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Identification of a small-molecule inhibitor of influenza virus via disrupting the subunits interaction of the viral polymerase

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ABSTRACT

Assembly of the heterotrimeric influenza virus polymerase complex from the individual subunits PB1, PA, and PB2 is a prerequisite for viral replication, in which the interaction between the C terminal of PA (PA_C) and the N-terminal of PB1 ($PB1_N$) may be a desired target for antiviral development. In this study, we compared the feasibility of high throughput screening by enzyme-linked immunosorbent assay (ELISA) and fluorescence polarization assay. Among the two, ELISA was demonstrated to own broader dynamic range so that it was used for screening inhibitors that blocked PA_C and $PB1_N$ interaction. Several binding inhibitors of PA_C-$PB1_N$ were identified and subsequently tested for the antiviral efficacy. Apparently, 3-(2-chlorophenyl)-6-ethyl-7-methyl[1,2,4]triazolo[4,3-a]pyrimidin-5-ol, designated ANA-1, was found to be a strong inhibitor of viral polymerase activity and act as a potent antiviral agent against the infections of multiple subtypes of influenza A virus, including H1N1, H3N2, H5N1, H7N7, H7N9 and H9N2 subtypes, in cell cultures. Intranasal administration of ANA-1 protected mice from lethal challenge and reduced lung viral loads in H1N1 virus infected BALB/c mice. Docking analyses predicted that ANA-1 bound to an allosteric site of PA_C, which might cause conformational changes thereby disrupting the PA_C-$PB1_N$ interaction. Overall, our study has identified a novel compound with potential to be developed as an anti-influenza drug.

1. Introduction

Influenza has caused morbidity and mortality in humans through routine seasonal epidemics and global pandemics (Hofer, 2014). The rapid mutability of influenza virus in conjunctions with genomic reassortment between viral strains promotes the virus' ability to evade vaccines and become resistant to antiviral drugs (Barik, 2012). Therefore, there is a continuing need for novel anti-influenza therapeutics using new targets and creative strategies.

The conserved protein–protein interaction sites have been suggested as potential targets in the anti-influenza drug discovery (Das et al., 2010). The RNA dependent RNA polymerase (RdRp) of influenza virus contains PB1, PB2 and PA subunits, which are responsible for the viral transcription and replication (Boivin et al., 2010). Before entering the host nucleus to exert functions, the three subunits bind each other non-covalently through a series of interactions that are essential for polymerase assembly (Reich et al., 2014). The primary protein–protein interactions are between the N terminus of PB1 ($PB1_N$) and the C terminus of PA (PA_C) (He et al., 2008; Obayashi et al., 2008) as well as between the C terminus of PB1 ($PB1_C$) and the N terminus of PB2 ($PB2_N$) (Sugiyama et al., 2009). The co-crystallization of PA_C-$PB1_N$ complex has revealed that the $PB1_N$ (residues 1–25 of PB1 subunit) clamps into the open 'jaws' of a dragon's head, which are hydrophobic grooves that formed by PA_C domain (residues 257–716 of PA subunit) (Yuan et al., 2009). These 'jaws' are, thus presenting a potential target for novel anti-influenza therapeutics. Importantly, alteration of the conserved residues contributing to PA_C–$PB1_N$ interaction restricts polymerase assembly and reduces polymerase activity (Wunderlich et al., 2010). In this regard, the emergence of escape mutants that induced by PA_C–$PB1_N$ inhibitors is expected to be minimized.

In the present study, we conceived that blocking of the polymerase assembly would abrogate its activity and therefore inhibit

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virus replication. Two widely utilized techniques in monitoring ligand–protein interactions, i.e. ELISA and fluorescence polarization (FP) assay, were compared for their capacity for effective screening. We demonstrated that ELISA exhibited a broader dynamic range than FP assay in the search of PA_C–$PB1_N$ inhibitors. A chemical library of 950 candidates (Kao et al., 2010) was utilized for ELISA screening and the selected compounds were tested *in vitro* and *in vivo* for antiviral efficacies. A potent virus replication inhibitor ANA-1 was identified, which acted by disrupting the polymerase formation probably through the inducing of PA_C conformational changes.

2. Materials and methods

2.1. Cells, viruses, peptides and chemical compounds

Madin–Darby canine kidney (MDCK) cells and human embryonic kidney 293T cells were cultured as described previously (Yuan et al., 2015). A total of 8 strains of influenza A virus with 6 subtypes, A/HK/415742/09(H1N1), A/Hong Kong/1/1968 (H3N2), A/Shenzhen/406H/2006 (H5N1), A/Hong Kong/156/97 (H5N1), A/Vietnam/1194/2004(H5N1), A/Netherlands/219/2003(H7N7), A/Anhui/1/2013(H7N9), and A/HK/1073/1999 (H9N2), were conserved by the P3 laboratory, University Pathology Building of Queen Mary Hospital and used in *in vitro* antiviral study. A mouse-adapted influenza virus strain, A/HK/415742Md/09 (H1N1), was utilized in mouse study. All experiments with live viruses were conducted in biosafety level 2 or 3 facilities as described previously (Zheng et al., 2010; 2008). All the testing compounds were purchased from ChemBridge Corporation (San Diego, USA). Peptides were synthesized by Cellmano Biotech Limited (Hefei, China) with >95% purity.

