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Comparison of the antiviral effect of solid-state copper and silver compounds

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HIGHLIGHTS

- Antiviral activities were comparatively evaluated using copper and silver compounds.
- Solid-state Cu_2O showed superior activity against enveloped and non-enveloped viruses.
- Exposure to Cu_2O preferentially inactivated infection ability of influenza viruses.
- Cu_2O has a unique antiviral mechanism mediated by direct contact.

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ABSTRACT

Antiviral activities of insoluble solid-state and soluble ionic copper and silver compounds were evaluated against influenza A virus (A/PR8/H1N1) possessing a viral envelope and bacteriophage Qβ lacking an envelope. The viral solutions were exposed on glass samples uniformly loaded with copper and silver compounds. Exposure to solid-state cuprous oxide (Cu_2O) efficiently inactivated both influenza A virus and bacteriophage Qβ, whereas solid-state cupric oxide (CuO) and silver sulfide (Ag_2S) showed little antiviral activity. Copper ions from copper chloride ($CuCl_2$) had little effect on the activity of bacteriophage Qβ in spite of the fact that copper ions strongly inactivate influenza A in previous studies. Silver ions from silver nitrate ($AgNO_3$) and silver(I) oxide (Ag_2O) in solution showed strong inactivation of influenza A and weak inactivation of bacteriophage Qβ. We also investigated the influence of the compounds on the function of two influenza viral proteins, hemagglutinin and neuraminidase. Silver ions from $AgNO_3$ and Ag_2O remarkably decreased enzymatic activity of neuraminidase through the breakage of disulfide (S—S) bonds, corresponding to the selective inactivation of influenza A virus. By contrast, exposure to Cu_2O markedly reduced the activity of hemagglutinin rather than neuraminidase. These findings suggest that solid-state Cu_2O disrupts host cell recognition by denaturing protein structures on viral surfaces, leading to the inactivation of viruses regardless of the presence of a viral envelope.

1. Introduction

Throughout history, infectious diseases caused by viruses have posed a serious threat to human populations worldwide. In recent times, Ebola virus caused more than 11,290 deaths in Africa [1], and over 160 people were infected with Middle East respiratory syndrome coronavirus in South Korea [2], illustrating the devastating effects of viruses on international communities. Influenza A virus,

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the causative agent of influenza in birds, can also lead to highly infectious respiratory disease in mammals. Viral genome mutations (antigenic drift) and periodic gene segment reassortment between humans and animals (antigenic shift) can result in the emergence of new viral pathogens, with the potential to cause serious pandemics. During the past 100 years, pandemic influenza occurred in 1918, 1957, 1968, and 2009 [3]. The most severe pandemic, in 1918, resulted in approximately 40 million deaths worldwide [4], while the most recent pandemic, the Swine flu pandemic of 2009, emerged in 214 countries and caused more than 18,000 deaths, according to a World Health Organization report [5]. Highly pathogenic H5N1 avian influenza was first identified in Hong Kong [3]. As the high pathogenicity of such a strain and the lack of host protective immunity against such viruses continue to threaten lives, effective infection control measures are crucial for the prevention of serious outbreaks.

Viral particles released from infected patients through coughing or sneezing can survive for prolonged periods in the air and on surfaces. Viral transmission occurs through direct person-to-person contact or through the uptake of contaminated airborne droplets or contact with contaminated surfaces [6]. The use of antiviral materials is an effective way to inactivate viral particles in the environment preventing viral transmission and thereby lowering the risk of infection [7,8]. For this purpose, solid-state inorganic compounds such as metal oxides are good candidates because of their chemical robustness and their feasibility for use as a coating material. Copper and silver have been recognized for their biocidal properties since ancient times [9,10]. However, unlike their ionic forms (Cu^{2+} and Ag^{+}), the biocidal properties of solid-state copper and silver compounds such as CuO, Cu_2O and Ag_2S, that are poorly soluble in water, remain to be fully elucidated.

We recently reported that solid-state copper(I) compounds, including copper(I) oxide (Cu_2O), sulfide (Cu_2S), and iodide (CuI), efficiently inactivated bacteriophages and bacteria [11]. The inactivation of bacteriophages is mainly mediated by direct contact with solid-state copper(I) compounds, rather than exposure to reactive oxygen species or leached copper ions. Here, we investigated the antiviral activities of four groups of compounds, solid-state copper compounds (Cu_2O and CuO), a copper ionic compound ($CuCl_2$), a solid-state silver compound (Ag_2S), and silver ionic compounds ($AgNO_3$ and Ag_2O), against pathogenic influenza A virus and bacteriophage Qβ. The effects of these compounds on the surface proteins of influenza viruses, hemagglutinin (HA), and neuraminidase (NA), were also investigated to reveal mechanistic insights into viral inactivation by these compounds.

