Discovery of Allopregnanolone Against Influenza Virus With Broad Spectrum in Vitro

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Research

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Abstract

Background: Influenza virus infection with seasonal or occasional but devastating morbidity and mortality, is a severe threat to public health. The frequent emergence of resistant viral strains limited application of current antivirals and posing an urgent need for novel antiviral therapies. Natural products offered a broad prospect in the screening and development of new influenza inhibitors.

Methods: In this research, a high-throughput antiviral screening for 891 natural products was performed based on a recombinant reporter influenza A virus. According to the cytotoxicity assay and dose-response relationship, alloprogesterone (ALLO), as the positive hit was selected, and verified by viral titer reduction assay and immunofluorescence using a wild-type virus. Followingly, we explored its antiviral potency of counteracting with IAV and IBV, and preliminary investigated the mechanism of ALLO through time-of-addition assay and mini-replicon system.

Results: Under the criteria of 80% inhibition and 70% cell viability, ALLO was screened out and confirmed antiviral activity in varied cells. The inhibitory effect of ALLO against influenza virus with a dose-dependent manner and significantly reduced viral yield of five different influenza viruses in the presence of 40 μ M ALLO, including oseltamivir-resistant virus. Moreover, ALLO exhibited no influence on IAV entry or release during the viral replication cycle, but obviously interfered with the genome replication regarding post-infection 2 hrs to 6 hrs, which is consistent with the evidence of decreased polymerase activity.

Conclusions: In summary, we firstly identified a new pharmacological activity of ALLO, as a broad spectrum inhibitor for treatment influenza infections, targeting viral replication stage and possessing great value of further development.

Background

Influenza virus is the main pathogen causing human acute respiratory tract diseases, which is prefer to induce seasonal infections and pandemic outbreaks with significant morbidity and mortality globally [1-3]. Clinical diagnosis analyses suggest that groups with low immunity including pregnant women, infants and the elderly, are susceptible to the influenza virus, and patients with chronic basic diseases have a higher risk of suffering more serious problems and even death [4-7]. Currently, the prevention and treatment therapy of influenza infection mainly focuses on vaccines and antivirals [8]. However, the application of vaccines is restricted by high costs, long cycle and the limited effectiveness caused by mismatching with circulating viral strains [9,10]. Besides, drug resistance and adverse reactions in the clinical usage of existing listed antivirals also urge us to find potential novel influenza viral inhibitors [11]. For instance, the ion channel blocker, amantadine, is no longer recommended for the treatment of influenza virus since its side effects of vertigo, insomnia, vomiting and others and no more than effective for most influenza viruses [12,13]. As the preferred drug for combating influenza diseases recommended by WHO, oseltamivir (Tamiflu®, NA inhibitor) also presented a declined efficacy to combat more than 20 relevant resistant viral strains in 2009, such as H274Y, D151G, K136Q, N386K, P431S, D334N, A193T and

so on [13-17]. Therefore, the urgent need for novel influenza-specific antiviral inhibitors is emphasized to control infection and reduce incidences of resistance.

The natural products with unique pharmacological activity and chemical structure remain popular in potential therapeutic value, of which the important source of new drug research and development [18]. For instance, 49% agents applied in clinically cancer therapy are derived from natural compounds [19]. And a series of traditional Chinese medicines or extracted active ingredients, not only in vitro antiviral experiments, but also in the clinical treatment of influenza, all displayed vital value and broad development prospects [20-22]. In this study, we performed a high-throughput antiviral screening of 891 natural products using a recombinant replication-competent reporter influenza virus, and Allopregnanolone (ALLO) was identified possessing the anti-influenza efficacy with broad spectrum, acting inhibition on the viral replication stage.

Materials And Methods

Cells, Viruses and natural compounds

Madin-Darby canine kidney (MDCK), human non small cell lung cancer (A549) and human embryonic kidney (293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Cellgro, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1,000 units/mL penicillin and 100 µg/ml of streptomycin (Invitrogen, USA). All cells were grown at 37 °C in 5% CO₂ incubator.

