Final Report

Virucidal Efficacy of Doxmed V-1 on SARS-CoV-2

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1. OBJECTIVE

This project was to evaluate the virucidal efficacy of Doxmed V-1, provided by Houchi Chemical Co., Ltd. (合記化學), on SARS-CoV-2 viruses according to the protocols modified from the peer-reviewed paper [1] and the British standard [2].

2. INTRODUCTION

The antiviral potential was tested by using two cell-based antiviral assays, infection rate determined by immunofluorescent assay and disinfection efficacy determined by median tissue culture infectious dose ($TCID_{50}$) on African green monkey kidney epithelial Vero-E6 cells. Cytotoxicity of the compounds on these cells were determined by using the CCK8 assay.

3. MATERIALS and METHODS

3.1. Vero-E6, ATCC CRL-1586,African green monkey kidney epithelial cells.

3.2. SARS-CoV-2, TCDC#4.

3.3. Human anti-SARS-CoV-2 N protein antibody (provided by Dr. An-Suei Yang in Genomics Research Center, Academia Sinica).

3.4. Cell Counting Kit-8 (CCK8) (Sigma).

3.5. Doxmed V-1 (Lot no.: 201907171D) was provided by Houchi Chemical Co., Ltd.

3.6. High-content imaging, ImageXpress Micro Imaging XL (Molecular Devices).

3.7. The Doxmed V-1, serially diluted with cell culture medium (2% Fetal Bovine Serum + Dulbecco's Modified Eagle Medium), were incubated with SARS-CoV-2 (TCDC#4, 100 PFU) at room temperature for 1 h. The mixtures were then added to Vero-E6 cells for 1 day incubation. Cells were fixed and immunostained with anti-SARS-CoV-2 N protein antibody and goat anti-human IgG-Alexa Fluor 488 (A11013, Invitrogen). Cell nucleus was stained with DAPI (4', 6-Diamidino-2-phenylindole

dihydrochloride, D1306, Invitrogen). To quantify viral infection, images were acquired and analyzed using an ImageXpress Micro XLS Wide field High-Content Analysis System. For cell viability test, Vero-E6 cells were treated with different dilutions of Doxmed V-1 with cell culture medium for 1 day at 37 °C. The cell viability was determined by Cell Counting Kit-8 (CCK-8). 50% inhibition concentration (IC50) and 50% cytotoxic concentration (CC50) were calculated by Prism software. [1]

3.8. The virus titer was determined by virus titration based on median tissue culture infectious dose ($TCID_{50}$). The Doxmed V-1, serially diluted with cell culture medium, was incubated with SARS-CoV-2 (TCDC#4) at room temperature for 1 hr. The mixtures were then 10-fold serially diluted with cell culture medium and added to Vero E6 cells for 4 day incubation. Cells were fixed with 10% formaldehyde for overnight and stained with 0.5% crystal violate for 20 min. The plates were washed with tap water and scored for infection. The $TCID_{50}$ was calculated by Reed and Muench Method [3].

3.9. The formula of log reduction value of virus titer is as follows.

Virus reduction for each step is:

$$LRV = \frac{Input\ virus\ titer/volume \times Input\ volume}{Output\ virus\ titer/volume \times Output\ volume}$$

4. RESULTS

The First Cell-based Antiviral Assay, Infection Rate Determined by Immunofluorescent Assay:

To test the antiviral potential of Doxmed V-1 from Houchi Chemical, SARS-CoV-2 pretreated with serially diluted Doxmed V-1 was added to Vero-E6 cells for incubation. The viral infection was quantified by SARS-CoV-2 N protein expression **(Figure 1A)** and cytotoxicity of the compound was determined by CCK-8 assay. The fluorescent signal was quantified by high-content imaging and calculated the IC50 and CC50 **(Figure 1B)**. Doxmed V-1 showed anti-SARS-CoV-2 activity with IC50 at 718.8-fold dilution, however high

cytotoxicity was noted with CC50 at 297.8-fold dilution.

The Second Cell-based Antiviral Assays, Disinfection Efficacy Determined by Median Tissue Culture Infectious Dose ($TCID_{50}$):

We further tested the disinfectant activity of Doxmed-V1. For untreated virus alone control, the SARS-CoV-2 titer was $10^{8.33}$ $TCID_{50}$/ml. For the test group, at 6.25-fold, 12.5-fold, and 25-fold dilution of Doxmed V-1, the virus titer was lower than 10^3 $TCID_{50}$/ml, partly due to the cytotoxicity of Doxmed V-1. At 50-fold dilution of Doxmed V-1, the virus titer was lower than 10^2 $TCID_{50}$/ml. At 100-fold, 200-fold, and 400-fold dilution of Doxmed V-1, the virus titers were $10^{3.33}$, $10^{4.67}$ and $10^{5.67}$ $TCID_{50}$/ml, respectively **(Figure 2)**. The Log-Reduction Value (LRV) of Dexmed-V1 at different dilution is shown in **Table 1**. For cytotoxicity test, different concentrations (100-fold and 300-fold dilution) of Doxmed V-1 were 10-fold serially diluted (10^0 ~ 10^{-7}), and added to Vero-E6 cells for 24 hrs. The cytotoxicity was monitored with cytopathic effect (CPE) as shown in **Table 2**.

