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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.

2.6. Viral replication inhibition assay

The antiviral spectrum evaluation was performed by infection of MDCK cells with other influenza A virus strains and influenza B virus in the presence of different drug concentrations. Cells were seeded in 96-well plates and infected with 100 $TCID_{50}$ of indicated influenza strains (stated in Section 2.1) after incubation at 37 °C for 18 h. Each drug candidate was displayed in the presence of 8 rounds of 2-fold serial dilutions at 4 °C for 1 h. After virus attachment, the cells were washed 3 times with phosphate buffered saline (PBS) and incubated with media containing the same serial concentrations of the studied compounds at 37 °C for 48 h. The supernatants were subjected to NA activity assay to confirm the dose-responsibility and to determine the efficacy in anti-influenza activity. Three independent experiments were performed in duplicate for the calculation of IC_{50}s using Prism v.5 software.

The antiviral activity of the 9 most potent drug candidates were also confirmed in A549 and DF1 cells challenging with 100 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1) just as described above.

2.7. Infectious virus yield reduction assay

MDCK cells seeded in 24-well plates were infected with 100 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1) in the presence of 6 concentrations of selected drug candidates. After adsorption at 37 °C for 1 h, the cells were washed twice with PBS and incubated in the same compound-containing medium at 37 °C for 48 h. The yields of the infectious virus in the supernatants were titrated through the $TCID_{50}$ assay, and the virus titre was calculated according to the method of Spearman–Karber (Kärber, 1931).

2.8. Indirect immunofluorescence assay (IFA)

MDCK were seeded in 96-well plates at a density of $0.8–1.0 \times 10^4$ cells per well and cultured for 18–24 h. Cells were washed with PBS and inoculated with medium alone (negative control), virus suspension (1000 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1)), or virus mixed with various concentrations of compound at 4 °C for 1 h. After attachment, the cells were washed and inoculated with the same medium for 6 h at 37 °C. Cells were washed with PBS and fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. The cells were then incubated in blocking buffer (PBS containing 3% BSA, 0.3% Triton X-100 and 10% FBS) for 30 min and then in binding buffer (PBS containing 3% BSA and 0.3% Triton X-100) with monoclonal antibodies against the nucleoprotein (1:50, Santa Cruz, CA, USA) for 1 h. After further washes, cells were incubated with TRITC-conjugated rabbit anti-mouse immunoglobulin G (1:200). The stained samples were then examined with a Nikon's Eclipse Ti inverted microscope (Nikon, Tokyo, Japan).

2.9. Time-of-addition experiment

To determine which stage(s) of IAV life cycle was abrogated by each selected candidate, a time-of-addition experiment was performed as previously reported (Chamoun-Emanuelli et al., 2013; Dai et al., 2012). The experiment mainly contained four tests: (1) Before infection, influenza virus stock (6.5×10^5 $TCID_{50}$/ml) was incubated with a medium containing each compound at 37 °C for 3 h, the virus-compound mixtures were diluted 100-fold and used to infect fresh MDCK cells. For controls, virus and drugs were separately incubated and diluted 50-fold and mixed before infecting cells (virucidal experiment). (2) Before infection, MDCK cells were incubated with a medium containing each compound at 37 °C for 2 h. Cells were thoroughly washed with PBS to remove residual drug (pre-treatment). (3) Compounds were added during viral adsorption at 4 °C for 1 h and removed by washing three times with PBS (adsorption). (4) After attachment, compounds were added at the indicated time points post infection (p.i.) and maintained until harvest (post-treatment). The cells were infected with 6.5×10^3 $TCID_{50}$ of influenza virus H1N1 (A/PuertoRico/8/1934) and treated with 5 IC_{50} of each compound throughout the experiment. After 12 h, the supernatants were collected and the viral yields were determined by NA activity assay. The inhibition of virus replication by oseltamivir was determined by TCID50 assay. For the virucidal experiment, tannic acid (10.0 μM), a compound with reported virucidal activity (Carson and Frisch, 1953), was used as a positive control. For the rest tests, ribavirin (50.0 μM) and oseltamivir (1.0 μM) were used as controls.

3. Results

3.1. Identification of 41 hit inhibitors of influenza A virus from the screen of an US Drug Collection Library

To search for novel inhibitors of influenza virus, we screened a chemical library composed of 1280 active compounds, mostly were approved for non-influenza therapeutic purposes. After primary screening, 72 compounds dose-dependent inhibited the NA activity and subjected to the second round confirmation, 41 of them showed a selective index over 4.0 and identified as "hits" (Table 1). The hit rate was 3.2% (41/1280). Of note, 32 of the 41 hits have been approved by the FDA. As expected, a few of the hit compounds have been reported to have antiviral activity against influenza virus. Those compounds were nitazoxanide (Rossignol et al., 2009), chlorophyllide (Dunham, 1954), tannic acid (Carson and Frisch, 1953), oseltamivir (Lew et al., 2000) and niclosamide (Jurgeit et al., 2012). Several other hit compounds can also be found in other drug screening lists but were not being studied further, which includes ketoconazole, miconazole nitrate, sulconazole nitrate, clotrimazole (Hung et al., 2009) and dicumarol (Hung et al., 2012). However, most of the hit compounds we identified here were reported to inhibit influenza virus replication for the first time.

These hit compounds can be divided into several classes through systematic analysis of their functions and targets. Some classes of drugs have been extensively studied and proved to be effective against influenza virus including anti-inflammatory agents (Fedson, 2009), antimicrobial agents (antibacterial or antifungal), and antilipemic agent (Budd et al., 2007; Fedson, 2013), our results expand the evidences of the link between these targets and influenza virus replication. Interestingly, some other classes of drugs attract our attention since there are few reports about their antiviral activity against influenza virus. These contain drugs used for the treatment of vasodilatation (penbutolol sulfate, nafronyl oxalate, buphenine and alprenolol), depression (trimipramine and venlafaxine), Parkinson's disease (procyclidine and biperiden) and spasm (dicyclomine and ethopropazine). Especially, the serotonin-norepinephrine reuptake inhibitor (SNRI), used for the treatment of depression, have been found recently to inhibit the replication of human enteroviruses and HIV (Benton et al., 2010; Ulferts et al., 2013; Zuo et al., 2012). Moreover, sertraline, another SNRI, can improve influenza virus induced-lung inflammation and mortality in animals when treated with oseltamivir (Sharma et al., 2013). These results suggest that SNRI may represent a promising class of antiviral drugs. In summary, we discovered several novel classes of inhibitors of influenza virus.

To further confirm the antiviral activity and efficacy, we purchased most of the hit compounds from other commercial resources and re-tested using the viral replication inhibition assay and cytotoxicity assay. All hit compounds showed similar dose-dependent inhibitory and cytotoxic effects.

Table 1
Hit compounds that showed a dose-dependent inhibitory effect against influenza virus in MDCK cells.

