Short communication

In vitro antiviral activity of doxycycline against SARS-CoV-2

Mathieu Gendrot[a,b,c#], Julien Andreani[c,d#], Priscilla Jardot[c,d], Sébastien Hutter[b,c], Manon Boxberger[c,d], Joel Mosnier[a,b,c,e], Marion Le Bideau[c,d], Isabelle Duflot[c,d], Isabelle Fonta[a,b,c,e], Clara Rolland[c,d], Hervé Bogreau[a,b,c], Bernard La Scola[c,d*], Bruno Pradines[a,b,c,e*]

[a]Unité Parasitologie et Entomologie, Département Microbiologie et Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Marseille, France.

[b]Aix Marseille Univ, IRD, SSA, AP-HM, VITROME, Marseille, France.

[c]IHU Méditerranée Infection, Marseille, France.

[d]Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France.

[e]Centre National de Référence du Paludisme, Marseille, France.

#Authors contributed equally to this work.

*Co-corresponding author. Present address: Unité Parasitologie et Entomologie, Institut de Recherche Biomédicale des Armées, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France. Tel.: +33 4 13 732 231.

E-mail address: bruno.pradines@gmail.com

MEPHI, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France.

E-mail address: bernard.la-scola@univ-amu.fr

Abstract

In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China. Despite containment measures, SARS-CoV-2 spread in Asia, Southern Europe, then in America and currently in Africa. Identifying effective antiviral drugs is urgently needed. An efficient approach to drug discovery is to evaluate whether existing approved drugs can be efficient against SARS-CoV-2. Doxycycline, which is a second-generation tetracycline with broad-spectrum antimicrobial, antimalarial and anti-inflammatory activities, showed *in vitro* activity against SARS-CoV-2 with median effective concentration (EC_{50}) of 5.6 ± 0.4 µM. Doxycycline, with its antiviral and anti-inflammatory activities, could be used in prophylaxis of COVID-19 at 100 mg day in combination with chloroquine, or in treatment at 200 mg day during 10 days in combination with hydroxychloroquine.

Keywords: COVID-19, SARS-CoV-2, Doxycycline, treatment, prophylaxis, antiviral, anti-inflammatory

1. Introduction

In December 2019, a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) causing coronavirus diseases 2019 (COVID-19) emerged in Wuhan, China [1]. Despite containment measures, SARS-CoV-2 spread in Asia, Southern Europe, then in America and currently in Africa. Currently, there is no antiviral treatment recommended by the French Health Ministry against SARS-CoV-2. Several drugs are tested in the context of the discovery trial [https://clinicaltrials.gov/ct2/show/NCT04315948] and a controversial protocol massively used worldwide associate a combination of hydroxychloroquine and azithromycin [2,3]. However, identifying effective low cost antiviral drugs with limited side effects affordable immediately especially for emerging countries is urgently needed. An efficient approach to drug discovery is drug repurposing that consists to evaluate whether existing approved drugs can be efficient against SARS-CoV-2.

Doxycycline is a second-generation tetracycline with broad-spectrum antimicrobial [4] and anti-inflammatory activities [5]. Additionally, doxycycline was approved as prophylaxis against malaria by the Food and Drug Administration in 1994 and has been used since 2006 at the dose of 100 mg/day by the French military forces deployed in malaria-endemic areas [6]. Doxycycline also shows antiviral activity *in vitro*. This tetracycline derivative significantly inhibited the replication of vesicular stomatis virus *in vitro* [7], dengue virus by inhibition of NS2B-NS3 serine protease [8-10]. Doxycycline showed inhibition of entry and replication of Chikungunya virus in Vero cell at 11 µM [11]. Using in-silico method, doxycycline might be a potential inhibitor of Crimean-Congo hemorrhagic fever virus nucleoprotein, an essential protein in virus replication [12]. Additionally, doxycycline inhibited the early-stage replication of porcine reproductive and respiratory syndrome virus, which causes respiratory disease, with EC_{50} (median effective concentration) of 0.25 µg/ml (about 0.5 µM) [13]. The current study evaluated the antiviral effect of doxycycline against SARS-CoV-2.

2. Methods & Materials

2.1. Agent, virus and cells

Stock solution of doxycycline hyclate (Sigma, Saint Louis, MO, USA) was prepared in methanol and diluted in Minimum Essential Media (MEM, Gibco, ThermoFischer) in order to have 7 final concentrations ranging from 0.1 µM to 100 µM. Chloroquine diphosphate (Sigma, Saint Louis, MO, USA) was used as comparator. The clinically isolated SARS-CoV-2 strain (IHUMI-3) [2] was maintained in production in Vero E6 cells (American type culture collection ATCC® CRL-1586™) in MEM with 4% of fetal bovine serum and 1% glutamine (complete medium).

