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Inhibitory and combinatorial effect of diphyllin, a v-ATPase blocker, on influenza viruses

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ABSTRACT

An influenza pandemic poses a serious threat to humans and animals. Conventional treatments against influenza include two classes of pathogen-targeting antivirals: M2 ion channel blockers (such as amantadine) and neuraminidase inhibitors (such as oseltamivir). Examination of the mechanism of influenza viral infection has shown that endosomal acidification plays a major role in facilitating the fusion between viral and endosomal membranes. This pathway has led to investigations on vacuolar ATPase (v-ATPase) activity, whose role as a regulating factor on influenza virus replication has been verified in extensive genome-wide screenings. Blocking v-ATPase activity thus presents the opportunity to interfere with influenza viral infection by preventing the pH-dependent membrane fusion between endosomes and virions. This study aims to apply diphyllin, a natural compound shown to be as a novel v-ATPase inhibitor, as a potential antiviral for various influenza virus strains using cell-based assays. The results show that diphyllin alters cellular susceptibility to influenza viruses through the inhibition of endosomal acidification, thus interfering with downstream virus replication, including that of known drug-resistant strains. In addition, combinatorial treatment of the host-targeting diphyllin with pathogen-targeting therapeutics (oseltamivir and amantadine) demonstrates enhanced antiviral effects and cell protection *in vitro*.

1. Introduction

An influenza pandemic poses a serious threat to humans and animals, and it has incurred great financial and societal cost for decades. The most common pathogens causing influenza in humans are type A influenza viruses (seasonal H1N1, pandemic H1N1 and H3N2) and type B influenza viruses. Conventional treatments against the disease include two classes of antivirals: M2 ion channel blockers adamantanes (amantadine and rimantadine) and neuraminidase (NA) inhibitors (oseltamivir, peramivir, zanamivir and laninamivir) (Ison, 2011; Lee and Yen, 2012). These two categories are designed to target viral proteins, thereby interfering with the virus' infection mechanisms. However, the emergence of new influenza viral strains carrying drug resistant mutations that can outpace the development of pathogen-targeting antivirals presents a major clinical challenge. Most circulating influenza A viruses show resistance to adamantanes (Ison, 2011; Jackson et al., 2011; Lee and Yen, 2012; Moscona, 2009), and intrinsic resistance to the compound has also been observed in Influenza B (Ison, 2011). In addition, beginning with the 2007–2008 influenza season, circulating seasonal H1N1 viruses possessing the oseltamivir resistance mutation (His275Tyr) have been observed (Lackenby et al., 2008). Since the first reported case of pandemic H1N1 in 2009, oseltamivir-resistant variant strains have also been identified (Baz et al., 2009; Leung et al., 2009; Speers et al., 2010; Storms et al., 2012). The rapid development of antiviral resistance highlights the need for alternative therapeutic strategies.

Influenza virus is an RNA virus that undergoes rapid mutations under the selective pressure of drug use. Pathogen-targeting antiviral drugs that interact with specific viral enzymes can therefore be rendered ineffective against a mutant population. In contrast, host-targeting therapeutics intervening with infection pathways offers the sustained therapeutic potential regardless of viral mutation.

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The fusion of viruses with host cellular endosomal membranes, facilitated by a low endosomal pH (Stertz and Shaw, 2011), is a major event of the influenza infection cascade. Vacuolar ATPase (v-ATPase) activity, which is responsible for pumping protons into endosomal compartments, has been identified as a requirement for influenza virus replication in previous studies (Guinea and Carrasco, 1995; Muller et al., 2011; Perez and Carrasco, 1994). V-ATPase-encoding genes have also been identified in several genome-wide screens for host factors regulating influenza virus replication, and the knockdown of v-ATPase subunits has been shown to result in significant inhibition of influenza virus replications (Chin and Brass, 2012; Hao et al., 2008; Karlas et al., 2010; Konig et al., 2010; Mehle and Doudna, 2010). Blocking v-ATPase activity, therefore, presents an opportunity to impede influenza infection by preventing the low pH-dependent membrane fusion between endosomes and virions. In addition to influenza viruses, flaviviruses (Pierson and Diamond, 2012), vaccinia viruses (Townsley et al., 2006), rhabdoviruses (Albertini et al., 2012), and coronaviruses (Belouzard et al., 2012) also enter target cells in a pH-dependent fashion.

Diphyllin, a natural compound isolated from *Cleistanthus collinus*, has recently been identified as a novel v-ATPase inhibitor that can inhibit lysosomal acidification in human osteoclasts (Sorensen et al., 2007) and reduce v-ATPase expression in gastric adenocarcinoma cells (Shen et al., 2011). This study aims to characterize the application of diphyllin as an antiviral for various influenza virus strains in two types of cell lines. Bafilomycin A1, a macrolide antibiotic and a specific inhibitor of vacuolar ATPase which inhibits growth of type A and type B human influenza viruses in MDCK cells (Ochiai et al., 1995) was included in key functional assays as a control. In addition, combinatorial effects between the diphyllin and pathogen-targeting therapeutics, including oseltamivir and amantadine, were assessed to evaluate diphyllin's potential in enhancing existing influenza therapies.