Chemical name	IC_{50} (μM)[a]	CC_{50} (μM)[b]	SI[c]	Approved of intend use[d]	Mechanism-of-action[e]
Enilconazole	10.1	192.9	19.1	Antifungal	Inhibitor of 14-alpha-demethylase,
Miconazole*	2.9	59.4	20.5	Antifungal	Inhibitor of 14-alpha-demethylase
Clotrimazole*	3.0	42.0	14.0	Antifungal	Inhibitor of 14-alpha-demethylase
Tioconazole*	4.4	34.7	7.9	Antifungal	Inhibitor of 14-alpha-demethylase
Sulconazole*	3.9	56.4	14.5	Antifungal	Inhibitor of 14-alpha-demethylase
Econazole*	4.0	36.6	9.2	antifungal	Inhibitor of 14-alpha-demethylase
Butoconazole*	5.0	45.2	9.0	Antifungal	Inhibitor of 14-alpha-demethylase
Ciclopirox olamine*	3.0	119.4	39.8	Antifungal	Chelating agent
Chloroxine*	2.9	51.3	17.1	Antifungal and antibacterial	Unknown
Monensin sodium	11.7	>100.0	>8.5	Antibacterial	Suppression intracellular protein transportation
Doxycycline*	22.1	>100.0	>4.5	Antibacterial and antimalarial	Inhibition bacterial protein synthesis
Niclosamide*	5.9	38.6	6.5	Anthelmintic, teniacide	Stimulation of ATPase activity
Nitazoxanide*	2.8	>100.0	>30.0	Antiprotozoal	Interference with the electron transfer reaction
Fenofibrate*	15.3	>500.0	>32.6	ANTILIPEMIC	PPAR-α agonist
Fluvastatin*	6.0	31.6	5.3	Antilipemic	HMG-CoA reductase inhibitor
Penbutolol sulfate*	18.7	>100.0	>5.3	Antihypertensive	Non-selective beta-adrenergic blocker
Nafronyl oxalate	12.3	235.3	19.1	Treatment of vasodilatation	5-HT_2 receptor antagonist
Buphenine*	4.0	>100.0	>25.0	Treatment of vasodilatation	Beta-adrenergic agonist
Alprenolol*	12.2	>100.0	>8.2	Antihypertensive and antiarrhythmic	Beta-adrenergic agonist
Trimipramine*	5.9	95.4	16.2	Antidepressant	Serotonin-norepinephrine reuptake inhibitor
Venlafaxine*	19.1	>100.0	>5.2	Antidepressant	Serotonin-norepinephrine reuptake inhibitor
Benzydamine	5.2	84.5	13.6	Antipyretic and anti-inflammatory	Prostaglandin synthetic inhibitor
Flufenamic acid	20.0	>400.0	>20.0	Antipyretic and anti-inflammatory	Prostaglandin synthetic inhibitor
Betamethasone*	18.2	145.3	8.0	Anti-asthmatic and anti-inflammatory	Glucocorticoid receptor agonist
Tannic acid	2.0	>100.0	>50.0	Food additive; antioxidant	Unknown
Erythrosine	20.6	>100.0	>4.8	Food coloring	Unknown
Chlorophyllide	3.9	276.5	71.0	Food coloring and anti-carcinogenic	Unknown
Anthralin*	4.5	>200.0	>44.4	Treatment of psoriasis	Impede DNA replication
Antazoline*	22.4	>100.0	>4.4	Antihistamine and anticholinergic	Histamine H1 antagonist
Brompheniramine*	18.7	>100.0	>5.3	Antihistamine	Histamine H1 antagonist
Hydroxyzine*	21.7	>100.0	>4.6	Antihistamine	Histamine H1 antagonist
Dicyclomine*	10.3	125.4	12.2	Antispasmodic	Specific anticholinergic and muscarinic antagonist
Ethopropazine*	2.6	65.7	25.3	Treatment of parkinson's disease	Specific anticholinergic and muscarinic antagonist
Procyclidine*	21.2	>100.0	>4.0	Treatment of parkinson's disease	A muscarinic antagonist
Biperiden*	22.8	>100.0	>4.0	Treatment of parkinson's disease	A muscarinic antagonist
Bepridil*	11.0	78.6	7.2	Antianginal	A calcium-blocking agent
Liothyronine*	22.4	>100.0	>5.0	Treatment of hypothyroidism	Thyroid hormone receptor agonist
Proadifen	15.4	72.4	>6.5	Prevent drug metabolism	Inhibitor of cytochrome P450 enzymes
Diethylstilbestrol*	4.5	63.5	14.3	Has banned by FDA	An endocrine disruptor
Dicumarol*	12.4	150.7	12.2	Anticoagulant	Interfere with the metabolism of vitamin K
Oseltamivir*	0.02	ND[f]		Anti-influenza drug	Influenza NA inhibitor

[a] IC_{50}: compound concentration required to inhibit virus production by 50%, as determined by NA activity. Values represent the mean of duplicate samples from two independent experiments.
[b] CC_{50}: compound concentration required to reduce cell viability by 50%, as determined by the alamarBlue® Assay. Values represent the mean of duplicate samples from two independent experiments.
[c] SI (Selective Index), ratio of CC_{50}/IC_{50}.
[d] Approved or intended use is the use for which the compound has received US FDA approval or for which it has been used in animal.
[e] Mechanism is the action attributed to the compound as stated on either its "label" or literature.
[f] ND: not determined.
* Compounds approved by the FDA.

3.2. Nine candidates showed broad-spectrum antiviral effect against human and avian influenza viruses

Broad-spectrum antiviral activity is a favorable feature for new antiviral agents. In order to obtain highly potent candidates with broad-spectrum of antiviral activity and to minimize the contribution of cytotoxicity in the antiviral effect, we selected 18 compounds from the 21 hits showing a SI ⩾ 10.0 (oseltamivir, nitazoxanide and tannic acid were excluded since they have been extensively studied) for the spectrum of antiviral activity experiments. The 18 selected compounds were tested for their antiviral activities against 7 other influenza viruses including an adamantane-resistant virus A/WSN/33/S31 N (H1N1) and an influenza B virus using the methods described above. As shown in Table 2 and 7 compounds (chlorophyllide, anthralin, ciclopirox, chloroxine, ethopropazine, nafronyl oxalate, and dicyclomine HCl) inhibited all the tested influenza strains with SIs over 5.0. Dicumarol was effective against all strains except A/Human/Hubei/3/2005 (H3N2). In addition to A/Human/Hubei/3/2005 (H3N2), enilconazole was also ineffective against A/Duck/Hubei/5/2010 (H6N6) even at the maximum concentration tested. Benzydamine, diethylstilbestrol, and trimipramine displayed significantly weaker antiviral activity against the 6 influenza A subtype strains (SIs < 5.0), however, they still strongly inhibited the replication of influenza B virus. The imidazoles (sulconazole, clotrimazole and miconazole) were ineffective against most of the tested virus strains except the seasonal A/human/Hubei/1/2009 (H1N1) subtype. The 9 compounds which were active against at least 5 influenza virus strains were categorized as broad-spectrum antivirals and subjected to further study. Their structures are shown in Fig. 1.

