnostic kits (USA).

**Spleen cellularity assay**

Single cell suspension of splenocytes was prepared according to Fararjeh et al. (19) with some modifications. Spleens collected from mice were teased in PBS using frosted-edge microscopic slides to remove connective tissue. The suspension was centrifuged at 1600 rpm for 7 min. RBCs present in the suspension were lysed at room temperature with RBC lysing buffer (0.83 % NH\(_4\)Cl in 0.0178 mol L\(^{-1}\) NaHCO\(_3\) and 0.127 mmol L\(^{-1}\) EDTA, adjusted pH 7.4),
then washed with PBS three times. The pellets were re-suspended in PBS and cells were counted using a hemocytometer under the light microscope. The mean difference in the splenocyte count was analyzed using one-way ANOVA on Graphpad Prism 6.

Delayed type hypersensitivity

Delayed-type hypersensitivity response (DTH) was determined using the method of Fararjeh et al. (19) with some modifications. On day 14 of the treatment, animals were injected subcutaneously with $1 \times 10^9$ sheep red blood cells (SRBCs) into the ventral flank. After five days of immunization (day 19), all animals were again challenged with a booster dose of $1 \times 10^8$ per 10 µL SRBCs in the left hind footpad. The right hind footpad was injected with the same volume of PBS to serve as trauma control for nonspecific swelling (19). The change in the left footpad volume was measured as the difference between SRBC-injected and PBS-injected hind footpad volumes 24 hours after the challenge with SRBCs. The difference between the left and right hind footpad volumes was calculated using a digital plethysmometer LE 7500 (Harvard, UK).

Hemagglutination assay

Five days before ending the treatment (day 15), mice were injected i.p. with $5 \times 10^8$ SRBCs in PBS. At the end of experiment (day 20), blood was collected from the retro-orbital plexus of each mouse before being sacrificed, where serum was separated and serially diluted in duplicates in PBS and placed in the wells of U-shape 96-microtiter plates. Aliquots (25 µL) of two-fold diluted sera in PBS were challenged with 25 µL of 1 % V/V SRBCs suspension and mixed. The plates were incubated at 37 °C for 1 h and then observed for hemagglutination. The log$_2$ of the highest dilution giving hemagglutination was taken as the antibody titer (18).

In vitro tigecycline cytotoxicity and cytokine response: Splenocyte culture preparation

Spleens from two female pathogen-free untreated Balb/c mice, aged 6–8 weeks, were extracted under sterile conditions and transferred to 15 mL of PBS in a 10-cm Petri dish. Single cell suspension of splenocytes was prepared as described in the spleen cellularity assay. Cultures were finally prepared using complete RPMI-1640 (cRPMI-1640) composed of 10 % FBS, 1 % penicillin-streptomycin, 1 mmol L$^{-1}$ sodium pyruvate, 1X NEAA and 0.05 mmol L$^{-1}$ of 2-ME.

Determination of tigecycline cytotoxicity

Splenocyte density was adjusted to $3 \times 10^6$ cell per 100 µL in cRPMI-1640 medium and placed in each well of a 96-well microtiter plate. An aliquot of 100 µL of TGC diluted in cRPMI-1640 to a concentration of 200 to 0.1 µmol L$^{-1}$ was added and incubated for 48 h at 37 °C (95 % humidity and 5 % CO$_2$). Two sets of plates were prepared; one was pretreated with 2 µg mL$^{-1}$ Con A mitogen while the other was left without mitogen. A MTT assay was done at the end of each treatment interval to determine cell viability. To each well, 20 µL of 5 mg mL$^{-1}$ MTT solution was added and the plates were incubated for 4 hours. Precipitated formazan crystals were dissolved in 200 µL DMSO and absorbance was measured at
570 nm/630 nm using a plate reader (Biorad, Japan). In this experiment, three wells were used for each concentration of TGC in the assigned concentration range (0.1–200 µmol L⁻¹). The whole experiment was repeated twice.

In vitro determination of cytokine release from splenocytes

Splenocyte count was adjusted to 5 × 10⁶ mL⁻¹ in cRPMI-1640 medium containing 1 % (V/V) mouse serum (cRPMI – 1 % mS). One milliliter of cell suspension was transferred to each well in a 24-well plate and incubated for 30 min at 37 °C (5 % CO₂ and 90 % humidity). Two-fold serial dilution of the drug was prepared (12.5 to 0.195 µmol L⁻¹) in cRPMI – 1 % mS and added to each well. Cells were pretreated with 2 µg mL⁻¹ Con A. After 72 h, the supernatant was collected, aliquoted and stored at −20 °C for later use. Murine interleukin (IL)-17 and IL-2 cytokines were measured using the ELISA assay according to the manufacturer's protocol (R&D Systems, USA and eBioscience, UK, resp.). All cytokine assays were calibrated against WHO standards using the manufacturer’s kit.

Statistical analysis

Data were presented as mean ± SD of the indicated number of experiments. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett’s post-hoc test to check for significant differences. A difference was considered significant at p < 0.05. All analyses were made using the GraphPad Prism 6 statistical software package.

