Short communication

Doxycycline inhibits SARS-CoV-2 replication *in vitro*

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ABSTRACT

Introduction and Aim: We examined the effect of pre- and/or post-infection doxycycline on human nasal epithelial cell viability and SARS-CoV-2 (clinical strain IHUMI-3) replication *in vitro*.

Materials and Methods: Human nasal epithelial cells, an *in vivo* SARS-CoV-2 target, were derived from healthy donor nasal epithelial stem/progenitor cells via *in vitro* differentiation. The cells were exposed to doxycycline at 0, 0.1, 0.5, 1, 5, 10, 50, and 100 μM before and/or after IHUMI-3 inoculation to determine the optimal inhibitory concentration. Viral replication was evaluated using quantitative reverse-transcription PCR, and doxycycline 50% cytotoxic concentration (CC_{50}) and half-maximal effective concentration (EC_{50}) were calculated. The peak serum concentration (Cmax) resulting from typical oral (100 or 200 mg) or intravenous (100 mg) doxycycline doses was estimated, and the Cmax/EC_{50} ratio was calculated as an index of potential clinical utility.

Results: Doxycycline exhibited low cytotoxicity (CC_{50} > 100 μM) in human nasal epithelial cells and inhibited SARS-CoV-2 replication (EC_{50}: 5.2 ± 3.3 μM) in a dose-dependent manner when administered pre- and/or post-infection. Reasonable oral or intravenous doses will help achieve effective concentrations *in vivo*.

Conclusion: Early administration of this well-characterized, safe, and accessible drug may limit person-to-person transmission and prevent progression to severe coronavirus disease.

Keywords: Doxycycline; COVID-19; SARS-CoV-2; *in vitro.*

INTRODUCTION

Current therapies to treat coronavirus disease (COVID-19) are largely supportive (ranging from simple symptom management to critical care), and effective novel or repurposed therapeutic agents are urgently required to decrease COVID-19-related morbidity and mortality until vaccines become widely available. Unfortunately, agents previously considered promising in this context (e.g., hydroxychloroquine (1) or lopinavir-ritonavir (2)) have caused unfavorable adverse effects or failed to exhibit significant efficacy.

Doxycycline exhibits broad-spectrum antimicrobial (including antiviral) activity and anti-inflammatory activity (including reversal of viral infection-associated cytokine storms) (3, 4). *In vitro*, doxycycline has been shown to inhibit the replication of vesicular stomatitis virus (5), dengue virus NS2B-NS3 serine protease activity (6), and Chikungunya virus entry and replication (7). However, information regarding the activity of doxycycline against severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) is currently limited. Therefore, in this study, we evaluated the cytotoxicity of doxycycline and its effect on SARS-CoV-2 *in vitro*.

MATERIALS AND METHODS

Human nasal epithelial cells (hNECs), a known *in vivo* SARS-CoV-2 target (8), were derived from healthy donor nasal epithelial stem/progenitor cells via *in vitro* differentiation. Isolated and enriched donor nasal epithelial stem/progenitor cells were expanded *in vitro* and then differentiated in 12-well plates with 0.4-μm Transwell inserts (Corning Inc., NY, USA), as previously described (9). The study protocols were approved by the International Higher School of Medicine ethics committee (Protocol no.

4114), all donors provided written informed consent prior to tissue biopsy, and data confidentiality was maintained.