3.3. Confirmation of the antiviral effect of the 9 drug candidates in DF1 and A549 cells

To further confirm the anti-influenza activities of the 9 broad-spectrum antivirals, we applied them to another two cell lines, chicken-originated DF1 and human-originated A549 cells. To exclude the possibility that the inhibitory effect was due to non-

Table 2
Inhibition of the replication of different influenza virus strains by the selected 18 hit compounds.

Chemical name	H1 N1[a]		H3N2[b]		A/WSN/S31N (H1N1)[c]		H6N6[d]		H7N8[e]		H9N2[f]		Influenza B virus[g]	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
Chlorophyllide	3.1	89.2	5.4	51.2	18.3	15.1	3.5	79.0	3.7	74.7	4.5	61.4	2.9	95.3
Anthralin	11.3	>17.7	14.3	>14.0	12.5	>16.0	8.9	>22.5	6.3	>31.7	12.5	>16.0	29.6	>6.7
Ciclopirox	5.4	22.1	6.3	19.0	2.0	59.7	3.5	34.1	5.2	22.9	6.8	17.6	3.2	37.3
Fenofibrate	28.9	>17.3	–[h]	–	–	–	–	–	157.5	3.2	–	–	18.9	>26.5
Chloroxine	3.2	16.0	6.3	8.2	2.3	22.3	1.6	32.0	1.2	42.8	5.9	8.7	6.5	8.0
Buphenine	ND[i]	ND	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND
Ethopropazine	5.3	12.4	3.8	17.3	8.7	7.6	7.4	8.9	9.2	7.1	6.4	10.2	3.2	20.5
Flufenamic acid	24.5	16.3	–	–	98.5	>4.1	–	–	150.3	>2.7	74.5	5.4	–	–
Miconazole	20.9	2.8	2.5	23.8	–	–	–	–	–	–	–	–	–	–
Nafronyl oxalate	11.5	20.5	45.3	5.2	34.7	6.8	28.3	8.3	19.5	12.1	18.3	12.8	12.4	19.0
Enilconazole	10.9	17.7	–	–	35.4	5.5	–	–	30.1	6.4	8.5	22.7	3.1	62.2
Trimipramine	8.6	11.1	45.2	2.1	30.1	3.2	22.4	4.3	9.8	9.7	6.2	15.4	10.5	9.1
Sulconazole	2.8	20.1	–	–	–	–	–	–	–	–	–	–	–	–
Diethylstilbestrol	3.2	19.8	–	–	20.2	3.1	6.3	10.0	3.2	19.8	13.2	4.8	1.6	40.0
Clotrimazole	2.5	16.8	–	–	–	–	15.3	2.7	6.3	6.7	–	–	4.5	9.3
Benzydamine	1.6	52.8	29.3	2.9	25.0	3.4	21.7	3.9	30.3	2.8	45.6	1.9	12.8	6.6
Dicumarol	13.3	11.3	–	–	16.4	9.2	25.4	6.0	11.3	13.3	4.8	31.4	5.9	25.5
DicyclomineHCL	5.9	25.5	23.6	5.3	14.7	8.5	40.2	3.1	24.1	5.2	17.2	7.3	5.6	22.3
Ribavirin[j]	13.6	17.3	12.5	18.8	16.7	14.1	9.8	24.0	17.8	13.2	14.7	16.0	12.5	18.8

[a] A/Human/Hubei/1/2009(H1N1).
[b] A/human/Hubei/3/2005(H3N2).
[c] A/human/WSN/33/(H1N1,S31 N,amantadine resistant).
[d] A/Duck/Hubei/5/2010(H6N6).
[e] A/Duck/Hubei/216/1983(H7N8).
[f] A/Chicken/Jiangsu/1/2005(H9N2).
[g] B/human/Hubei/1/2007.
[h] "–" lower than 50% inhibition or no effective.
[i] ND: not determined.
[j] Ribavirin was used as a positive control. Data were shown as the mean of duplicate samples from three independent experiments.

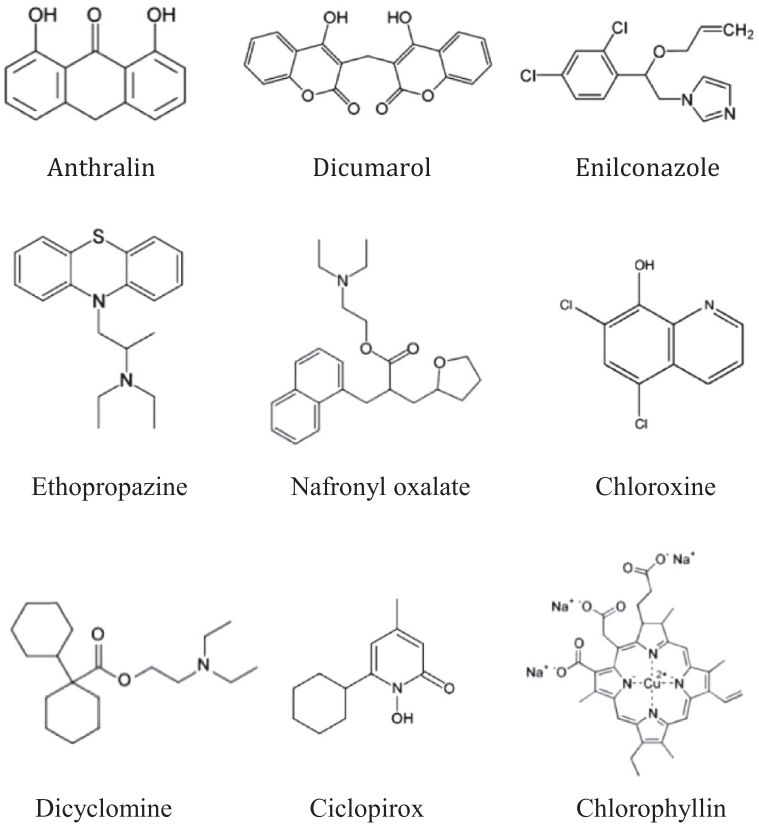

Fig. 1. Chemical structures of drug candidates selected for further analysis.

specific cytotoxicity, cell viability assays were performed in parallel. As shown in Table 3, the IC_{50}s and SIs of tested candidates in A549 and DF1 were similar to those in MDCK cells ranging from 1.0 μM to 50.0 μM and 9.0 to 100, respectively, indicating that

Table 3
The antiviral activity and cytotoxicity of each drug candidate in MDCK, A549 and DF1 cells.

