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INHIBITION OF AVIAN INFLUENZA A VIRUS H5N1 BY USING METHYLENE BLUE WITH LASER TREATMENT *IN VITRO*

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ABSTRACT

The avian influenza virus H5N1 causes the death of millions of avians, including the risk of viral infection in humans. In photodynamic therapy, photosensitizers with light and molecular oxygen might result in the death of cells by generating cytotoxic molecules. Photosensitizers such as methylene blue and laser treatment have been demonstrated to have high efficacy against several types of viruses. The anti-H5N1 effects of several photosensitized compounds, such as methylene blue with laser treatment, are not known. We found that the titer of the ST-2009 virus was 10×10^7 pfu/mL via the MDCK plaque assay. Methylene blue treatment at 0.1 to 1.0 µM had safe effects on MDCK cells. In this study, we demonstrated that the titers of the ST-2009 virus were 3.9 log10 pfu/mL, 5.7 log10 pfu/mL and 6.8 log10 pfu/mL without the methylene blue treatment, but no ST-2009 virus titer was detected after treatment with 0.5 µM methylene blue. In addition, the survival of the ST-2009-infected MDCK cells was significantly greater when the cells were treated with 0.5 to 1.0 µM methylene blue in combination with a laser for 80 s at 16 J/cm^2 than in the nontreated control. The survival rates of the MDCK cells were 98.1 ± 0.2 and 96.7 ± 0.4% when the laser treatment (80 s and 16 J/cm^2) was combined with 0.5 and 1.0 µM methylene blue, respectively. In addition, without laser treatment or the addition of 0.5 or 1.0 µM methylene blue, the percentages of living cells significantly decreased to 8.3 ± 0.3 and 6.3 ± 0.2%, respectively. The survival of MDCK cells without the addition of methylene blue was 7.5 ± 0.4%, whereas that without laser treatment was 4.6 ± 0.5%. In summary, photosensitizers such as methylene blue with laser treatment can be suitable therapies for the inhibition of avian influenza A virus H5N1.

Keywords: laser, MDCK assay, methylene blue, ST-2009 virus, virus titer

INTRODUCTION

The highly pathogenic avian influenza (HPAI) H5N1 virus, which causes pandemics in poultry, continues to circulate and represents a significant threat to poultry, mammals and even human health. Avian influenza A viruses are enveloped RNA viruses belonging to the Orthomyxoviridae family, featuring a single-stranded, negative-sense genome composed of eight segments (Bouvier *et al*., 2008). From 2023 to the beginning of 2024, there were nearly 900 human cases of infection with influenza A (H5N1) in the world, including 463 deaths. Almost all cases of human infection with avian influenza A (H5N1) have been linked to infected live or dead birds (WHO, 2024). With respect to the formation of clades, notably, in the population of pathogenic viruses in Vietnam, there have been great fluctuations, with the appearance and disappearance of several strains of H5N1 viruses belonging to different clades (Nguyen *et al.*, 2022).

Several drugs against influenza A viruses, such as oseltamivir, are approved for the treatment of these viruses (Hooke *et al.*, 2021). In addition, various photosensitizers, such as neutral red, toluidine blue O, and methylene blue, are used in the inactivation of herpesvirus or in the treatment of oral infections without causing toxicity to humans (Embleton *et al*., 2005; Marotti *et al*., 2010). Photodynamic therapy (PDT) has recently been used to treat several viruses. PDT can target HSV-1, VSV, HIV-1, coronaviruses, etc. (Lim *et al*., 2011; Abe *et al*., 1995; Wainwright *et al*., 2000; Vzorov *et al*., 2002). The basic step of PDT is the formation of free radicals (ROS), which are harmful to bacteria or viruses.

In this study, we describe a novel promising approach utilizing methylene blue, either alone or in combination with laser, to inhibit recombinant avian influenza A virus that presents a potential therapeutic strategy for this type of virus.

MATERIALS AND METHODS

Virus strain

The recombinant virus accommodating HA and NA from A/duck/Vietnam/ST0907/ 2009 H5N1 (clade 1.1.2) (namely, ST-2009) produced on the PR8 background was previously described and stored in a freezer at -70°C for subsequent experiments (Thu Hang *et al.*, 2018; Hoang *et al*., 2020). The 50% tissue culture infectious dose ($TCID_{50}$) in the cell culture supernatant was identified by endpoint titration in MDCK cells, as described previously (Siegers *et al*., 2019). The virus stock was adapted after one passage in embryonated chicken eggs at a titer of approximately 10^7 $TCID_{50}$. All laboratory work involving H5N1 viruses was performed in biosafety facilities in accordance with the regulations of the Institute of Biotechnology (IBT, Vietnam).