2. Materials and methods

2.1. Compounds

Diphyllin (ChemBridge, San Diego, CA) (Charlton et al., 1996; Fukamiya and Lee, 1986) was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific), and oseltamivir carboxylate and amantadine hydrochloride (Sigma) were dissolved in sterile water. For all three compounds, 10 mM primary stocks and 100 μM working stocks were made in respective solvents and stored at −20 °C. Bafilomycin A1 (Sigma) was dissolved in DMSO to make a 10 μM working stock. Right before each experiment, compounds were freshly diluted in culture media to achieve desired concentrations.

2.2. Cells and viruses

Mardin-Darby canine kidney (MDCK) cells and A549 cells (both from ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the influenza virus infection experiments in MDCK cells, cells were overlaid with DMEM supplemented with 0.2% BSA, 25 mM HEPES buffer, and 2 μg/ml TPCK-treated trypsin. Reagents for cell culture were purchased from Invitrogen. All incubation and infection steps were carried out at 37 °C and with 5% CO_2 unless otherwise specified.

NS1-GFP virus, with a background of A/PuertoRico/8/34(H1N1) (Manicassamy et al., 2010) was kindly provided by Dr. Aldolfo Garcia-Sastre at Mount Sinai School of Medicine, New York. Two reference influenza virus strains A/Aichi/2/68(H3N2) (VR-547) and B/Taiwan/2/62 (VR-1735) were purchased from ATCC. In addition, three human influenza virus isolates, A/San Diego/21/2008(H1N1), A/San Diego/61/2008(H1N1), and A/San Diego/1/2009(H1N1 pdm09) were used in this study. Avian influenza virus A/Duck/Yilan/2904/99(H6N1) was isolated from duck in Yilan, Taiwan. All types of influenza viruses were propagated in MDCK cells and titrated with plaque assays as previously described (Szretter et al., 2006). The dengue virus serotype 2 (DENV2) strain S221, a triple-plaque-purified clone from a clinical isolate, was cultured and titrated with plaque assays as previously described (Yauch et al., 2009).

2.3. In vitro cytotoxicity assay of diphyllin

MDCK cells and A549 cells were grown in a 96-well clear polystyrene microplate (Corning) at a density of 10,000 cells per well 1 day prior to experiment. Diphyllin was twofold serially diluted in cell media and added to the cell monolayer in four replicates. The final DMSO concentration was no more than 0.5% in all wells. After 3 days, the culture supernatant was removed and 100 μl of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 1 mg/ml in PBS) was added to each well and incubated at 37 °C for 3 h. Next, MTT was removed and 100 μl of DMSO was added to each well to solubilize the purple formazan crystals. Absorbance values were measured at 570 nm with a reference wavelength of 650 nm on a Tecan Infinite M200 reader (Tecan Group, Männedorf, Switzerland). Control cell wells (without diphyllin treatment) were assumed to represent 100% cell viability. Normalized cell viability data were plotted against diphyllin concentrations and fitted to a non-linear regression curve using Graphpad Prism (GraphPad Software, San Diego, CA). The 50% cytotoxicity concentration (CC_{50}, the concentration of diphyllin at which cellular viability was reduced to 50%) was obtained accordingly.

2.4. Acridine orange labeling

Confluent cultures of MDCK cells or A549 cells in 96-well black polystyrene clear bottom microplates (Corning) were incubated with bafilomycin A1 or various concentrations of diphyllin in four replicates at 37 °C for 20 min. Acridine orange (1 μg/ml in media) (Molecular probes) was then distributed to each well and incubated at 37 °C for an additional 10 min before wash. Fluorescence images were obtained and data was quantified on iCys Research Imaging Cytometer (Compucyte, Westwood, MA) using 488 nm excitation/532 nm emission filters for green fluorescence and 560 nm excitation/610 nm emission filters for red fluorescence.

2.5. Time-of-addition assay of diphyllin

MDCK cells were seeded in a 12-well plate (200,000 cells/well) 1 day before experiment to obtain cultures with 80% confluency. Diphyllin (2 μM) was added to the cells at three different time points relative to virus infection: 1 h prior to infection, same time as infection, or 1 h after infection. Untreated wells were used as controls. In this experiment, NS1-GFP virus at a multiplicity of infection (MOI) of 0.01 was used to infect the cells. After a 1-h period of infection, all test cells were washed and overlaid with fresh media containing 2 μM of diphyllin. After 24 h, the cells were washed and lysed to analyze viral nucleoprotein (NP) expression using Western blotting (Section 2.11).

2.6. In vitro antiviral activity assay of diphyllin

The following method regarding cellular incubation with diphyllin and virus infection was used throughout this study to examine the antiviral effect of diphyllin. Various concentrations