First, hNECs were exposed to doxycycline at various concentrations (0, 0.1, 0.5, 1, 5, 10, 50, and 100 µM) before and/or after inoculation of a clinical SARS-CoV-2 isolate (strain IHUMI-3) to determine the optimal inhibitory concentration. In subsequent experiments, we assessed the effect of 5 µM doxycycline on hNEC viability and viral replication. Cells were seeded into 96-well conical-bottom plates (Sigma-Aldrich, MO, USA) at 7.5×10^4 cells/well in 100 µL of complete medium (RPMI 1640 supplemented with 4% fetal bovine serum and 1% glutamine (all from Sigma-Aldrich)). The plates were incubated at 37 °C in the presence of 5% CO_2 for four weeks, and the media were replaced every 48 h. IHUMI-3 was inoculated at various multiplicities of infection (0.5 µM). Doxycycline hyclate (100 µL of a stock solution prepared in dimethyl sulfoxide (DMSO; Ivesco, Iowa, USA)) was dissolved in methanol and diluted in minimum essential medium (Sigma-Aldrich) to produce final in-well concentrations of 0 to 100 µM. As a vehicle control, a concentration of DMSO equivalent to that required to achieve the highest tested in-well doxycycline concentration was added to untreated cells. The activity of doxycycline (5 µM) against IHUMI-3 was then evaluated. Cells were subjected to three doxycycline treatment schedules: (1) doxycycline treatment started 2 h pre-infection and maintained for 48 h, (2) doxycycline treatment started 2 h pre-infection, followed by medium replacement 4 h post-infection, and (3) doxycycline treatment started 4 h post-infection and maintained for 48 h. Pre-infection treatment aimed to examine the effect of doxycycline on viral attachment and entry, whereas post-infection treatment used to assess doxycycline effect on viral replication.

Incubation proceeded for another 48 h post-doxycycline addition. Then, the cells were triturated, harvested into 96-well conical-bottom plates (Sigma-Aldrich), diluted 10-fold using complete medium, pelleted by centrifugation (500 × *g*, 10 min, 37 °C), and supernatants (200 µL/well) were used to evaluate viral replication using quantitative reverse-transcription (RT-q) PCR. Briefly, viral RNA was purified using a MagNA Pure 24 system (Roche, Basel, Switzerland) and qPCRs were run using a LightMix Modular SARS-CoV-2 RdRP gene kit (TIB MOLBIOL, Berlin, Germany) in conjunction with an RNA Process Control kit (Roche). All assays were performed according to the manufacturers' instructions.

For all conditions, the 50% cytotoxic concentration (CC_{50}) and half-maximal effective concentration (EC_{50}) of doxycycline were calculated using a sigmoid inhibitory E_{max} model provided by the online R-based tool ICEstimator v.1.2. (10), and model parameters were estimated using nonlinear regression analysis. All experiments were performed at least in quintuplicate (and up to octuplicate), and values are presented as the mean ± standard deviation. In addition, the peak serum concentration (C_{max}) that would result from typical oral (100 or 200 mg) or intravenous (100 mg) doxycycline doses was estimated, and the $C_{max}/EC50$ ratio was calculated as an index of potential clinical utility. All statistical analyses were performed using GraphPad Prism v.9.0.0 (GraphPad Software, CA, USA).

RESULTS

The results of the doxycycline cytotoxicity analysis (after 48 h of exposure) revealed a CC_{50} exceeding 100 µM, and those of the doxycycline efficacy analysis (after 48 h of exposure) showed that it inhibited SARS-CoV-2 replication dose-dependently (Fig. 1), with an EC_{50} of 5.2 ± 3.3 µM. In addition, doxycycline significantly inhibited SARS-CoV-2 replication when administered prior to or following hNEC entry (Fig. 2). Finally, calculated C_{max}/EC_{50} ratios for 100 mg (oral), 200 mg (oral), and 100 mg (intravenous) doxycycline were 0.81, 2.32, and 1.36, respectively.

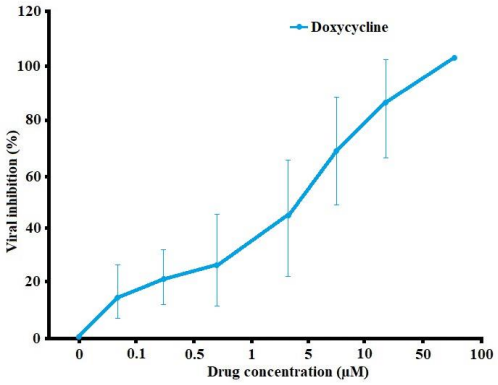

Fig. 1: Doxycycline dose-dependently inhibits SARS-CoV-2 (strain IHUMI-3) replication *in vitro*