MDCK plaque assay

Madin-Darby canine kidney (MDCK) cells were purchased from ATCC (VA, USA) and cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma). The plaque assay was performed in 6-well plates containing 80% confluent MDCK cell monolayers as previously described (Hung *et al.,* 2009). The cells were inoculated with the ST-2009 virus at a titer of 10^7 $TCID_{50}$. Following a 1-hour incubation at 35°C, the infection media were

aspirated. Each well in the plate was overlaid with 3 mL of agar media containing various concentrations of methylene blue. The plates were then incubated for 2 days at 35°C in a CO_2 incubator. After incubation, the cells were fixed by adding 1 mL of 10% formaldehyde and left at room temperature for 1 hour. Once the formaldehyde was removed, the cells were stained with 0.5% crystal violet for 15 minutes at room temperature. The plates were subsequently washed and dried, and the plaque-forming units (pfu)/mL titer was determined as previously described (Baer *et al*., 2014).

$$\text{Pfu/mL} = \frac{The\ number\ of\ plaques}{D\ x\ V}$$

D: dilution factor, V: volume of diluted virus added to the well

Laser treatment assay

Light exposure of virus-infected MDCK cell culture monolayers was performed via photodynamic equipment (PDT-LAS-01) designed by the Institute of Materials Science (Vietnam Academy of Science and Technology), operating at a wavelength of λ = 660 nm. This experiment was conducted in a box containing plastic tubes and culture vessels with a bottom area of 40 cm^2, which supported the cell culture monolayers. A light energy dose of approximately 16 J/cm^2 was applied for 80 s via PDT-LAS-01, following a previously described protocol with minor modifications. (Svyatchenko *et al*., 2021).

Plaque reduction assay

Ten microliters of methylene blue were added to 190 µL of ST-2009 dilutions at various titers (10^3, 10^5, 10^7, and 10^8 pfu/mL) to obtain final concentrations of 0, 0.5, 1.0, 1.5, and 2.0 µM. The mixtures were then incubated in the dark at 35°C for 1 hour, after which the infectious viruses were quantified via a plaque reduction assay (Wei *et al.,* 2024).

Cell viability assay

The viability of MDCK cells treated with methylene blue was assessed via the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were counted and adjusted to a final concentration of 5×10^5 cells/mL. A volume of 1 mL of the cell suspension was added to each well of a 24-well plate, followed by incubation at 37°C and 5% CO_2 for 1 hour. Various concentrations of methylene blue were subsequently added to the wells. The test plate included control wells containing only 10% DMSO and blank wells containing only medium. The plate was incubated at 37°C and 5% CO_2 for 24 hours. After that, MTT solution (0.5 mg/mL) was added to each well and incubated for 4 hours. The plate was read by a microplate reader, and the absorbance of the samples was measured at 540 nm. Cell viability was determined via a previously described method (Luz *et al.*, 2022).

$$\%\ \text{Cell viability} = \left(\frac{OD_{\text{treated sample}} - OD_{\text{blank}}}{OD_{\text{control}} - OD_{\text{blank}}}\right) X\ 100\ (\%)$$

Statistical analysis

The experiments were performed two or three times in duplicate. The data are shown as the mean ± standard deviation (SD). The statistical analysis was performed via

Student's *t*-test, and $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Investigation of the virus titer of the ST-2009 strain via the MDCK plaque assay

To explore the virus titer of ST-2009, the virus was diluted in each well from 10^{-1} to 10^{-9} in the MDCK plaque assay (Figure 1). Figure 1 shows that with virus dilutions ranging from 10^{-1} to 10^{-6}, many cell lysates appeared, and the virus concentration could not be determined. The number of cell lysates gradually decreased and was determined at a 10^{-7} dilution. Therefore, the appropriate virus concentration for plaque assays ranges from 10^{-7}. This dilution was used for subsequent studies. At a virus dilution of 10^{-7}, the number of plaques was counted, and approximately 100 plaques formed. The titer of ST-2009 strain stocks passaged in eggs and titrated by plaque assays using MDCK cells is 10×10^7 pfu/mL.