A recombinant influenza A reporter virus (PR8-PB2-Gluc) that had been engineered to express *Gaussia luciferase* of which inserted into the PB2 segment, and the wild-type influenza A/Puerto Rico/8/34 (A/H1N1/PR8) were rescued and generated as previously described¹⁶⁻¹⁹. Oseltamivir-resistant influenza A/H1N1/pdm(09) virus containing NA/H274Y mutant site was provided by Beijing CDC, China, influenza A/Brisbane/10/2007(H3N2) was provided by Chinese Academy of Medical Sciences, and influenza B-Yamagata-like and B-Victoria-like strains were provided by Shandong CDC (Jinan, China). Infections were performed in Opti-MEM containing 1.5 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St. Louis, MO, USA).

The compound library containing 891 natural products dissolved in 100 µL DMSO at a stored concentration of 10 mM, was purchased from MedChemExpress (MCE, Monmouth Junction, NJ, USA). Samples were arranged in 96-well plates and diluted with Opti-MEM containing 1.5 µg/mL TPCK-trypsin to 20 µM screened concentration.

Cell-Based High-throughput antiviral screening

The replication-competent reporter IAV carrying *Gaussia luciferase* gene (PR8-PB2-Gluc) has been reported as a sensitive and convenient tool to characterize the virus titer [23]. A cell-based phenotypic high-throughput screening (HTS) that allows for multicycle viral replication to identify potential inhibitors was carried out as previously described [24,25]. Briefly, the adherent growth of monolayer MDCK cells

growing in white, flat-bottom, 96-well culture plates (PerkinElmer, Waltham, MA) were infected with PR8-PB2-Gluc virus at 0.01 multiplicities of infection (MOI) in presence of test samples of 20 μ M. After incubation 36 hours (hrs), infections were determined through the Guassia luciferase activity which was measured using Pierce Guassia luciferase glow assay kit (Thermo scientific, Rockford, IL, USA) following the manufacturer's instructions. Mock infected cells were used as blank control. DMSO and baloxavir acid (BA, diluted to 20 nM concentration) were set as negative and positive control, respectively. The compounds with inhibition rate above 80% were selected for detection of cytotoxicity.

Cytotoxicity assay

The cytotoxicity analysis was performed according to the manufacturer's instructions of CCK-8 kit (MCE). As described previously, MDCK cells at a density of 10000 cells/well grown in 96-well assay plates were treated with specified screening concentration and incubated for 36 hrs. Cell viability was assessed by measuring the absorbance at a wavelength of 450 nm of a [microplate reader](#).

Dose-response assay

The dose-effect relationship was verified for the selected active natural product. Serial diluted samples were added into infected cells and mock cells, separately to obtain IC₅₀ (50% inhibiting concentration) and CC₅₀ (50% cytotoxic concentration) by fitting dose-response curves with a four-parameter logistic regression to the data in GraphPad Prism software (version 5.02, La Jolla, CA, United States), and the selectivity index (SI) was further analyzed.

Viral Titer Reduction Assay

Viral yield reduction assay was performed as previously described²². Briefly, MDCK cells growing in 24-well plates were infected with A/H1N1/PR8, oseltamivirresistant influenza A/H1N1/pdm(09), A/H3N2/Brisbane, B-Yamagata-like, and B-Victoria-like strains at MOI of 0.01 with or without various concentrations of ALLO, respectively. The culture supernatants were harvested at 36 hrs post-infection (p.i.), and virus titers (TCID₅₀/mL) were determined using [cytopathic effect](#) (CPE) experiment. DMSO (0.2%) and uninfected MDCK cells were used as the negative control and the blank control, BA at different concentrations was designed as an antiviral positive drug control group.