Overall, at 100, 200 and 400-fold dilution, Doxmed V-1 could inactive SARS-CoV-2 with a LRV at 5, 3.66 and 2.66, respectively. The immunofluorescent assay also showed 200-fold and 400-fold dilution completely blocked SARS-CoV-2 infection. The best inhibition against SARS-CoV-2 was noted at 200~400-fold dilution in this cell-based study condition.

5. FIGURES and TABLES

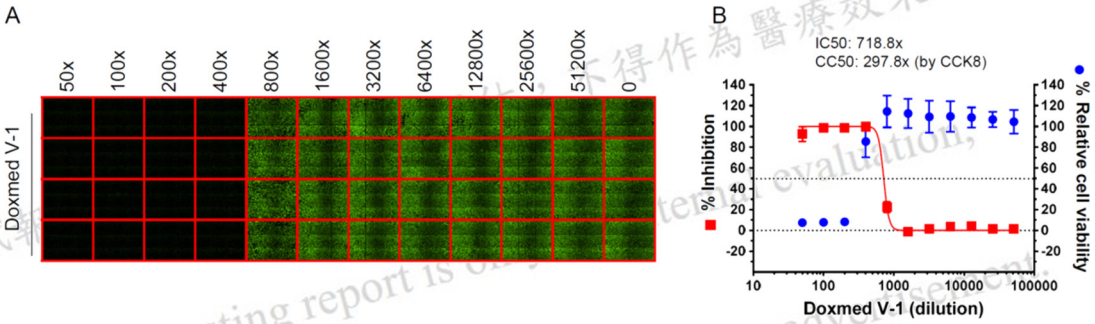

Figure 1. Anti-SARS-CoV-2 activity of Doxmed V-1 by immunofluorescent assay (IFA). (A) The serially diluted Doxmed V-1 was incubated with 100 PFU SARS-CoV-2 at room temperature for 1 h. The mixtures were then added to Vero-E6 cells for 1 day incubation. Cells were fixed and immunostained with anti-SARS-CoV-2 N protein antibody and goat anti-human IgG-Alexa Fluor 488. (B) SARS-CoV-2 N protein expression levels were acquired and analyzed by using a High-Content Analysis System. Cell viability were determined by CCK-8 assay. 50% inhibition concentration (IC50) and 50% cytotoxic concentration (CC50) were calculated by Prism software.

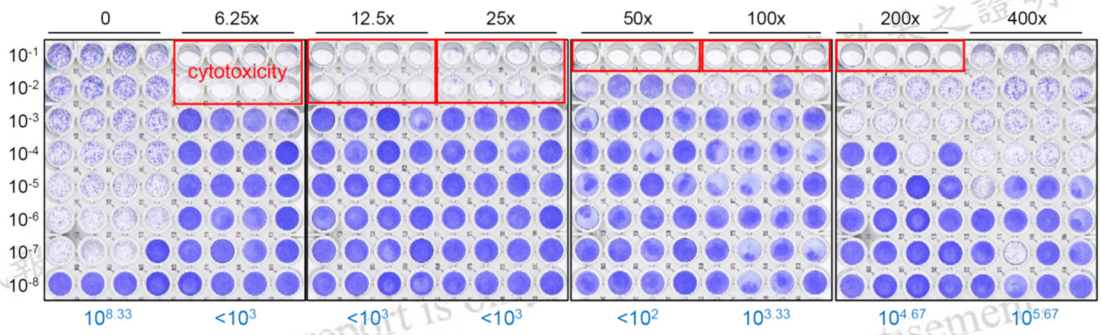

Figure 2. Disinfection efficacy of Doxmed V-1 was determined by $TCID_{50}$. The serially diluted Doxmed V-1 was incubated with SARS-CoV-2 (TCDC#4) at room temperature for 1 hr. The mixtures were then 10-fold serially diluted and added to Vero-E6 cells for 4 day incubation. Cells were fixed with 10% formaldehyde and stained with 0.5% crystal violate for 20 min. The plates were washed with tap water and scored for infection. The red boxes indicate wells with cytotoxicity. $TCID_{50}$ was calculated by Reed and Muench Method [3].

Table 1

	LRV of Doxmed V-1 at different dilution						
Fold of dilution	6.25x	12.5x	25x	50x	100x	200x	400x
Log-reduction value	>5.33	>5.33	>5.33	>6.33	5	3.66	2.66

Table 2

Cytotoxicity of Doxmed V-1									
Fold of dilution		10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
V1	100x	+ (0.059%)	+ (71.7%)	- (93.5%)	- (94.5%)	- (93.5%)	- (93.2%)	- (94.2%)	- (94.0%)
	300x	+ (13.29%)	- (90.77%)	- (96.8%)	- (98.1%)	- (96.5%)	- (96.5%)	- (98.8%)	- (99.9%)

The number in brackets indicates the ratio of surviving cells.

6. REFERENCES

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2. British standard. Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of virucidal activity in the medical area. Test method and requirements (phase 2, step 1). UK. 2019. (BS EN 14476:2013+A2:2019).
3. Reed, L. J., and H. Muench. 1938. A simple method of estimating 50 per cent end-points. Amer. Jour. Hyg., 27: 493-497.