2.2. Cytotoxicity assay

In vitro cell viability evaluation on the VERO E6 cell line was performed according to the method described by Mosmann with slight modifications [14]. Briefly, 10^5 cells in 200 µl of complete medium were added to each well of 96-well plates and incubated at 37 °C in a humidified 5% CO_2. After 24 h incubation, 25 µl of each concentration of doxycycline and chloroquine were added and the plates were incubated 48h at 37 °C. After removal of the surpernatant, 100 µL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide, Sigma Aldrich, France) solution (0.5 mg/ml in MEM without FBS) were then added to each well. Cells were incubated for 2 h at 37 °C. After incubation, the MTT solution was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Then, plates were shaken at 700 rpm for 10 min at 37 °C. The absorbance was measured at 570 nm using a TECAN Infinite F200 Microplate Reader using DMSO was used as blank. The 50% cytotoxicity concentration (CC_{50}) was calculated with the inhibitory sigmoid E_{max} model, which estimated the CC_{50} through nonlinear regression by using a

standard function of the R software (ICEstimator version 1.2, http://www.antimalarial-icestimator.net). CC_{50} value resulted in the mean of 5 different experimentations.

2.3. Antiviral activity assay

Briefly, 96-well plates were prepared with 5.10^5 cells/mL of Vero E6 (200µL per well), as previously described [15]. Doxycycline and chloroquine concentrations were added 4 h before infection. Vero E Cells were infected with IHUMI-3 at an MOI of 0.25. After 48h post-infection, the replication was estimated by RT-PCR using the Superscrit III platinum one step with Rox kit (Invitrogene) after extraction with the BIoExtract SuperBall kit (Biosellal, Dardilly, France). The primers used were previously described [16]. The median effective concentration (EC_{50}) was calculated with the inhibitory sigmoid E_{max} model, which estimated the EC_{50} through nonlinear regression by using a standard function of the R software (ICEstimator version 1.2). CC_{50} value resulted in the mean of 4 different experimentations.

3. Results and discussion

The cytotoxicity evaluation of doxycycline and chloroquine showed that the CC_{50} values were > 100 µM for 48h. The CC_{50} value of chloroquine is consistant with those previously described [17,18]. The median effective concentration (EC_{50}) was 5.6 ± 0.4 µM for doxycycline and 2.1 ± 0.8 µM for chloroquine (Figure 1). These EC_{50} values depend on several methodological conditions like MOI, duration of incubation [17,19]. The EC_{50} value for chloroquine is consistent previous results on Vero E6 cells at MOI of 0.2 [17]. Doxycycline was found to decrease the SARS-CoV-2 replication in a concentration-dependent manner. Besides its antiviral activity, doxycycline has anti-inflammatory effects by decreasing the expression of various pro-inflammatory cytokines including interleukins 1, 6 and 8 and tumor necrosis factor-alpha by macrophages [5] and chemokines including

monocyte chemotactic protein 1, macrophage inflammatory protein 1α and 1β [20]. The immunimodulatory activity of doxycycline improved survival of septic mice with pulmonary inflammation [21]. Doxycycline concentration was twice as high in lungs than in serum [22]. Doxycycline, used as malaria prophylaxis, was well tolerated at the dose of 100 mg/day during several months [23]. Additionally, doxycycline has been recommended at 100 mg twice a day during 10 days for post-exposure prophylaxis of anthrax. This dose can even been taken by children below 8 years old [24]. Doxycycline could be used in prophylaxis of COVID-19 at 100 mg day in combination with chloroquine at 100 mg day, which inhibits the entry of SARS-CoV-1 in cells [25], or in treatment at 200 mg day during 10 days in combination with hydroxychloroquine. At these doses currently used for other indications, doxycycline could be rapidly evaluated in clinical trials in COVID-19.

Declarations

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03.

Competiting interests: The authors have no conflict of interest to declare.

Ethical approval: Not required.

References

[1] Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. Nature 2020; 579: 365-9.

[2] Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, Mailhe M, et al. Hydroxychloroquine and azithromycin as a treatment of COVID-19: results of an open-

label non-randomized clinical trial. Int J Antimicrob Agents 2020 17 Mar. doi: 10.1016/j.ijantimicag.2020.105949.

[3] Gautret P, Lagier JC, Parola P, Hoang VT, Meddeb L, SevestreJ, et al. Clinical and microbiological effect of a combination of hydroxychloroquine and azithromycin in 80 COVID-19 patients with at least a six-day follow up: an observational study. https://www.mediterranee-infection.com/wp-content/uploads/2020/03/COVID-IHU-2-1.pdf.

[4] Michalopoulos A. A clinical and laboratory study of doxycycline ('Vibramycin'): a broad-spectrum antibiotic. Curr Med Res Opin 1973; 1: 445-55.

[5] Cazalis J, Bodet C, Gagnon G, Grenier D. Doxycycline reduces lipopolysaccharide-induced inflammatory mediator secretion in macrophage and ex vivo human whoole blood models. J Periodontol 2008; 79: 1762-8.

