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# Screening and identification of inhibitors against influenza A virus from a US drug collection of 1280 drugs

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ABSTRACT

Infection with influenza A virus is still a global concern since it causes significant mortality, morbidity and economic loss. New burst pandemics and rapid emergence of drug-resistance strains in recent years call for novel antiviral therapies. One promising way to overcome this problem is searching new inhibitors among thousands of drugs approved in the clinic for the treatment of different diseases or approved to be safe by clinical trials. In the present work, a collection of 1280 compounds, most of which have been clinically used in human or animal, were screened for anti-influenza activity and 41 hits (SI > 4.0) were obtained. Next the 18 hit compounds with SI >10.0 were tested for antiviral activity against 7 other influenza virus strains in canine-originated MDCK cells, 9 compounds exhibited broad antiviral spectrum. The antiviral effects of the 9 compounds were also confirmed in human-originated A549 cells and chicken-originated DF1cells, by infectious virus yield reduction assay and indirect immunofluorescent assay. Results from the time of addition assay showed that the 9 candidates impaired different stages of influenza virus life cycle, indicating they are novel inhibitors with different mechanisms compared with the existing M2 ion-channel blockers or neuraminidase (NA) inhibitors. Taken together, our findings provide 9 novel drug candidates for the treatment of influenza virus infection. Further mechanism of action study of these inhibitors may lead to the discovery of new anti-influenza targets and structure–activity relationship (SAR) study can be initiated to improve the efficacy of these new classes of influenza inhibitors.


## 1. Introduction

Influenza A virus is still an important human pathogen that causes yearly epidemics and periodic pandemics worldwide. The latest pandemic emerged in Mexico in April 2009 accounted for an estimated 284,500 deaths around the world (Dawood et al., 2012). In May 2013, H7N9 was first reported to infect human in eastern China with a relative high death rate (45 deaths in 139 confirmed human cases), which caught global attention about the highly pathogenic avian influenza (Gao et al., 2013; Li et al., 2013; Uyeki and Cox, 2013). Currently, there are only two classes of antiviral drugs approved by the FDA for the treatment and prophylaxis of influenza infection, namely M2 ion-channel [M2] blockers (amantadine and rimantadine) and the neuraminidase (NA) inhibitors (NAIs) (oseltamivir and zanamivir) (De Clercq, 2006). However, the rapid emergence of drug-resistance influenza A virus strains highlights the urgent need to identify novel drug targets and develop new classes of antiviral drugs.

High cost and lengthy approval process restricts the development of new antiviral drugs for clinical use. An innovative strategy to combat these problems is selective optimization of side activities of drug molecules (the SOSA approach), which searches new pharmacological targets using old drugs (Wermuth, 2004, 2006). Since the safety and pharmacokinetics profiles of these old drugs have already been assessed in human or animals, the potential hits can be directly tested in the clinic or as pre-drugs for further development. This strategy has been applied for searching new antimalarial, antibacterial and antiviral drugs in the last few years (Gastaminza et al., 2010; Imperi et al., 2013; Lamontagne et al., 2013; van Cleef et al., 2013; Weisman et al., 2006). To be more specifically, a number of clinical-used or investigational drugs like probenecid (Perwitasari et al., 2013b), paracetamol (Lauder et al., 2011), aminobisphosphonates pamidronate (PAM) (Tu et al., 2011), eritoran (Shirey et al., 2013), niclosamide (Jurgeit et al., 2012) and bortezomib (Dudek et al., 2010) had been found to inhibit influenza replication both *in vitro* and *in vivo* with various mechanisms of action. Nitazoxanide, a drug licensed by the FDA

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for treating the parasites *Cryptosporidium* and *Giardia* in children and adults (Wright, 2012), had been assessed in adults and adolescents with acute uncomplicated influenza in a phase II/III study since it first reported to inhibit the influenza virus replication in 2009 (Rossignol et al., 2009). Another Phase III study is starting to further demonstrate its efficacy, making it a promising anti-influenza drug in the next 10 years (Hurt et al., 2012). All the results suggest that SOSA approach is an effective strategy to identify new inhibitors of influenza virus from drug library consisting of old drugs or pre-drugs.

Recently, using a cell-based screening assay that covers the complete life cycle of influenza virus, we identified germacrone as a potent inhibitor of influenza virus from a small molecular library of traditional Chinese herbal medicines (Liao et al., 2013). In the present study, the MicroSource (Gaylordsville, CT) compound library, a US drug collection of 1280 compounds, has been screened for anti-influenza activities using the cell-based assay system. Most of the compounds in this library are approved for animal or human use and their pharmacological and toxicological profiles have been defined and published. Each compound has been assigned USAN or USP status and is included in the USP Dictionary (U.S. Pharmacopeia), the authorized list of established names for drugs in the USA. Our findings reported here the identification of a panel of novel anti-influenza agents with diverse mechanisms of action and the potential use in the treatment of influenza.