2.2. Expression and purification of His–PA_C and GST-$PB1_N$ protein

His–PA_C and GST–$PB1_N$ protein were expressed according to reports published previously (Li et al., 2012; Muratore et al., 2012). The target protein was detected by SDS-PAGE or western blot as described previously (Zheng et al., 2001) using anti-His (for His–PA_C) or anti-GST (for GST–$PB1_N$) antibody.

2.3. ELISA

ELISA was conducted in 96-well black micro-plates (Greiner Bio-One). For ELISA, which was performed according to a previous report with modifications (Muratore et al., 2012). Briefly, plates were coated with 400 ng/well His–PA_C at 4 °C for overnight and blocked with 5% (wt/vol) skimmed milk (Sigma) in phosphate buffer saline (PBS) containing 0.1% Tween-20 (PBST) at 37 °C for 1 h. After washing, 800 ng/well of the GST–$PB1_N$ protein were added with or without the testing compounds and incubated at 37 °C for 3 h. Subsequently, intensive wash were carried out, followed by the addition of anti-GST primary antibody, secondary antibody and substrate. Buffer that contains 20 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM DTT, and 1% (vol/vol) Triton X-100 was used in either protein dilution or reaction.

2.4. Small-molecule compounds screening

ELISA was applied in the primary screening of a library of 950 compounds, in which each was tested at the concentration of 10 μg/ml. Next, a dose-response analysis was done to identify the 'active' compounds that consistently blocked the PA_C–$PB1_N$ binding from the primary hits. Afterwards, a secondary screening was performed with plaque reduction assay (Zheng et al., 2010) to evaluate the *in vitro* anti-H1N1 efficacies of the 'active' compounds. Selected compounds were serial-diluted (10, 5, 2.5, 1.25, and 0.625 μM) and tested on MDCK cells.

2.5. The evaluation of cross-protection

The antiviral effect of compound was tested against multiple subtypes of influenza virus as mentioned above. Briefly, MDCK cells were infected with the viruses at multiplicity of infection (MOI) of 0.002. One hour after virus inoculation, the inoculum was removed and replaced by fresh MEM medium containing serial-diluted compound. The culture supernatants were collected at 24 h post-infection (p.i.) and titrated by RT-qPCR. Briefly, viral RNA (vRNA) was first extracted from the cell supernatants by QIAamp viral RNA mini kit (Qiagen), then reverse transcribed using influenza specific primer Uni12: 5′-AGCAAAAGCAGG-3′ (Hoffmann et al., 2001) and PrimeScript II reverse transcriptase (Takara). Transcriptions were determined by LightCycler® 96 Real-Time PCR System (Roche) using 480 SYBR Green I master mix with universal primers of M gene that designed for all the subtypes of influenza virus: 5′-CTTCTAACCGAGGTCGAAACG-3′ (forward) and 5′-GGCATTTTGGACAAAGCGTCTA-3′ (reverse).

2.6. In vivo evaluation of antiviral effects

BALB/c female mice, 6–8 weeks old, were kept in biosafety level-2 housing and given access to standard pellet feed and water *ad libitum*. All experimental protocols followed the standard operating procedures of the approved biosafety level-2 animal facilities and were approved by the Animal Ethics Committee in the University of Hong Kong. After anesthesia, a total of 56 mice in four groups were inoculated with 60% lethal dose (LD_{60}, 300 pfu/mouse) of mouse-adapted influenza H1N1 virus. The therapeutic treatment initiated at 6 h post-virus-challenge by intranasal route. The mice were anesthetized by intraperitoneal injection of ketamine-xylazine (50/5 mg/kg) and then intranasally received 20 μl of 2.5 mg/ml of ANA-1 or PAC-3 or zanamivir. One group of mice was administrated with PBS as an untreated control. The treatment continued for 3 days with 2 doses/day. Animal survival, body weight and general conditions were monitored for 21 days or until death. A body weight loss of 25% was set as the humane endpoint. Four mice in each group were euthanized on day 4 post-challenge. Half of the mouse lungs were collected for virus titration or for histopathologic examination as described previously (Peiris et al., 2004).

2.7. Multi-cycle virus growth assay

MDCK cells were infected with the influenza A viruses at multiplicity of infection (MOI) of 0.002 and then maintained in the presence or absence of the selected compounds (20 μM). Culture supernatants were collected at 0, 6, 21, 25, 32, 47 and 54 h p.i. and titrated by plaque assay.

2.8. The investigation of antiviral mechanism

To investigate which stage of virus life cycle was interfered by the compound, MDCK cells were inoculated with influenza H1N1 virus at MOI of 2 and with ANA-1 (20 μM) addition during the time of virus absorption (−1 h) or at 1 h p.i.. For the former, infectious inoculum containing the tested compound was replaced by fresh medium without the compound after virus entry. For the later, the compound was maintained in the medium after virus internalization. Virus yield in the cells or supernatants was determined at 6 h p.i. by RT-qPCR. To determine whether the compound inhibit the virus replication or transcription, intracellular viral mRNA and