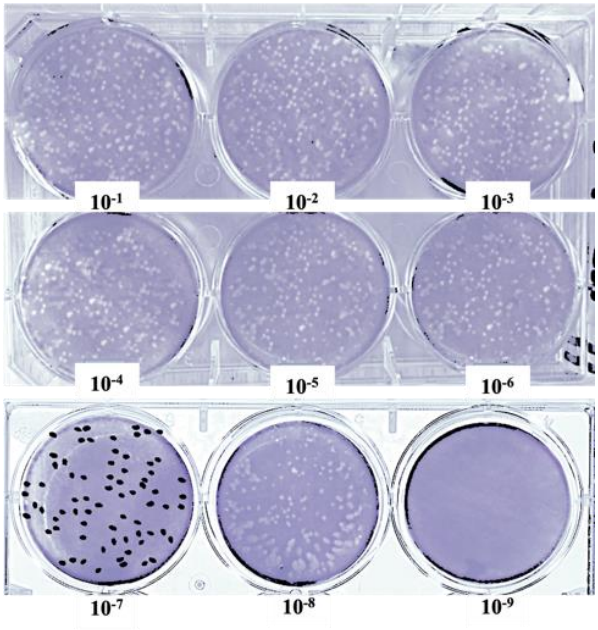

Figure 1. Titer of the ST-2009 virus according to the MDCK plaque assay. Plaque assays were performed using MDCK cells under standard conditions with crystal violet staining. Monolayers of MDCK cells were infected with dilutions of viruses ranging from 10^{-1} to 10^{-9}. The virus lysates (plaques) can be quantified at dilutions of 10^{-7}. The data are representative of three independent experiments.

Cytotoxicity of methylene blue

Photosensitizers such as methylene blue should have energy levels that allow for easy generation of ROS upon stimulation while maintaining nontoxicity to normal tissues and cells. Before the antiviral activities were examined, the effects of methylene blue on cell growth and viability were tested in MDCK cells via the MTT assay. Compared with 0 μM methylene blue, 0.1–1.0 μM methylene blue did not induce cytotoxicity. The percentage viability of MDCK cells after treatment with various concentrations of methylene blue (0, 0.1, 0.2, 0.3, 0.5, and 1.0 μM) was 99, 98, 97, 96, 94, and 92%,

respectively (Figure 2). Therefore, this tested range of methylene blue concentrations was used for subsequent experiments.

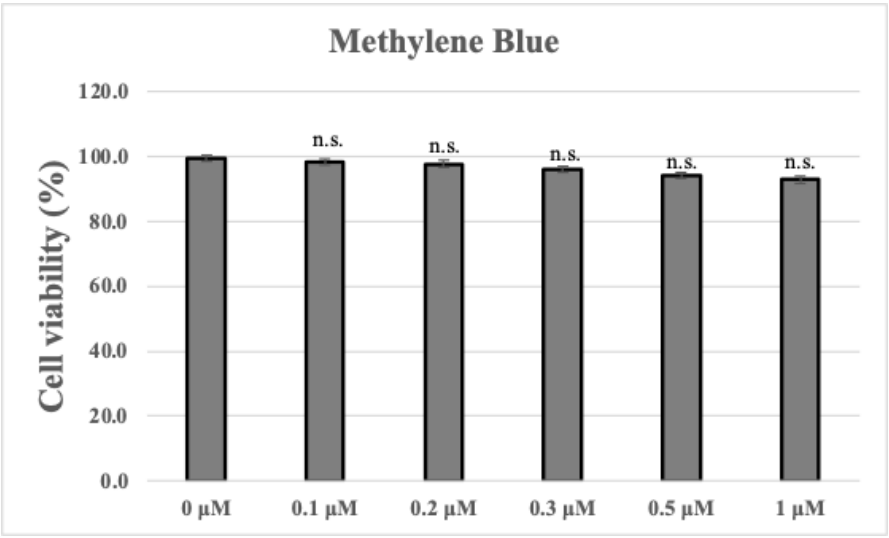

Figure 2. Cytotoxicity of methylene blue in MDCK cells. The data are presented as the means ± SDs of three independent experiments. Cell viability was determined via the MTT assay. Statistical analysis was performed using the Student's *t*-test. All data are presented as means ± SDs from three independent experiments. n.s. indicates not significant ($p > 0.05$) vs. control (0 µM treated) sample.