RESULTS AND DISCUSSION

For direct immunotoxicity effect of tigecycline in vivo, we investigated its effect on the total body mass, liver and kidney masses, spleen cellularity, differential WBCs, delayed type hypersensitivity and hemagglutination.

Body and metabolic organs’ masses

As alteration of body mass and internal organs’ mass is a primary sign of immunotoxicity, mean body masses were determined before and after the treatment as well as absolute liver and kidney masses (Table I). Tigecycline caused no significant change in the total body mass with three different doses compared to the control group (Table I). Our results are in line with the results obtained from retrospective studies and animal models that showed body mass of healthy animals maintained upon treatment with parent compounds (20).

Biochemical markers of acute liver and kidney injuries

As TGC is eliminated mainly by hepatic metabolism via glucuronidation and to a limited extent by renal excretion, we investigated the changes in liver and kidney functions after the treatments. Groups treated with TGC showed no significant change in ALT and AST liver enzymes, which are reasonably sensitive indicators of acute liver damage or
injury (Table II). Kidney function tests showed no significant changes upon treatment with TGC. This is consistent with pharmacokinetic studies and case reports of its use in acute kidney injury because of urosepsis (21, 22) and might indicate that the drug is not toxic to liver or kidneys.
Fig. 1. Spleen cellularity in vivo after treatment with 0.25X, 1X and 2X tigecycline or NS (control) for 20 days via i.p. injection. Cell count is expressed as mean ± SD, n = 7; NS – normal saline; 0.25X – 12.5 mg per 70 kg per dose; 1X – 50 mg per 70 kg per dose; 2X – 100 mg per 70 kg per dose.

Fig. 2. The effect of tigecycline on delayed type hypersensitivity (DTH) measured as the change in footpad volume. Data are mean ± SD, n = 7. Significant difference versus control animals (NS): *p < 0.05; NS – normal saline; for dosing see Fig. 1.

White blood cell indices

Leukocytosis and/or leucopenia are other rare side-effects (less than 1 %) of TGC. Preclinical studies in rats, dogs and monkeys indicated the safety of TGC for blood cells (4) and so did our results. Tigecycline slightly increased the WBC count and percentage of peripheral blood neutrophils and monocytes at therapeutic (1X) and toxic doses (2X), but not significantly (Table III). Although TGC did not significantly change the WBC count or relative counts of neutrophils, lymphocytes and monocytes in vivo, it showed a slight increase in relative neutrophil counts with a slight decrease in relative lymphocyte count in a dose-dependent manner. Unlike macrolides that accelerate neutrophil apoptosis and
impair neutrophil migration (23), a previous study showed that TGC penetrates neutrophils and maintains their intracellular concentration for a relatively long time. Besides, it enhances the proliferation and proinflammatory activities of neutrophils in vitro (9).

**Spleen cellularity**

Spleen is a secondary lymphoid organ where B cells mature to become plasma cells that produce antibodies. Immunotoxic drugs affect B lymphocytes by altering spleen's cellular structure and lymphocyte function. TGC has been reported to show a variety of immunotoxic effects, such as delayed neutrophil engraftment (6) and enhanced neutrophil activity and proliferation (9) but few reports have explained TGC immunotoxicity to lymphocytes and neutrophils. In this study, tigecycline showed no significant change in splenocyte count at all three doses compared to the control group (as shown in Fig. 1), although the splenocyte count at 1X was lower than the control and the other treatments at the therapeutic dose.

**Effect of TGC on delayed type hypersensitivity (DTH)**

Alteration in leukocyte balance opens the possibility that the drug might have an effect on adaptive immunity, so we investigated the induction of DTH to confirm the TGC immunomodulatory effects. All doses of TGC showed an increase in the DTH response 24 h after secondary injection of antigen (SRBCs) in a dose-dependent manner. However, it was significant only when treated with the toxic 2X dose of TGC ($p < 0.05$) (Fig. 2). Induction of DTH reaction by TGC could be a result of the availability of factors responsible for the T cell maintenance and proliferation and those required for chemotaxis of macrophages (24). As there are no previous studies that investigated the effect of TGC on lymphocytes, the underlying mechanism of such effect is still unknown. Theoretically, the observed increase of footpad thickness and edema might be a response to increased macrophages, DC and T-lymphocyte migration in addition to the proliferation of lymphocytes.

![Fig. 3. The effect of tigecycline on anti-SRBCs antibody production assessed by hemagglutination titer. Data are mean ± SD, n = 7. Significant difference vs. control animals (NS): *$p < 0.05$; NS – normal saline; SRBC – sheep red blood cell; for dosing see Fig. 1.](image-url)
or antigen-antibody complex reaction at the footpad. This effect might lead to induction of an inflammatory response, which is associated with increased vascular permeability, immune cell migration and edema.