Compound	MDCK			A549			DF1		
	CC_{50}(μM)	IC_{50}(μM)	SI	CC_{50}(μM)	IC_{50}(μM)	SI	CC_{50}(μM)	IC_{50}(μM)	SI
Anthralin	>200.0	4.5	>44.4	>200.0	5.8	>30.7	>200.0	52.3	>9.6
Ciclopirox	119.4	3.0	39.8	210.5	6.7	31.4	97.8	7.1	13.8
Chloroxine	51.3	1.6	32.0	85.4	5.4	15.8	29.8	3.8	7.8
Chlorophyllide	276.5	3.9	71.0	125.1	6.3	19.8	267.4	9.5	28.1
Dicumarol	150.7	12.4	12.2	143.2	48.9	3.0	164.3	10.1	16.3
Dicyclomine HCl	125.4	10.3	12.2	120.1	8.0	12.0	106.4	10.5	10.1
Ethopropazine	65.7	2.6	25.3	98.4	10.4	9.5	73.2	17.5	4.2
Nafronyl oxalate	235.3	12.3	19.1	252.4	6.0	42.0	300.0	8.5	35.3
Enilconazole	192.9	10.1	19.1	195.7	9.4	20.8	186.2	12.3	15.1

All the three cells lines were challenged with 100 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1). Values represent the mean of duplicate samples from three independent experiments.

their antiviral activities were not cell-type specific. Of note, dicumarol in A549 cells (IC_{50} = 48.9, SI = 3) and anthralin in DF1 cells (IC_{50} = 52.3, SI > 9.6) were less effective compared with in MDCK cells.

3.4. Confirmation of the antiviral effect of the 9 drug candidates by infectious virus yield reduction assay

To evaluate the antiviral activities of the drug candidates on the production of infectious influenza viruses, the infectious virus yield reduction assay was performed. As shown in Fig. 2, all the 9 candidates can reduce the production of virus particles in dose-dependent manners compared with the DMSO control. Chlorophyllide showed the best inhibitory effect with an IC_{50} of 1.6 μM. Actually, we did not detect any production of infectious virus particles at the maximum non-cytotoxic concentration of each candidate (data not shown). These results further confirmed the antiviral activities of the 9 broad-spectrum antiviral candidates.

3.5. Confirmation of the antiviral effect of the 9 drug candidates by IFA

Next, IFA was used to detect the effect of the 9 broad-spectrum antiviral candidates on the expression of NP protein in single cycle infection assay. Seven compounds (chlorophyllide, ciclopirox,

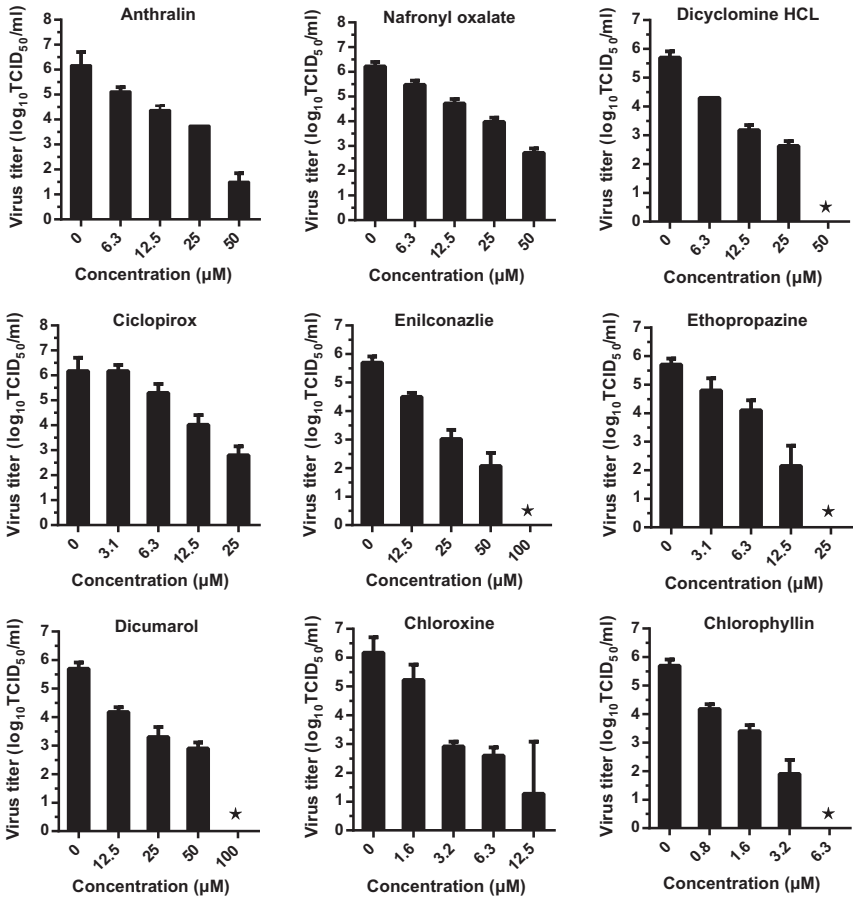

Fig. 2. The antiviral effects of the 9 broad-spectrum antiviral drug candidates determined by infectious virus yield reduction assay. MDCK cells were propagated with 100 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1) in the presence of different concentrations of compounds and incubated at 37 °C for 48 h. The supernatants were harvested and the virus titer was determined by $TCID_{50}$ assay. Virus titers were calculated according to Spearman–Karber method as described above in Section 2.7. The values represent the means ± S.D. of duplicate samples from three independent experiments. Black star indicates the values which are below the limit of detection of the method.