The extracellular virucidal activity of methylene blue

To investigate the antiviral activity of methylene blue in vitro, we initially employed a plaque assay to test whether methylene blue exhibits extracellular virucidal activity through a plaque reduction assay in the dark. Different concentrations of methylene blue (0, 0.5, 1.0, 1.5, and 2.0 µM) were incubated with different concentrations of ST-2009 (10^3, 10^5, 10^7, and 10^8 pfu/mL) at 35°C for 1 h, after which the infectious virus particles were quantified via a plaque assay after serial dilution (Figure 3). Infectious titers were not determined after treatment with 0.5 µM methylene blue. The titers of the ST-2009 virus were 3.9 log10 pfu/mL, 5.7 log10 pfu/mL and 6.8 log10 pfu/mL without methylene blue treatment, as shown in Figures 3A, 3B, and 3C, respectively, but no virus titer was detected after treatment with methylene blue at a concentration of 0.5 µM. The titer of ST-2009 at 7.9 log10 pfu/mL decreased to 3.3 log10 pfu/mL after treatment with 0.5 µM methylene blue, and infectious titers were not determined after treatment with 1.0 µM methylene blue, as shown in Figure 3D. These findings suggest that methylene blue has significant extracellular virucidal activity.

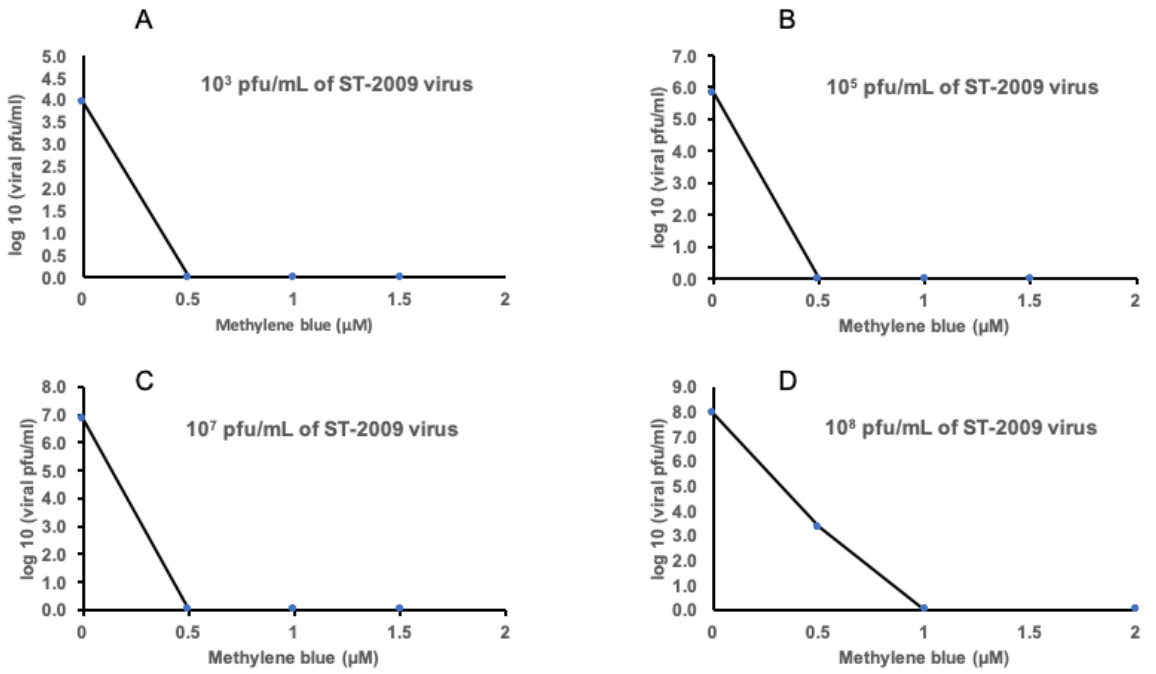

Figure 3. Virucidal activity of methylene blue outside of the cells. Different concentrations (0, 0.5, 1.0, 1.5, and 2.0 μM) of methylene blue were incubated with different titers of ST-2009 (10^3, 10^5, 10^7, and 10^8 pfu/mL) for 1 hour at 35°C, followed by titration via a plaque assay in the dark. A, B, and C show no infectious titers after treatment with 0.5 μM methylene blue. D indicates that no virus was detected after treatment with 1.0 μM methylene blue.