2. Experiments

2.1. Preparation of copper and silver compounds

All cuprous, copper and silver compounds were obtained from Wako Pure Chemicals (Tokyo, Japan). The diameters of the particles were in the range of 0.5–5 μm in cuprous oxide (Cu_2O), 0.5–40 μm in cupric oxide (CuO), and 3–60 μm in silver sulfide (Ag_2S). The measured BET surface areas of Cu_2O, CuO, and Ag_2S particles were 1.23, 1.20, and 0.14 m^2/g, respectively. Ethanol suspensions of the compound particles were uniformly spreading on glass slides (2.5 cm × 2.5 cm), followed by heating at 120 °C for 3 h for sterilization. The particles were spread at 2.1 μmol metal (copper or silver), corresponding to 0.24 g/m^2 for Cu_2O, 0.27 g/m^2 for CuO, 0.36 g/m^2 for Ag_2O, and 0.42 g/m^2 for Ag_2S. Although Ag_2O is moderately water-soluble, glass samples were prepared with the same procedures as other solid-state compounds. In the case of water-soluble compounds, $CuCl_2$ and $AgNO_3$, the dried particles (0.45 g/m^2 for $CuCl_2$ and 0.57 g/m^2 for $AgNO_3$) from the ethanol solution on the glass substrate at room temperature were used to avoid oxidation by heating treatment.

2.2. Virus strains

The human influenza A virus reference strain (A/PR8/H1N1) used in this study was purchased from the American Type Culture Collection (Manassas, VA, USA). For viral propagation, 11-day-old embryonated chicken eggs were inoculated with the virus, incubated at 35.5 °C for 48 h, chilled at 4 °C for 12 h, and virus was then harvested. Harvested virus particles were purified and concentrated using a combination of depth filters (5.0 μm polypropylene depth filters, GE Infrastructure, USA) and membrane filters (0.45 μm polypropylene mesh microfiltration and cross-flow ultrafiltration polysulfone hollow-fiber filters, 750-kDa cutoff; GE Infrastructure, USA), as previously described [12]. The concentrated virus particles were further purified by sucrose density gradient centrifugation using a 10–60% linear sucrose gradient [13]. The purified samples contained 10 mg/mL protein, as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Virus stocks were stored at −80 °C between all experimental manipulations.

Viral stocks were thawed for the titer assay, described below, and each 200 μL experimental sample was placed in a presterilized centrifuge tube. All experimental samples were adjusted to 0.1 mg/mL protein and were supplemented with 0.01% bovine serum albumin as a stabilizer prior to performing measurements.

Bacteriophage Qβ (NBRC 20012) was also used to evaluate the antiviral activities of the copper and silver compounds. The stock viral suspension was prepared according to a previous report [11]. A suspension of the bacteriophage was mixed with *Escherichia coli* (NBRC 13965) cells at 35 °C for 10 min. The mixture was then plated onto agar medium to form a double agar layer. After overnight incubation at 35 °C, the bacteriophage from the top agar layer was extracted into SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 50 mM Tris–HCl [pH 7.5], and 0.1% gelatin) at 4 °C overnight. The extract solution was centrifuged (8000 × *g*, 4 °C, 20 min), and the supernatant was collected and filtered using a Millex filter (ϕ = 0.22 μm; Millipore, MA, USA). The stock suspension of bacteriophage Qβ was stored at −80 °C prior to use in the evaluation experiment.

2.3. Virus inactivation assay

To evaluate the effects of these compounds on influenza A virus or bacteriophage Qβ, viral suspensions were inoculated onto sample glass slides at room temperature, and incubated in the dark over a time range between 10 min and 1 h. After incubation, the viruses were harvested by shaking in a plastic case (ϕ 60 mm) in phosphate-buffered saline (PBS) buffer. Influenza A virus was titrated using Madin–Darby canine kidney cells, and a 50% tissue culture infective dose ($TCID_{50}$)/mL was determined according to the method of Reed and Muench [14]. The viral titers of bacteriophage Qβ were also determined from the number of plaques formed using the double-layer method, as described above.

2.4. Hemagglutination assay

The denaturation of HA after exposure to Cu_2O, CuO, $AgNO_3$, and Ag_2O was investigated. Twenty-five microliters of purified HA H1 (0.125 ng/μL; Abcam, Cambridge, UK) was mixed with an equal volume of copper or silver compound suspension and then incubated in the dark at room temperature. Solids were then removed by centrifugation at 5000 × *g* for 3 min using ultra-free tubes (ϕ = 0.22 μm; Millipore). Hemagglutinin samples were serially 2-fold diluted in a 96-well microplate and mixed with 25 μL of 2% chicken red blood cells (Nihon BD, Tokyo, Japan) washed in PBS.