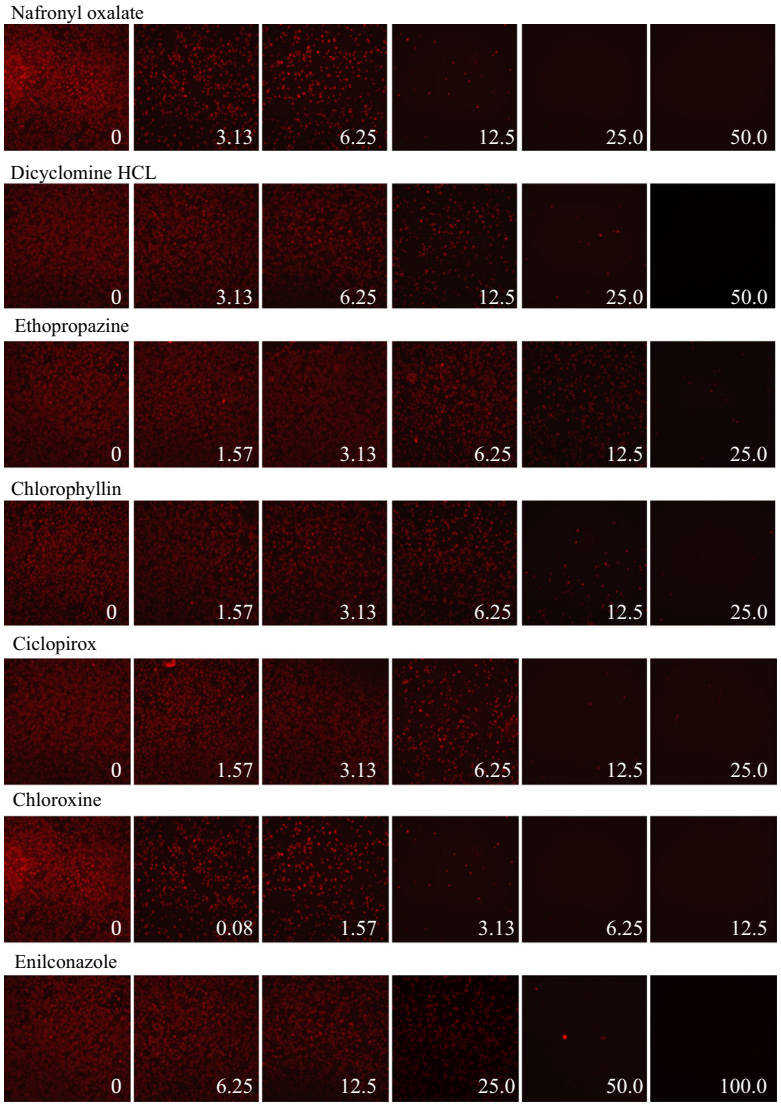

Fig. 3. Seven candidates reduced the influenza virus protein synthesis in A549 cells. Confluent A549 cells were infected with 1000 $TCID_{50}$ A/PuertoRico/8/1934 (H1N1) virus at 4 °C for 1 h. The medium was removed, and the cells were treated with various concentrations of the indicated compounds. At 6 h p.i., the cells were fixed with paraformaldehyde and the influenza virus NP expression was detected by IFA using a monoclonal NP antibody. Results shown are representative images.

enilconazole, chloroxine, ethopropazine, nafronyl oxalate, and dicyclomine HCl) dose-dependent inhibited the NP protein expression both in A549 (Fig. 3) and MDCK cells (Fig. S1). The IC_{50} of each drug were comparable to the data in Table 3 that were determined by a multiple-cycle replication assay. Interestingly, the other 2 compounds, dicumarol and anthralin, did not exhibit any inhibitory activities even at the highest non-cytotoxic concentrations both in A549 and MDCK cells (Fig. S2), implying that they may impeded late stages of the influenza life cycle. In fact, dicumarol was recently reported as an NP inhibitor that can induce conformational changes on NP structure in an anti-influenza drug screening assay (Hung et al., 2012), which was also consistent with our result that it did not affect the NP expression.

3.6. The 9 potent candidates act at different stages of influenza virus life cycle

In order to determine which stage(s) of the virus life cycle are affected by the 9 candidates, we performed a time-of-addition experiment as described in Materials and Methods. Firstly, to determine whether these compounds are virucidal, influenza virus was mixed with each candidate for 3 h and diluted 100 fold (pretreatment), or each component was separately diluted and then mixed (control). The infectivity of influenza virus pretreated with each candidate was similar to the control samples (DMSO) in spite of tannic acid, the positive control, reduced influenza virus infectivity >80% during the same period (Fig. 4A). These results indicate that these compounds do not inhibit the influenza virus replication by inactivating the virus directly. Meantime, the cells pretreated with compounds or added during adsorption period were also sensitive to virus infection (Fig. 4B *dashed bar and empty bar*), suggesting that they had no influence on the host cells and the adsorption period of influenza virus.

Next, we administrated the drug candidates at various time points (0, 2, 4, 6, 8 h) post infection. As shown in Fig. 4B (*filled bar*), dicyclomine HCl and nafronyl oxalate exerted fully inhibitory effect only when they were added just after attachment (0 h p.i.), indicating that they may impede a very early event of the virus replication. Enilconazole, ethopropazine and chlorophyllide can still reach 50% inhibition rates when added 2 h p.i., implying that

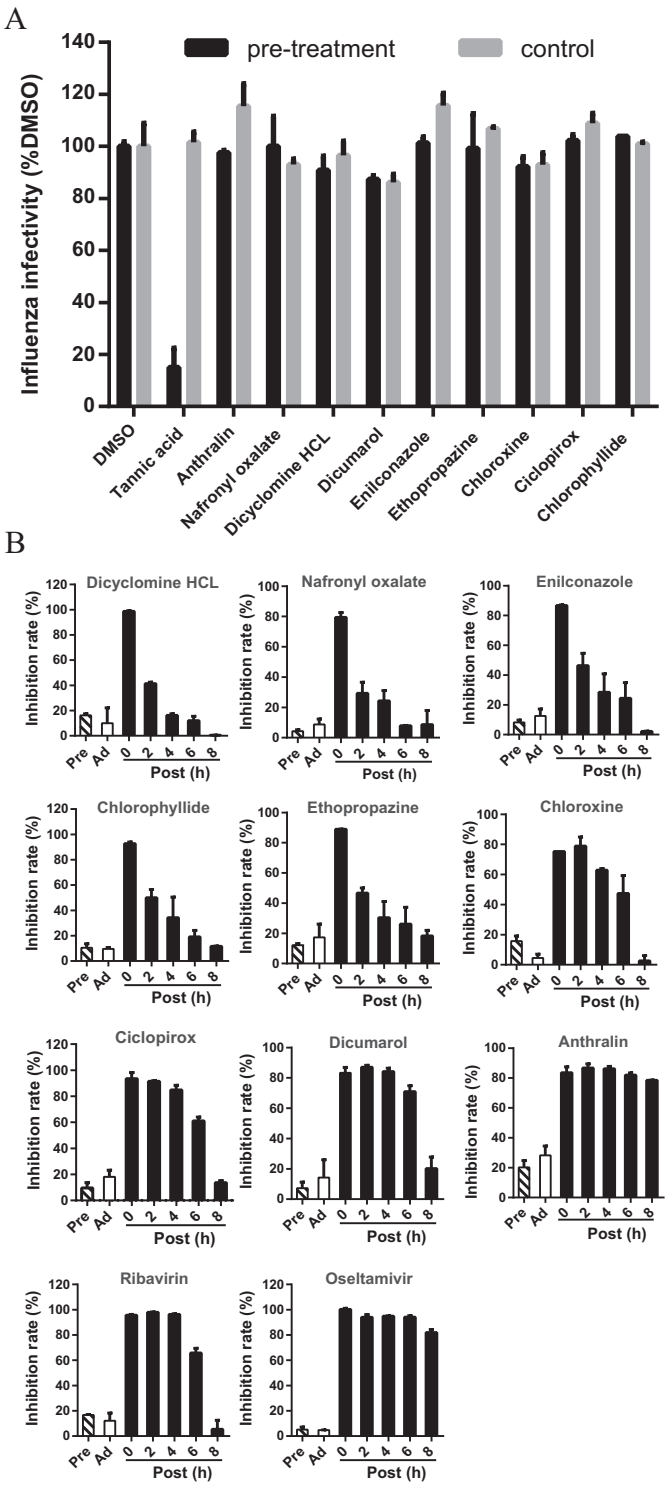

Fig. 4. Time-of-addition experiment of the selected 9 compounds. (A) Influenza virus A/PuertoRico/8/1934 (6.5×10^5 $TCID^{50}$/ml) was incubated with 5 IC_{50} of each candidate at 37 °C for 3 h and then diluted 100-fold and used to infect MDCK cells. In the control groups, the same amounts of virus and drugs were incubated separately at 37 °C for 3 h, diluted 50-fold, and then mixed to infect cells. The final concentrations of candidates were identical between two groups and were ineffective against influenza virus replication. (B) Confluent MDCK cells were treated with 5 IC_{50} of each candidate at 37 °C for 2 h before infection (*pre, dashed bar*), only during the absorption period at 4 °C for 1 h (*co, empty bar*) or at the indicated times immediately after the absorption period (*post, filled bar*). At 12 h p.i., the NA activity in the supernatants was measured. For oseltamivir, $TCID_{50}$ assay was performed to determine the virus titers in the supernatants. The values represent the means ± S.D. of duplicate samples from three independent experiments.