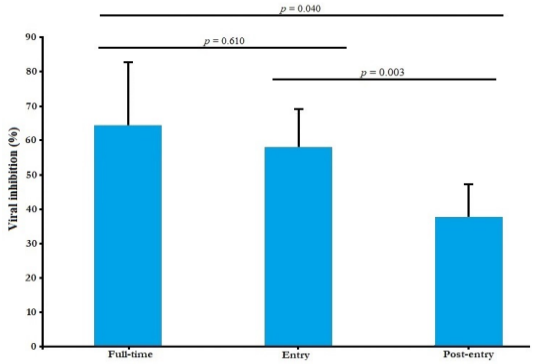

Fig. 2: Exposure to doxycycline (5 µM) pre- and/or post-infection inhibits SARS-CoV-2 (strain IHUMI-3) replication *in vitro*. Doxycycline treatment schedules were as follows: (1) doxycycline treatment started 2h pre-infection and maintained for 48 h, (2) doxycycline treatment started 2h pre-infection, followed by medium replacement 4h post-infection, and (3)

doxycycline treatment started 4 h post-infection and maintained for 48 h.

DISCUSSION

The extremely high CC_{50} (> 100 μM) and the relatively low EC_{50} (5.2 ± 3.3 μM) determined in the present study are consistent with those reported in a recent study evaluating doxycycline-mediated inhibition of SARS-CoV-2 replication in Vero E6 renal cells (CC_{50} > 100 μM, EC_{50} 5.6 ± 0.4 μM) (11). A CC_{50} of an order of magnitude greater than the EC_{50} indicates that the doxycycline level at which SARS-CoV-2 replication is effectively inhibited will likely not produce cytotoxicity. Doxycycline effectively inhibited viral replication regardless of administration in pre- or post-infection which indicates its potential in both prophylactic and therapeutic use. Finally, C_{max}/EC_{50} ratios exceeding 1 are favorable and indicate that the C_{max} achievable following oral or intravenous administration exceeds the concentrations required to decrease viral replication by 50%.

Doxycycline is a lipophilic molecule that chelates zinc ions, which are required by mammalian matrix metalloproteinases (MMPs) (8). An *in vitro* study demonstrated that murine coronaviruses depend on MMPs for host cell membrane fusion, entry, and replication (12), and prophylactic doxycycline treatment (100 mg/day) in combination with chloroquine (100 mg/day) or hydroxychloroquine (200 mg/day for ten days) inhibits entry of SARS-CoV-1 into target cells *in vitro* (13). In addition, prophylactic doxycycline treatment prevents acute lung injury during murine influenza H3N2 virus infection (14).

Although COVID-19 causes mild to moderate illness in many patients, certain patients develop severe disease (5) in association with cytokine storm syndrome, in which interleukin (IL)-6 plays an essential role (15). Doxycycline significantly decreases the levels of IL-6 and tumor necrosis factor-α in patients with dengue hemorrhagic fever (16). Importantly, doxycycline exhibits pulmonary protective properties during the clinical treatment of lung injury (17, 18). Acute respiratory distress syndrome is common among severely ill COVID-19 patients, and MMP inhibition may help repair lung tissue and promote respiratory recovery (19).

Region-specific evidence-based treatment guidelines already mandate the provision of empirical doxycycline therapy for community-acquired and atypical pneumonias (20). In a small preliminary study, 89 COVID-19 patients at particular risk for progression to moderate or severe illness received early (within 12 h) oral or intravenous doxycycline (100 mg/day for seven days), and this was associated with symptom amelioration and decreased hospitalization and mortality rates (21).

CONCLUSION

The present study demonstrated that doxycycline exposure pre- and/or post-infection significantly inhibits SARS-CoV-2 replication in hNECs *in vitro* at non-cytotoxic concentrations that are readily achievable *in vivo* via oral or intravenous doxycycline administration. These findings provide pre-clinical evidence supporting the clinical potential of this well-characterized, safe, and accessible drug as a prophylactic or therapeutic agent in the context of COVID-19. Prophylactic or early-stage administration of effective antiviral agents could limit person-to-person transmission and prevent progression to severe COVID-19, thereby improving both public health and individual patient outcomes. The results of the present study also support the notion that the repurposing of existing drugs represents a parallel and complementary strategy to novel antiviral drug development.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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