[6] Gaillard T, Madamet M, Pradines B. Tetracyclines in malaria. Malar J 2015; 14: 445.

[7] Wu ZC, Wang X, Wei JC, Li BB, Shao DH, Li YM, et al. Antiviral activity of doxycycline against vesicular stomatis virus in vitro. FEMS Microbiol Lett 2015; 362: fnv195.

[8] Yang JM, Chen YF, Tu YY, Yen KR, Yang YL. Combinatorial computational approaches to identify tetracycline derivatives as flavivirus inhibitors. PLoS One 2007; 2: e428.

[9] Rohan HA, Buckle MJ, Ammar YA, Mohammadjavad P, Shatrah O, Noorsaadah AR, et al. Study the antiviral activity of some derivatives of tetracycline and non-steroid anti inflammatory drugs towards dengue virus. Trop Biomed 2013; 30: 681-90.

[10] Rohan HA, Mohamed Z, Paydar M, Rahman NA, Yusof R. Inhibitory effect of doxycycline against dengue virus replication in vitro. Arch Virol 2014; 159: 711-8.

[11] Rohan HA, Bahrani H, Mohamed Z, Teoh TC, Shankar EM, Rahman NA, et al. A combination of doxycycline and ribavirin alleviated Chikungunya infection. PLoS One 2015; 10: e0126360.

[12] Sharifi A, Amanlou A, Moosavi-Movahedi F, Golestanian S, Amanlou M. Tetracyclines as a potential antiviral therapy against Crimean Congo hemmrrhagic fever virus: Docking and molecular dynamic studies. Comput Biol Chem 2017; 70: 1-6.

[13] Li Y, Wu Z, Liu K, Qi P, Xu J, Wei J, et al. Doxycycline enhances adsorption and inhibits early-stage replication of porcine reproductive and respiratory syndrome virus in vitro. FEMS Microbiol Lett 2017; 364: fnx170.

[14] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, J Immunol Methods 1983; 65: 55-63. https://doi.org/10.1016/0022-1759(83)90303-4.

[15] Andreani J, Le Bideau M, Duflot I, Jardot P, Rolland C, Boxberger M, et al. In vitro testing of hydroxychloroquine and azithromycin on SARS-CoV-2 shows synergistic effect. https://www.mediterranee-infection.com/wp-content/uploads/2020/03/Andreani-et-al.-Pre-print-V2.pdf

[16] Amrane S, Tissot-Dupont H, Doudier B, Eldin C, Hocquart M, Mailhe M, et al. Rapid viral diagnosis and ambulatory management of suspected COVID-19 cases presenting at the infection diseases referral hospital in Marseille, France, -January 31st to March 1st, 2020: A respiratory virus snapshot. Travel Med Infect Dis. 2020 Mar 20:101632. doi: 10.1016/j.tmaid.2020.101632.

[17] Liu J, Cao R, Xu M, Wang X, Zhang H, Hu H, et al. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection in vitro. Cell Discovery 2020; 6: 16.

[18] Wang M, Cao R, Zhang L, Yang X, Liu J, Xu M, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCov) in vitro. Cell Res 2020; 30: 269-71.

[19] Yao X, Ye F, Zhang M, Cui C, Huang B, Niu P, et al. In vitro antiviral activity and projection of optimized dosing design of hydroxychloroquine for the treatment of severe acute respiratory syndrome coronavirus (SARS-CoV-2. Clin Infect Dis 2020 Mar 9. pii: ciaa237. doi: 10.1093/cid/ciaa237.

[20] Krakauer T, Buckley M. Doxycycline is anti-inflammatory and inhibits staphylococcal exotin-induced cytokines and chemokines. Antimicrob Agents Chemother 2003; 47: 3630-3.

[21] Patel A, Khande H, Periasamy H, Mokale S. Immunomodulatory effect of doxycycline ameliorates systemic and pulmonary inflammation in a murine polymicrobial sepsis model. Inflammation 2020 Jan 18. doi: 10.1007/s10753-020-01188-y.

[22] Vargas-Estrada D, Gutirrez L, Juarez-Rodriguez I, Sumano H. Pharmacokinetics of doxycycline and tissue concentrations of an experimental long-acting parenteral formulation of doxycycline in Wistar rats. Arzneimittelforschung 2008; 58: 310-5.

[23] Michel R, Bardot S, Queyriaux B, Boutin JP, Touze JE. Doxycycline-chloroquine vs. Doxycycline-placebo for malaria prophylaxis in nonimmune soldiers: a double-blind randomized field trial in sub-Saharan Africa. Trans R Soc Trop Med Hyg 2010104: 290-7.

[24] Gaillard T, Briolant S, Madamet M, Pradines B. The end of a dogma: the safety of doxycycline use in young children for malaria treatment. Malar J 2017; 16: 148.

[25] Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. Virol J 2005; 2: 69.

Figure: Comparative antiviral efficacy of doxycycline and chloroquine against SARS-CoV-2 infection *in vitro*