Immunofluorescence (IF) analysis

The procedures used for NP immunofluorescence staining were described previously [26]. In a brief, MDCK cells were grown on coverslips and incubated in DMEM containing 2% fetal bovine serum followed by the infection with A/H1N1/PR8 (MOI = 5). After added compound 8 hrs, cells were fixed with 4% PFA for 30 min at room temperature and then were permeabilized with 0.25% TritonX-100 (Sigma-Aldrich, USA). Influenza nucleoprotein (NP) in the cells were detected using anti-NP antibodies (Gene Tex, USA) followed by CoraLite488-conjugated affinipure goat anti-rabbit IgG (H+L) (Proteintech, USA). Nuclear staining with DAPI (40,6-diamidino-2-phenylindole, Solarbio, Beijing) was also performed.

Time of Addition Assay

A classic experiment to further investigate which stage of viral life cycle ALLO disturbed was performed as previously reported [27]. MDCK cells seeded in 24-well plates at a density of 1×10^5 cells/well were infected with 0.1 MOI PR8-wt virus, and interfered with 20 μ M compounds at indicated time (-2, 0, 2, 4, 6, 8) to investigate the period of the viral life cycle targeted by ALLO. At 12 hrs pi, the expression of newly produced virion was analyzed by viral titer test.

Mini-replicon System

The polymerase activity assay was determined by using viral ribonucleoprotein complex (vRNP) minigenome system in A549 cells as previously described [26, 28,29]. In a brief, A549 cells grown in 6-well plates were transfected with viral polymerase plasmids (PB2/pCAGGS, PB1/pCAGGS, PA/pCAGGS, and NP/pCAGGS) and pPoll-Fluc (a firefly luciferase reporter plasmid) together with hRluc-TK (Renilla luciferase plasmid) using Lipofectamine 2000 reagent. At 5 hrs co-transfection, the cells were washed with PBS, re-suspended with fresh DMEM (phenol red free) and seeded into white, 96-well plates at a density of 10,000 cells per well, with various concentrations of ALLO or 0.5% DMSO. After incubation at 37 °C for 24 hrs, luciferase activity was measured by dual-luciferase reporter system (Promega, Madison, WI USA) according to manufacturer's instruction.

Statistical Analysis

In order to quantify the robustness of the screen, Z' factor was calculated from the normalized signals from positive and negative control wells on each plate as follows: $Z' = 1 - 3 \times (\text{SD of positive control} + \text{SD of negative control}) / (\text{mean of negative control} - \text{mean of positive control})$. SD represents the standard deviation. Z' value between 0.5 and 1.0 is regarded as appropriate for an HTS assay [30].

The antiviral inhibition rate was calculated according measured luciferase expression with the following equation: inhibition rate = (signal of negative control - signal of tested compound) / (signal of negative control - signal of positive control) $\times$ 100%.

Results

Influenza Virus Inhibitors Identification Screen Assay

A cell-based HTS assay used a recombinant influenza A/Puerto Rico/8/34 virus, of which the open reading frame for the viral PB2 gene was replaced with that of *Gaussia* luciferase in silent mutations of the original packaging signal. This screening assay allows for the purpose of capturing potential inhibitors targeting viral overall life cycle. During screening procedure, infected cells administered with 0.01 MOI PR8-PB2-Gluc virus were treated with compounds at 20 μ M final concentration to assess the inhibition rate by measuring luciferase activity value after 36 hrs p.i.. A novel influenza cap-endonuclease inhibitor, baloxavir acid was solubilized in DMSO and diluted to 20 nM as the positive control well.

In this study, 891 samples were included in the primary screen, 96 hits that met the cutoff of 80% inhibition were then proceeded in the toxicity screen to exclude cytotoxicity caused false positive ones. Under the screening threshold of 80% inhibition and 30% cytotoxicity dual authentication, it finally yielded one selected hit which continued to carry out the dose-response assay to confirm antiviral activity of candidate inhibitor. The stability and quality of primary HTS system was evaluated by the Z' values of each screening plate, which ranges from 0.54 to 0.91, indicating our preliminary screen was robust and reliable.