early to middle stages of the life cycle were affected by these candidates. The patterns of ciclopirox, chloroxine and dicumarol are similar to ribavirin, which is still effective when added 6 h p.i. The three compounds may impact events related with RNA transcription or translation since ribavirin had been proved to act on ribonucleoprotein synthesis (Wray et al., 1985). Anthralin, however, is similar to oseltamivir may act on very late stage of life cycle because it still restrained the influenza virus replication when added 8 h p.i. In summary, our results showed that the 9 potent candidates impede the influenza virus replication at different stages of life cycle.

4. Discussion

Currently, many high-throughput screening (HTS) assays either host-targeted or virus-targeted have been developed for searching novel inhibitors of influenza virus (Hsu et al., 2012; Hung et al., 2012; Mao et al., 2013; Maroto et al., 2008; Ortigoza et al., 2012; Perwitasari et al., 2013a; Severson et al., 2008). Usually, these assays were performed at a single fixed concentration for each candidate that is prone to false-positive or false-negative due to the cytotoxicity and the single drug concentration. These target-based approaches are limited to the development of novel treatments for existed targets. However, it is of great significance to search for innovative inhibitors of new targets especially host-oriented targets correlating to all stages of the complete life cycle of influenza virus.

In the present study, using an unbiased screening technology and wide range of drug concentrations (0.41–100.0 μM), we identified 41 candidates with antiviral activity against influenza virus from screening a safe drug library (Table 1). Several classes of drugs were firstly discovered as anti-influenza agents. Further analysis revealed 9 candidates as broad–spectrum antivirals (Table 2). Their antiviral activities were further characterized by cell specificity (Table 3), the infectious virus production inhibition (Fig. 2) as well as the NP expression reduction (Fig. 3). At last, we found that they acted on the different stages of influenza virus life cycle (Fig. 4).

Chlorophyllide is not a drug but is extensively used as a food additive for coloration. It has been reported to be effective against a variety of enveloped viruses including influenza virus, but was ineffective with non-enveloped viruses (Benati et al., 2009; Dunham, 1954; Guo et al., 2011). It is speculated that chlorophyllide is acting by physically inactivating virus particles considering its broad antiviral spectrum. However, in the present study, chlorophyllide showed litter effect on inactivation influenza virus (Fig. 4A). Indeed, since the influenza virus envelopes are derived from the cell membrane and chlorophyllide is well tolerated in MDCK cells (CC_{50} > 250.0 μM), it is unlikely that chlorophyllide inhibits the influenza virus replication by disrupting the virus particle, but events in early stage of virus infection may be affected (Fig. 4B). In addition to antiviral activity, chlorophyllide has also been shown to be effective against liver cancer (Reinbothe et al., 2006). Importantly, chlorophyllide had been investigated in clinical trials for anticancer activity and proved to be safe in human (>300 mg/d for 4 months) (Kensler et al., 2004). In our study, the replication of influenza virus was completely inhibited at 6.3 μM of chlorophyllide (Fig. 2), making it a ideal candidate to further study *in vivo*.

Anthralin (also called dithranol) is a synthetic compound for the treatment of psoriasis. Its mechanism of anti-psoriatic action is thought to be linked with its anti-proliferative and anti-inflammatory activities and abilities to induce lipid peroxidation and reduce levels of endothelial adhesion molecules (Ashton et al., 1983). Ciclopirox is currently approved as a topical antifungal cream for the

dermatologic treatment of superficial mycoses. Ciclopirox may exert its effect by altering cell permeability or acting through chelation with polyvalent metal cations such as Fe^{3+} and Al^{3+} (Gupta and Skinner, 2003). Intriguingly, ciclopirox has been reported to inhibit HIV-1 gene expression by disrupting eIF5A maturation and kill the HIV-infected cells by activating apoptosis (Hanauske-Abel et al., 2013; Hoque et al., 2009). Chloroxine, another dermatological agent approved by the FDA, is mainly used in the treatment of dandruff and seborrheic dermatitis of the scalp. Enilconazole is a fungicide widely used in agriculture and veterinary medicine as an inhibitor of 14-alpha-demethylase. Since the four active compounds are all used in topical in the form of cream or shampoo, the translation of our results to the clinic should be taken cautiously as they may exhibit human toxicity when used systemically for the treatment of influenza infection. For these compounds, structure–activity relationship (SAR) study should be initiated to reduce the toxicity of these new classes of influenza inhibitors.

However, the other four drugs are used in the clinic by systemically through oral or intravenous injection, their drug toxicity is low and tolerance is well in human. Ethopropazine and dicyclomine HCl are two muscarinic receptor antagonists (MRA) used for the treatment of Parkinson's disease. In the present study, these two MRAs were shown to impair the early stage of virus life cycle (Fig. 4B), which is similar to benztropine mesylate, another MRA, described as a HCV entry inhibitor (Mingorance et al., 2014). Dicumarol is effective for the treatment of deep venous thrombosis by acting as a competitive inhibitor of vitamin K epoxide reductase. Nafronyl oxalate, a 5-HT_2 receptor antagonist, has been sold in many countries for the treatment of vasodilatation in spite of still not receiving FDA approval. For these compounds, the specific antiviral mechanism of each candidate and antiviral effect *in vivo* need to be further explored. A drug-based drug discovery program, based on these compounds, can also be launched to determine the potential of such agents in clinical trials of influenza-infected patients.

In summary, with the aid of a powerful unbiased cell-based screening technology, we screened the US Drug Collection library and indentified a number of FDA-approved drugs as novel inhibitors of influenza virus. Our further study revealed 9 compounds are potent drug candidates with broad spectrum of antiviral activity and target various aspects of influenza virus life cycle. Despite certain limitations, we believe these 9 compounds deserve further investigation as potential antivirals for treatment of influenza infection. Ethopropazine and dicyclomine HCl, the two muscarinic receptor antagonists, can serve as top priority candidates for further analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.06.007.

References

Ashton, R.E., Andre, P., Lowe, N.J., Whitefield, M., 1983. Anthralin: historical and current perspectives. J. Am. Acad. Dermatol. 9, 173–192.