**Serum antibody hemagglutination (HA) titer**

Hemagglutination titer is measured as agglutination of non-specific antibodies against sheep RBC (SRBC). The log$_2$ of the highest dilution is used as the titer of the antibodies. It shows the ability of lymphocytes to generate antibodies against non-specific SRBC antigens. Hemagglutination titer at the 2X dose (1428 µg kg$^{-1}$ bm) showed significant reduction ($p < 0.05$) in the concentration of the anti-SRBC antibodies expressed as antibody titer (Fig. 3), whereas sub-therapeutic and therapeutic doses were unable to produce a significant change compared to the control (group I). Tigecycline significantly depressed the HA titer at 2X compared to the control. As antibodies are produced by B lymphocytes, which mature in the spleen, the reduction in antibody production compared to the control group is consistent with the reduction in lymphocytes in this study. Previous studies reported varying impacts of TGC on adaptive immunity, with various experimental models. *In vitro* treatment of PBMCs with TGC and bacterial antigens reported an inhibitory activity on antibody production (13); yet another study, which used *in vivo* pneumonia murine, found that TGC did not alter antibody production (11). The reduction in peripheral lymphocytes, spleen cellularity and antibody production are indicators of increased body’s susceptibility to infections as well as altered immunity.

**Effect of TGC on splenocyte proliferation in vitro**

Tigecycline achieves maximum blood concentration ($c_{\text{max}}$) of 1.5 µmol L$^{-1}$ after a 50-mg *i.v.* twice-daily dose; however, it has a minimum inhibitory concentration (MIC) of 0.25–1 mg L$^{-1}$ (1–2 µmol L$^{-1}$) against multidrug resistant bacteria *in vitro* (25). Therefore, higher doses are used clinically. In our study, splenocyte viability *in vitro* was significantly re-
duced after incubation with concentrations higher than 3.125 µmol L\(^{-1}\) of tigecycline for 48 hours. TGC effectively inhibited the growth of splenocytes at concentrations above 1 µmol L\(^{-1}\) (\(p < 0.05\)). The \(IC_{50}\) value was 3.39 µmol L\(^{-1}\) with Con A and 2.92 µmol L\(^{-1}\) without Con A (Fig. 4). Increasing tigecycline concentration above \(IC_{50}\) showed an obvious reduction in splenocyte viability \textit{in vitro}; this further complies with the \textit{in vivo} results of decreased HA titer and altered splenocyte count at the 2X dose. When Con A-pretreated splenocytes were treated with 1.56 µmol L\(^{-1}\) of TGC (\(c_{\text{max}}\) of TGC from the pharmacokinetic study) or less, they significantly increased proliferation leading to a plateau, but not higher than the control cells (22). Although tigecycline is relatively safe at the regular dose (1X, 50 mg \textit{i.v.} twice daily for humans), increasing the dose might be accompanied with alteration of the immune response and immune system components.

**Effect of TGC on IL-17 and IL-2 release in Con A pretreated splenocytes**

Interleukin-2 and IL-17 have a pronounced role in the non-specific immune response and T cell differentiation. IL-2 is a regulatory cytokine produced by Th-1 cells that activates
macrophages against intracellular antigens (26), since it mediates acute inflammation and DTH (27). IL-17 is a proinflammatory cytokine produced by Th-17, neutrophils and mast cells and is required for Th-17 development (28). Th-17 cells were found to have some immunotoxic effects (27).

Due to the pronounced role of IL-2 and IL-17 in the non-specific immune response and T cell differentiation, we tested the effect of tigecycline on their release from splenocytes after pretreatment with Con A. A dose-dependent effect on the release of the tested cytokines is shown in Fig. 5. Above IC$_{50}$, the concentration of IL-2 increased significantly (Fig. 5b, $p < 0.001$): at concentrations higher than 6.25 µmol L$^{-1}$ by about 5 to 6 times, while IL-17 significantly decreased ($p < 0.05$) at the same concentrations (Fig. 5a). Our results are consistent with previous studies where tetracycline treatment induced IL-2 release (29). To the best of our knowledge, no previous studies investigated the release of IL-17 and IL-2 from splenocytes. Theoretically, naive CD4+ T cells differentiate into either Th-1 or Th-17 according to the cytokines in the milieu and the present antigen by APCs. Enhanced release of IL-2 is known to suppress IL-4/Th-2 and promote the Th-1 and Treg development. This increase in IL-2 could inhibit IL-17 and consequently Th-17 differentiation (30, 31). In vivo model of mouse pneumonia reported an inhibitory activity of TGC on IL-2; alteration of cytokine release and reduced cell viability provided further evidence of the immunomodulatory effects of tigecycline (11).

**CONCLUSIONS**

Although tigecycline was approved by the FDA for complicated infections in critically ill patients, its usage has been associated with increased mortality. As a new drug, investigation of the drug’s toxicity on the immune system and metabolic organs is of great importance. Our data showed that FDA-approved doses of tigecycline caused no toxicity to the metabolic and immunological functions. However, exceeding these doses would increase the potential immunomodulatory effects of TGC. Doubling of the dose caused alteration in adaptive immunity components as well as differential leukocytes in blood. It is worth considering the alteration that is exerted by the infection itself on the immune system. Therefore, further studies are needed to investigate the immunotoxicity of TGC in models of susceptible infections.

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