ALLO inhibits viral replication in a dose - dependent manner

Throughout antiviral screening and validation assay, allopregnanolone (ALLO, structure indicated in Fig. 2a), as a progesterone metabolite, was selected out from natural product library. We tested a 6-point diluted concentrations of ALLO in the presence of reporter virus or in the absence of virus for inhibition and cytotoxicity dose-response assay in varied cells. The results in MDCK, A549 and 293T cells, all showed that the antiviral activity was regulated by ALLO amount with a positive correlation reflected in the fitted dose-response curves (Fig. 2b). ALLO significantly inhibited IAV replication in a dose-dependent manner with IC₅₀ ranged from 3.01 to 10.64 μM among three cell lines, and all exhibited inconsiderable cytotoxicity besides relatively sensitive 293T. Calculated selective index (SI) of ALLO against influenza virus in MDCK, A549 and 293T cells were 77.4, more than 47 and 21.7 respectively (Table 1).

Table 1
The selective index (SI) of ALLO against PR8-PB2-
Gluc virus in varied cells.

Cell	IC ₅₀ (μM)	CC ₅₀ (μM)	SI*
MDCK	6.27	485.5	77.4
A549	10.64	> 500	> 47.0
293T	3.01	65.29	21.7
* represents the selective index by CC ₅₀ over IC ₅₀ .			

The significant antiviral effect of ALLO was further reassessed in wild-type IAV (A/H1N1/PR8) replication by monitoring the reduction of viral titer and immunofluorescence phenomenon of viral protein expression after compounds treatment. As a result, ALLO at 40 μM concentration greatly decreased viral generation over 5 log compared to the viral load of negative group, which had a comparable antiviral effect with 40 nM baloxavir acid (viral titer reductions of > 6 log). In the meanwhile, IF observed the expression of viral protein stained 8 hrs p.i. was inversely proportional to the drug concentration, that is, the higher ALLO dose, the less fluorescence amount of NP protein particle and the weaker fluorescence intensity (Fig. 4a, MDCK cells). When treated with a concentration of 40 μM, ALLO also almost completely suppressed viral replication in wt-PR8 virus (MOI = 1) infected A549 cells (Fig. 4b).

ALLO is effective to inhibit influenza A and B virus

To further explore the broad-spectrum antiviral potency of ALLO, IAV-pdm09 (H1N1), which was resistant to oseltamivir with NA/H274Y mutations, IAV-Brisbane (H3N2), IBV-Yamagata and IBV-Victoria strains were carried out for viral yield reduction assay. Each viral strain was inoculated with different concentrations of ALLO for 36 hrs, the supernatants were harvested and the virus titers were determined. All groups with triplicate measurements. As indicated in Fig. 5, ALLO at 40 μ M concentration could inhibit the replication of influenza A and B virus, especially H3N2 virus (viral yield decreased > 6 log) and oseltamivir-resistant virus (viral yield decreased > 5 log), showing more sensitivity to ALLO, which compared to IBV two strains (> 3 log decreased in Yamagata, about 2 log decreased in Victoria). Thus, we concluded that ALLO exhibits a broad spectrum of anti-influenza virus activity.

ALLO exhibits inhibitory effect on the stage of the viral replication

We further investigated which stage of viral life cycle was disturbed by ALLO according classic time-of-addition assay as previously reported [27]. Over the single viral replication cycle, viral yield did not appear significant reduction when cells were treated with 20 μ M ALLO at -2-0h and 8-12h intervals. Instead, infected cells administrated with ALLO at 0h, 2h, 4h and 6h, during viral replication proceeding, the viral titer was inhibited. It is indicated that the antiviral effect of ALLO is associated with viral genome replication rather than virus entry and release periods. In the meanwhile, polymerase, the vital replicated machine, was disturbed by ALLO at active concentrations, with a decline of the fluorescence value ranging from 50–90% using the mini-replicon system.