## 2. Materials and methods

### 2.1. Cell lines and virus strains

The Madin–Darby Canine Kidney (MDCK) cells (ATCC CCL-34) and chicken embryonic fibroblast DF1 cells (ATCC CRL-12203) were cultured in Dulbecco's modified Eagle's medium (DMEM). The Human Pulmonary Epithelial (A549) cells (ATCC CCL-185) were maintained in Minimal Essential Medium (MEM). Both DMEM and MEM were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin. Virus-infected cells were grown in the media containing 0.3% bovine serum albumin (BSA) and 2.5 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin. All these cells were maintained at 37 °C in a 5% $CO_2$ incubator.

All the influenza virus strains, A/PuertoRico/8/1934 (H1N1), A/human/Hubei/1/2009 (H1N1), A/Human/Hubei/3/2005 (H3N2), A/Human/WSN/33 (H1N1, S31N amantadine resistant), A/Duck/Hubei/216/1983 (H7N8), A/Duck/Hubei/5/2010 (H6N6), A/Chicken/Jiangsu/1/2005 (H9N2) and B/Human/Hubei/1/2007, were originally provided from the virus collection at Wuhan Institute of Virology, Chinese Academy of Sciences, China. Virus stocks were prepared in 10-day-old embryonated chicken eggs. The virus titres were determined through a hemagglutination test (HA) and the 50% tissue culture infective dose ($TCID_{50}$) assay in MDCK cells using the method developed by Reed and Muench (1938).

### 2.2. Chemicals

The compounds applied in the library screening were purchased from MicroSource Discovery Systems, Inc. (Gaylordsville, CT, USA). The library consists of 1280 drugs and bioactive compounds, divided into 16 plates of 80 each. All compounds had a >95% purity and provided as DMSO stock solution at a concentration of 10 mM. To our knowledge, not all drugs in this library are FDA-approved but all have known biological activity. In addition to the FDA-approved drugs, many are approved for clinical use in other countries but have not received FDA approval.

In the rest of studies, fenofibrate, benzydamine, anthralin, diethylstilbestrol, clotrimazole, dicumarol, monensin sodium, trimipramine, chlorophyllin, flufenamic acid, miconazole, ciclopirox and chloroxine were purchased from Gold Wheat Biological Technology Co., Ltd. (Shanghai, China). Proadifen, penbutolol sulfate, nafronyl oxalate, ethopropazine, enilconazole, fluvastatin and betamethasone were purchased from J&K Scientific Ltd. (Beijing, China). Dicyclomine HCl and ribavirin were purchased from Sigma Chemical Company (Sigma-Aldrich, MO, USA). Oseltamivir (GS 4071) was purchased from Toronto Research Chemicals (Toronto, Canada). All test compounds were initially dissolved in DMSO.

### 2.3. Library screening

MDCK cells were plated at a density of 1.0–1.5 × $10^4$ cells per well in 96-well plates and incubated for 24 h at 37 °C in 5% $CO_2$ prior to drug addiction. Then cells were infected with 100 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1) in the presence of 6 concentrations of 3-fold serial dilutions starting at 100.0 μM for each candidate at 37 °C for 48 h. The inhibition of viral replication was measured by the modified neuraminidase activity (NA) assay (Ivachtchenko et al., 2013). Briefly, the supernatants were transferred to 96 black well plates and incubated with 20 μM 2-(4-Methylumbelliferyl-α-D-N-acetylneuraminic acid sodium salt (MUNANA, Sigma, cat. No M8639), dissolved in 33 mM 2-[N-morpholino]ethanesulfonic acid (pH 6.5) and 4 mM $CaCl_2$, at 37 °C for 1 h. The reaction was terminated by adding 0.14 M NaOH in 83% ethanol. The fluorescence intensity was measured at an excitation wavelength of 355 nm and an emission wavelength of 485 nm using a multi-label plate reader (Wallac Envision, PerkinElmer, MA, USA). Ribavirin was used as a positive control.

Primary hits were identified as those reducing the NA activity in dose-dependent manners, and no apparent cytotoxicity was observed under microscope. Primary hits were subsequently re-screened with the viral replication inhibition assay and cytotoxicity assay described as below. $IC_{50}$ (drug concentration required to inhibit virus production by 50%) and $CC_{50}$ (drug concentration required to reduce cell viability by 50%) of each compound was calculated using Prism v.5 software (Graphpad software, San Diego, CA). Compounds displaying selective index (SI) over 4.0 were further considered as "hits" in this study.

### 2.4. Confirmation of hits with re-ordered compounds

After determining the hits from library screening, we purchased most of hits from different commercial vendors as described in Section 2.2 and re-tested their antiviral activities and drug cytotoxicities in MDCK cells just exactly following the protocol described in Section 2.5 and 2.6.

### 2.5. Cytotoxicity assay

Compound toxicity was determined by alamarBlue® Assay (Invitrogen). MDCK, A549 and DF1 cells were seeded in 96-well plates at 5000 cells per well and cultured for 24 h. Then the cells were treated with compounds serially diluted with fresh medium and further incubated at 37 °C for 72 h. Cells were washed two times with PBS and 0.1 ml alamarBlue (10%) completely diluted in DMEM or MEM was added in the cell culture and incubated at 37 °C for 1 h. The fluorescence intensity was read with excitation and emission wavelengths of 570 nm and 585 nm, respectively. Three independent experiments were performed in duplicate for the calculation of $CC_{50}$ using Prism v.5 software.