Laser treatment is a potent antiviral agent against influenza A virus

Finally, we investigated the survival of virus-infected MDCK cells in response to methylene blue and laser treatment. We infected the cells with 10×10^7 pfu/mL ST-2009 virus along with a combination of different methylene blue treatments (0.5 and 1.0 μM) with laser exposure for 80 s at a 16 J/cm^2 dose. The antiviral efficacy was defined as the viability of the cells at the end point, as measured by the MTT assay (Figure 4). Finally, we investigated the survival of 10×10^7 pfu/mL ST-2009 virus-infected MDCK cells in response to methylene blue and laser treatment. In the presence of laser treatment at 80 s at a 16 J/cm^2 dose and the addition of 0.5 and 1.0 μM methylene blue, 98.1 ± 0.2% and 96.7 ± 0.4% of the cells, respectively, survived. In addition, with the addition of 0.5 and 1.0 μM methylene blue and without laser treatment, the percentages of living cells significantly decreased to 8.3 ± 0.3% and 6.3 ± 0.2%, respectively. In the absence of methylene blue, the percentage of living cells following laser treatment was 7.5 ± 0.4%, which was greater than that without laser treatment (4.6 ± 0.5%). These findings demonstrated the virus inhibitory ability of the combination of PS and laser treatment and thus suggested the potential for the combined use of these two methods as virus inhibitors. Whether methylene blue and lasers inhibit the ST-2009 H5N1 virus by affecting nucleic acid, leading to a loss of virus replication ability, inhibiting the virus's reverse transcriptase

enzyme, affecting virus envelope proteins, leading to the inhibition of virus attachment to host cells or oxidizing the virus with singlet oxygen, should be further investigated.

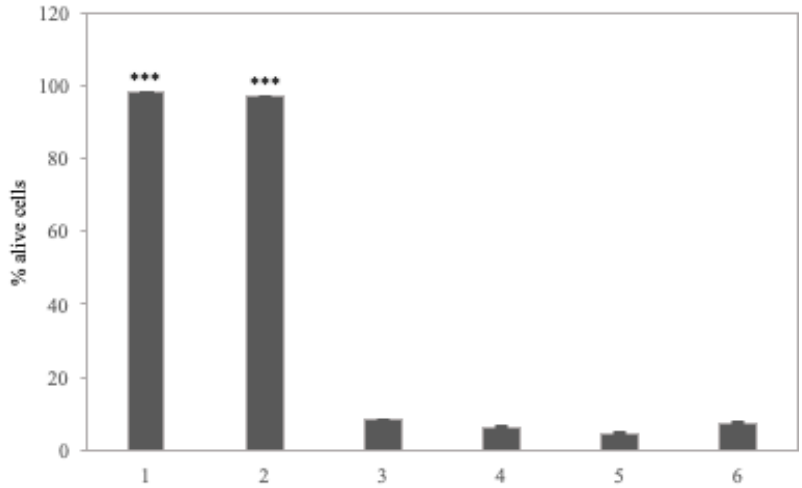

Figure 4. Efficacy of methylene blue and laser irradiation on MDCK cells infected with the ST-2009 virus. The data are presented as the means ± SDs of two independent MTT tests. MDCK cells were infected with the ST-2009 virus and then treated with different concentrations of methylene blue and a laser (16 J/cm^2, 80 s). Cell viability was determined by the MTT assay. **1.** 10 × 10^7 pfu/mL ST-2009 virus and the addition of methylene blue at a concentration of 0.5 µM, followed by laser treatment for 80 s at a 16 J/cm^2 dose; **2.** 10 × 10^7 pfu/mL ST-2009 virus and the addition of methylene blue at a concentration of 1.0 µM, followed by laser treatment for 80 s at a 16 J/cm^2 dose; **3**. 10 × 10^7 pfu/mL ST-2009 virus and the addition of methylene blue at a concentration of 0.5 µM without laser treatment; **4.** 10 × 10^7 pfu/mL ST-2009 virus and the addition of methylene blue at a concentration of 1.0 µM without laser treatment; **5.** 10 × 10^7 pfu/mL ST-2009 virus and no addition of methylene blue without laser treatment; **6.** 10 × 10^7 pfu/mL ST-2009 virus and no addition of methylene blue, following laser treatment for 80 s at a 16 J/cm^2 dose. Statistical analysis was performed using the Student's *t*-test. All data are presented as means ± SDs from three independent experiments. ***p < 0.001 vs. non-treated samples.

CONCLUSION

In conclusion, 0.5 µM methylene blue can decrease the titer of the ST-2009 virus at 10^5 to 10^7 viral pfu/mL, whereas 1.0 µM methylene blue can decrease the titer of the ST-2009 virus at 10^8 viral pfu/mL. In addition, the combination of methylene blue (0.5 and 1.0 µM) and laser treatment for 80 s at 16 J/cm^2 significantly inhibited the presence of the ST-2009 virus in the MDCK plaque assay. PDT, including a photosensitizer such as methylene blue with a laser, can be a potential treatment for avian influenza virus H5N1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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