Benati, F., Lauretti, F., Faccin, L., Nodari, B., Ferri, D., Mantovani, M., Linhares, R., Nozawa, C., 2009. Effects of chlorophyllin on replication of poliovirus and bovine herpesvirus in vitro. Lett. Appl. Microbiol. 49, 791–795.

Benton, T., Lynch, K., Dubé, B., Gettes, D.R., Tustin, N.B., Lai, J.P., Metzger, D.S., Blume, J., Douglas, S.D., Evans, D.L., 2010. Selective serotonin reuptake inhibitor suppression of HIV infectivity and replication. Psychosom. Med. 72, 925–932.

Budd, A., Alleva, L., Alsharifi, M., Koskinen, A., Smythe, V., Müllbacher, A., Wood, J., Clark, I., 2007. Increased survival after gemfibrozil treatment of severe mouse influenza. Antimicrob. Agents Chemother. 51, 2965–2968.

Carson, R.S., Frisch, A.W., 1953. The inactivation of influenza viruses by tannic acid and related compounds. J. Bacteriol. 66, 572.

Chamoun-Emanuelli, A.M., Pecheur, E.-I., Simeon, R.L., Huang, D., Cremer, P.S., Chen, Z., 2013. Phenothiazines inhibit hepatitis C virus entry, likely by increasing the fluidity of cholesterol-rich membranes. Antimicrob. Agents Chemother. 57, 2571–2581.

Dai, J., Wang, G., Li, W., Zhang, L., Yang, J., Zhao, X., Chen, X., Xu, Y., Li, K., 2012. High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy. J. Biomol. Screen. 17, 605–617.

Dawood, F.S., Iuliano, A.D., Reed, C., Meltzer, M.I., Shay, D.K., Cheng, P.-Y., Bandaranayake, D., Breiman, R.F., Brooks, W.A., Buchy, P., 2012. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. Lancet Infect. Dis 12, 687–695.

De Clercq, E., 2006. Antiviral agents active against influenza A viruses. Nat. Rev. Drug Discovery 5, 1015–1025.

Dudek, S.E., Luig, C., Pauli, E.-K., Schubert, U., Ludwig, S., 2010. The clinically approved proteasome inhibitor PS-341 efficiently blocks influenza A virus and vesicular stomatitis virus propagation by establishing an antiviral state. J. Virol. 84, 9439–9451.

Dunham, W.B., 1954. Differential inhibition of virus hemagglutination by chlorophyllin. Exp. Biol. Med. 87, 431–433.

Fedson, D.S., 2009. Confronting the next influenza pandemic with anti-inflammatory and immunomodulatory agents: why they are needed and how they might work. Influenza Other Respir. Viruses 3, 129–142.

Fedson, D.S., 2013. Treating influenza with statins and other immunomodulatory agents. Antiviral Res. 99, 417–435.

Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H., Xu, K., 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N. Engl. J. Med. 368, 1888–1897.

Gastaminza, P., Whitten-Bauer, C., Chisari, F.V., 2010. Unbiased probing of the entire hepatitis C virus life cycle identifies clinical compounds that target multiple aspects of the infection. Proc. Natl. Acad. Sci. 107, 291–296.

Guo, H., Pan, X., Mao, R., Zhang, X., Wang, L., Lu, X., Chang, J., Guo, J.-T., Passic, S., Krebs, F.C., 2011. Alkylated porphyrins have broad antiviral activity against hepadnaviruses, flaviviruses, filoviruses, and arenaviruses. Antimicrob. Agents Chemother. 55, 478–486.

Gupta, A.K., Skinner, A.R., 2003. Ciclopirox for the treatment of superficial fungal infections: a review. Int. J. Dermatol. 42, 3–9.

Hanauske-Abel, H.M., Saxena, D., Palumbo, P.E., Hanauske, A.-R., Luchessi, A.D., Cambiaghi, T.D., Hoque, M., Spino, M., Gandolfi, D.D.A., Heller, D.S., 2013. Drug-induced reactivation of apoptosis abrogates HIV-1 infection. PLoS One 8, e74414.

Hoque, M., Hanauske-Abel, H.M., Palumbo, P., Saxena, D., D'Alliessi Gandolfi, D., Park, M.H., Pe'ery, T., Mathews, M.B., 2009. Inhibition of HIV-1 gene expression by Ciclopirox and Deferiprone, drugs that prevent hypusination of eukaryotic initiation factor 5A. Retrovirology 6, 1742–4690.

Hsu, J.T.-A., Yeh, J.-Y., Lin, T.-J., Li, M.-L., Wu, M.-S., Hsieh, C.-F., Chou, Y.C., Tang, W.-F., Lau, K.S., Hung, H.-C., 2012. Identification of BPR3P0128 as an inhibitor of cap-snatching activities of influenza virus. Antimicrob. Agents Chemother. 56, 647–657.

Hung, H.-C., Tseng, C.-P., Yang, J.-M., Ju, Y.-W., Tseng, S.-N., Chen, Y.-F., Chao, Y.-S., Hsieh, H.-P., Shih, S.-R., Hsu, J.T.-A., 2009. Aurintricarboxylic acid inhibits influenza virus neuraminidase. Antiviral Res. 81, 123–131.

Hung, H.-C., Liu, C.-L., Hsu, J.T.-A., Horng, J.-T., Fang, M.-Y., Wu, S.-Y., Ueng, S.-H., Wang, M.-Y., Yaw, C.-W., Hou, M.-H., 2012. Development of an anti-influenza drug screening assay targeting nucleoproteins with tryptophan fluorescence quenching. Anal. Chem. 84, 6391–6399.

Hurt, A.C., Butler, J., Kelso, A., Barr, I.G., 2012. Influenza antivirals and resistance. the next 10 years? Expert Rev. Anti Infect. Ther. 10, 1221–1223.

Imperi, F., Massai, F., Pillai, C.R., Longo, F., Zennaro, E., Rampioni, G., Visca, P., Leoni, L., 2013. New life for an old drug: the anthelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing. Antimicrob. Agents Chemother. 57, 996–1005.

Ivachtchenko, A.V., Ivanenkov, Y.A., Mitkin, O.D., Yamanushkin, P.M., Bichko, V.V., Leneva, I.A., Borisova, O.V., 2013. A novel influenza virus neuraminidase inhibitor AV5027. Antiviral Res. 100, 698–708.

Jurgeit, A., McDowell, R., Moese, S., Meldrum, E., Schwendener, R., Greber, U.F., 2012. Niclosamide is a proton carrier and targets acidic endosomes with broad antiviral effects. PLoS Pathog. 8, e1002976.

Kärber, G., 1931. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche. Naunyn Schmiedebergs Arch. Exp. Pathol. Pharmakol. 162, 480–483.

Kensler, T.W., Egner, P.A., Wang, J.-B., Zhu, Y.-R., Zhang, B.-C., Lu, P.-X., Chen, J.-G., Qian, G.-S., Kuang, S.-Y., Jackson, P.E., 2004. Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas. Gastroenterology 127, S310–S318.