Taken together, although the more detailed antiviral mechanism is in progress, these results already suggested that the inhibitory effect of ALLO through acting on the stage of viral replication, and influenced the expression of polymerase.

Discussion

Influenza virus is a member of the *Orthomyxoviridae* family, being segmented, single stranded, negative sense RNA viruses [31]. It mainly adheres to respiratory tract epithelium leading viral pneumonia with typically clinical manifestations of cellular infiltration, diffuse inflammation and ground-glass opacities, following highly morbidity not only in immunocompromised individuals and patients with underlying comorbid conditions, but also in young healthy adults [32]. Natural products occupy an important position in the development of new antiviral inhibitors to cope with the widespread occurrence of drug resistance events in existing listed agents.

In this research, we employed a recombinant reporter virus expressing *Gaussia luciferase* to carry out the antiviral HTS and eventually obtained one potent compound named ALLO (Allopregnanolone, the metabolite of progesterone). It could dose-dependently decrease the viral luciferase expression among MDCK, A549 and 293T cells, indicating that ALLO exerted an inhibitory effect on influenza virus without cell specificity. Additionally, immunofluorescence of viral NP protein confirmed that ALLO inhibited viral replication in a dose-dependent manner. We further explored the inhibitory effect of ALLO on various subtypes of influenza viruses in a titer reduction assay. The viral yields in the infected MDCK cells could

be remarkably reduced by 40 μ M ALLO, implying a broad spectrum of anti-IAV and IBV activity. Moreover, it was preliminarily concluded that the mode of action of ALLO by blocking viral replication stage according a single-cycle replication assay and a mini-replicon system.

It has been reported that ALLO is an allosteric regulator of GABA receptors playing a role in depression and anxiety [33], as well as a variety of neurodegenerative diseases such as diabetic neuropathy [34], Alzheimer's disease [35] and Parkinson's disease [36]. It has been investigated for the treatment of Severe Postpartum Depression as the main ingredient of Zulresso (the first FDA-authorized PPD inhibitor) [37]. Eminently, our finding is the first time revealing the novel pharmacological activity of ALLO against influenza virus with broad-spectrum and identified ALLO playing an antiviral role in the replication period in vitro. Interestingly, another reported anti-influenza ingredient acting on downregulating NF- κ B expression, cirsimaritin, derived from a flavone of *A. scoparia*, can bind weakly with GABAA receptors with antidepressant and anxiolytic activity [38, 39]. The commonality of these two influenza inhibitors' pharmacological activity suggests whether ALLO can also play an indirect antiviral role by stimulating the immune function. The specific of treating PPD of ALLO makes us suppose that the antiviral activity of ALLO whether or not be associated with clinical changes in progesterone levels in patients with influenza during pregnancy and postpartum. Also, the further mechanism research and in vivo antiviral study of ALLO are under way, with respect to the activity of its analogues and synergy effect in combination with current inhibitors are also deserved to be explored. In conclusion, this article provided a new perspective on potential activity of ALLO.

Conclusions

In this study, we provided a novel insight about anti-influenza virus potency of ALLO. The promising prospect of ALLO with broad-spectrum antiviral activity in vitro, rendering us further in vivo study and detailed mechanism research are necessary. And it is of great significance to be an alternative antiviral agent against influenza viruses in the future.

Abbreviations

ALLO: Allopregnanolone; BA: baloxavir acid; OA: Oseltamivir Acid; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's modified Eagle's medium; MDCK: Madin-Darby canine kidney; A549: human non small cell lung cancer; 293T: human embryonic kidney; IAV: Influenza A virus; IBV: Influenza B virus; Gluc: Gaussia luciferase; HTS: high-throughput screening; MOI: Multiplicity of infection; SD: Standard deviation; IC₅₀: 50% inhibiting concentration; CC₅₀: 50% cytotoxic concentration; SI: Selection index; IF: Immunofluorescence.