Lamontagne, J., Mills, C., Mao, R., Goddard, C., Cai, D., Guo, H., Cuconati, A., Block, T., Lu, X., 2013. Screening and identification of compounds with antiviral activity against hepatitis B virus using a safe compound library and novel real-time immune-absorbance PCR-based high throughput system. Antiviral Res.

Lauder, S.N., Taylor, P.R., Clark, S.R., Evans, R.L., Hindley, J.P., Smart, K., Leach, H., Kidd, E.J., Broadley, K.J., Jones, S.A., 2011. Paracetamol reduces influenza-induced immunopathology in a mouse model of infection without compromising virus clearance or the generation of protective immunity. Thorax 66, 368–374.

Lew, W., Chen, X., Kim, C.U., 2000. Discovery and development of GS 4104 (oseltamivir) an orally active influenza neuraminidase inhibitor. Curr. Med. Chem. 7, 663–672.

Li, Q., Zhou, L., Zhou, M., Chen, Z., Li, F., Wu, H., Xiang, N., Chen, E., Tang, F., Wang, D., 2013. Preliminary report: epidemiology of the avian influenza A (H7N9) outbreak in China. N. Engl. J. Med..

Liao, Q., Qian, Z., Liu, R., An, L., Chen, X., 2013. Germacrone inhibits early stages of influenza virus infection. Antiviral Res. 100, 578–588.

Mao, L., Wang, J., DeGrado, W.F., Inouye, M., 2013. An assay suitable for high throughput screening of anti-influenza drugs. PLoS One 8, e54070.

Maroto, M., Fernandez, Y., Ortin, J., Pelaez, F., Cabello, M.A., 2008. Development of an HTS assay for the search of anti-influenza agents targeting the interaction of viral RNA with the NS1 protein. J. Biomol. Screen. 13, 581–590.

Mingorance, L., Friesland, M., Coto-Llerena, M., Pérez-del-Pulgar, S., Boix, L., López-Oliva, J.M., Bruix, J., Forns, X., Gastaminza, P., 2014. Selective inhibition of hepatitis c virus infection by hydroxyzine and benztropine. Antimicrob. Agents Chemother. (02619-02614).

Ortigoza, M.B., Dibben, O., Maamary, J., Martinez-Gil, L., Leyva-Grado, V.H., Abreu Jr, P., Ayllon, J., Palese, P., Shaw, M.L., 2012. A novel small molecule inhibitor of influenza a viruses that targets polymerase function and indirectly induces interferon. PLoS Pathog. 8, e1002668.

Perwitasari, O., Torrecilhas, A.C., Yan, X., Johnson, S., White, C., Tompkins, S.M., Tripp, R.A., 2013a. Targeting cell division cycle 25 homolog B to regulate influenza virus replication. J. Virol. 87, 13775–13784.

Perwitasari, O., Yan, X., Johnson, S., White, C., Brooks, P., Tompkins, S.M., Tripp, R.A., 2013b. Targeting organic anion transporter 3 with probenecid as a novel anti-influenza a virus strategy. Antimicrob. Agents Chemother. 57, 475–483.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. 27, 493–497.

Reinbothe, C., Bartsch, S., Eggink, L.L., Hoober, J.K., Brusslan, J., Andrade-Paz, R., Monnet, J., Reinbothe, S., 2006. A role for chlorophyllide a oxygenase in the regulated import and stabilization of light-harvesting chlorophyll a/b proteins. Proc. Natl. Acad. Sci. U.S.A. 103, 4777–4782.

Rossignol, J.F., La Frazia, S., Chiappa, L., Ciucci, A., Santoro, M.G., 2009. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. J. Biol. Chem. 284, 29798–29808.

Severson, W.E., McDowell, M., Ananthan, S., Chung, D.-H., Rasmussen, L., Sosa, M.I., White, E.L., Noah, J., Jonsson, C.B., 2008. High-throughput screening of a 100,000-compound library for inhibitors of influenza A virus (H3N2). J. Biomol. Screen. 13, 879–887.

Sharma, G., Sharma, D.C., Fen, L.H., Pathak, M., Bethur, N., Pendharkar, V., Peiris, M., Altmeyer, R., 2013. Reduction of influenza virus-induced lung inflammation and mortality in animals treated with a phosophodisestrase-4 inhibitor and a selective serotonin reuptake inhibitor. Emerg. Microbes Infect. 2, e54.

Shirey, K.A., Lai, W., Scott, A.J., Lipsky, M., Mistry, P., Pletneva, L.M., Karp, C.L., McAlees, J., Gioannini, T.L., Weiss, J., 2013. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature 497, 498–502.

Tu, W., Zheng, J., Liu, Y., Sia, S.F., Liu, M., Qin, G., Ng, I.H., Xiang, Z., Lam, K.-T., Peiris, J.M., 2011. The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a γδ T cell population in humanized mice. J. Exp. Med. 208, 1511–1522.

Ulferts, R., van der Linden, L., Thibaut, H.J., Lanke, K.H., Leyssen, P., Coutard, B., De Palma, A.M., Canard, B., Neyts, J., van Kuppeveld, F.J., 2013. Selective serotonin reuptake inhibitor fluoxetine inhibits replication of human enteroviruses B and D by targeting viral protein 2C. Antimicrob. Agents Chemother. 57, 1952–1956.

Uyeki, T.M., Cox, N.J., 2013. Global concerns regarding novel influenza A (H7N9) virus infections. N. Engl. J. Med. 368, 1862–1864.

van Cleef, K.W., Overheul, G.J., Thomassen, M.C., Kaptein, S.J., Davidson, A.D., Jacobs, M., Neyts, J., van Kuppeveld, F.J., van Rij, R.P., 2013. Identification of a new dengue virus inhibitor that targets the viral NS4B protein and restricts genomic RNA replication. Antiviral Res..

Weisman, J.L., Liou, A.P., Shelat, A.A., Cohen, F.E., Kiplin Guy, R., DeRisi, J.L., 2006. Searching for new antimalarial therapeutics amongst known drugs. Chem. Biol. Drug Des. 67, 409–416.

Wermuth, C.G., 2004. Selective optimization of side activities: another way for drug discovery. J. Med. Chem. 47, 1303–1314.

Wermuth, C.G., 2006. Selective optimization of side activities: the SOSA approach. Drug Discovery Today 11, 160–164.

Wray, S.K., Gilbert, B.E., Noall, M.W., Knight, V., 1985. Mode of action of ribavirin: effect of nucleotide pool alterations on influenza virus ribonucleoprotein synthesis. Antiviral Res. 5, 29–37.

Wright, S.G., 2012. Protozoan infections of the gastrointestinal tract. Infect. Dis. Clin. North Am. 26, 323–339.

Zuo, J., Quinn, K.K., Kye, S., Cooper, P., Damoiseaux, R., Krogstad, P., 2012. Fluoxetine is a potent inhibitor of coxsackievirus replication. Antimicrob. Agents Chemother. 56, 4838–4844.