Declarations

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Authors' contributions

YQW and YYW designed and performed the experiments and draft the paper. YYW and HC contributed to the data analyses. HC and YQW approved the final version. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The flowchart of antiviral screening based on a recombinant reporter IAV. (a) Schematic of the screening process. Screened samples were added into MDCK cells infected with PR8-PB2-Gluc virus (MOI=0.01) to perform a phenotypic-based antiviral HTS. Secondary hits were defined as meeting the screening criteria of 80% inhibition and 70% cell viability. The selected positive hit was carried out dose-response

validation. (b) Results from the HTS of 891 compounds indicating the number of primary hits (96), the hits confirmed in counter cytotoxicity (5), and the selection of hit for revalidation (1).

Figure 2

ALLO exhibited the activity of anti-influenza virus. (a) Chemical structure of ALLO and its molecular weight (M.W). (b) Dose-response analysis of ALLO against PR8-Gluc virus in varied cells. R² of three fitted curves by Graphpad Prism5 software are 0.95 in MDCK, 0.99 in A549 and 0.88 in 293T. Data were standardized with three independent experiments.

Figure 3

ALLO reduced viral titer of PR8-wt virus in a dose-response manner. Confluent monolayers MDCK cells inoculated in 24-well plates at 10⁴ cells per well for 24 hrs before infection. After challenged with PR8-wt virus (MOI=0.01) 2hrs, MDCK cells were treated with Opti-MEM (1.5 μ g/mL TPCK-trypsin) containing different concentrations of baloxavir acid or ALLO for 36 hrs and viral yield of culture supernatants were measured. Each point represents the mean and standard deviation of three independent experiments.

Figure 4

IF observation of ALLO inhibiting the expression of influenza viral nucleoprotein. (a) MDCK cells were infected with PR8-wt virus at 1 MOI and treated with serial dilutions of ALLO for 8 hrs. (b) Infected A549 cells were incubated with ALLO at 40 μM concentration and observed at 8 hrs p.i. under original magnification of 10x, 20x and 40x. Nuc (Nucleozin, 1 μM) as an influenza NP inhibitor was set as positive control. The expression of viral NP protein was detected by immunofluorescence microscopy (OLYMPUS-

IX73, Japan). Cells stained with DAPI and anti-influenza NP antibody were used to define the nucleus and influenza particle, respectively, with colored blue and green spots.

Figure 5

Antiviral activity of ALLO against various subtypes of influenza viruses in a yield reduction assay. MDCK cells in 24-well plates were infected with (a) oseltamivir-resistant IAV-pdm09 (H1N1), (b) IAV-Brisbane (H3N2), (c) IBV-Yamagata, and (d) IBV-Victoria at an MOI of 0.01. The culture supernatants were collected at 36 hrs p.i., and virus titers (TCID₅₀/mL) were determined using MDCK cells. BA at 40 nM was used as the positive control (not shown in picture), and DMSO as the negative control. Results are the means $\pm$ standard deviations of three independent experiments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 6

ALLO exert an inhibitory activity on the influenza virus replication cycle. (a) Time-of-addition assay for inhibition of wt-PR8 virus by ALLO. The infected MDCK cells were treated with 20 μM ALLO or 2 μM OA (Oseltamivir Acid) at indicated time intervals (-2-12, -2-0, 0-12, 2-12, 4-12, 6-12, and 8-12 h), and viral titer was determined after 12 hrs p.i.. (b) Inhibitory effect of ALLO on viral polymerase activity was tested by a minireplicon assay. Transfected A549 cells by four constituent plasmids of vRNP (PB2/pCAGGS, PB1/pCAGGS, PA/pCAGGS, and NP/pCAGGS) and two internal reference plasmids (pPol-Fluc and hRluc-TK), was added with serially diluted ALLO from 200 μM to 4 μM after transfection 5 hrs. Incubation of 24 hrs later, the luciferase activity was measured and DMSO was employed as negative control. Data were expressed as means ± SD (n = 3). *p < 0.05; **P < 0.01; ***p < 0.001.