Antiviral Research 116 (2015) 55–61

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A monoclonal antibody binds to threonine 49 in the non-structural 1 protein of influenza A virus and interferes with its ability to modulate viral replication

Bhaskar Barnwal [a], Chee-Keng Mok [a], Jianping Wu [a], Mandakhalikar Kedar Diwakar [a], Garvita Gupta [b], Qi Zeng [c], Vincent Tak Kwong Chow [a], Jianxing Song [b], Y. Adam Yuan [b,d], Yee-Joo Tan [a,c,*]

[a] Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, Singapore
[b] Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore
[c] Institute of Molecular and Cell Biology, A*STAR (Agency for Science, Technology and Research), Singapore
[d] National University of Singapore (Suzhou) Research Institute, Suzhou Industrial Park, Jiangsu 215123, China

ARTICLE INFO

Article history:
Received 18 July 2014
Revised 10 December 2014
Accepted 30 January 2015
Available online 7 February 2015

Keywords:
Non-structural 1 protein
Monoclonal antibody
Influenza A virus

ABSTRACT

The emergence of resistant influenza A viruses highlights the continuous requirement of new antiviral drugs that can treat the viral infection. Non-structural 1 (NS1) protein, an indispensable component for efficient virus replication, can be used as a potential target for generating new antiviral agents. Here, we study the interaction of 2H6 monoclonal antibody with NS1 protein and also determine whether influenza virus replication can be inhibited by blocking NS1. The 2H6-antigen binding fragment (Fab) forms a multimeric complex with the NS1 RNA-binding domain (RBD). T49, a residue which forms a direct hydrogen bond with double stranded RNA, in NS1 protein was found to be critical for its interaction with 2H6 antibody. NS1(RBD) has high affinity to 2H6 with K_D of 43.5 ± 4.24 nM whereas NS1(RBD)-T49A has more than 250 times lower affinity towards 2H6. Interestingly, the intracellular expression of 2H6-single-chain variable fragment (scFv) in mammalian cells caused a reduction in viral growth and the M1 viral protein level was significantly reduced in 2H6-scFv transfected cells in comparison to vector transfected cells at 12 h post infection. These results indicate that the tight binding of 2H6 to NS1 could lead to reduction in viral replication and release of progeny virus. In future, 2H6 antibody in combination with other neutralizing antibodies can be used to increase the potency of viral inhibition.

1. Introduction

Influenza A virus (IAV), a member of *Orthomyxoviridae* family, is still a threat to human health and a burden on the health services (Salomon and Webster, 2009). Despite many advances, IAVs are still a challenge for the scientists. IAVs are highly contagious and causative agents of seasonal flu epidemics resulting in morbidity, mortality and huge economic losses. Based on the circulating strains, seasonal influenza vaccines are developed annually or biannually, if needed, by WHO but the immunity provided is short-lived due to continuous change in the virus strains. Therefore, vaccination is usually required every year to be protected from seasonal flu that leads to increase in vaccine cost along with shortage of vaccines in developing countries. But the two main problems with vaccination are the time required to select, manufacture and deliver vaccine and the variable annual immunization rates (Couch, 2008). Besides vaccination, the antiviral agents are the therapeutic options to treat the infection. Antivirals against M2 protein and neuraminidase are available but their irrational use has led to the emergence of resistant strains (Agrawal et al., 2010; Hayden and Hay, 1992; Poland et al., 2009). Thus, there is a continued requirement of new antiviral agents against IAV.

The non-structural protein NS1 of IAV is a multifunctional protein associated with various viral functions including mRNA processing regulation via interactions with the cleavage and

Abbreviations: IAV, influenza A virus; NS1, non-structural 1; RBD, RNA binding domain; CPSF30, cleavage and polyadenylation and specificity factor 30; PI3K, phosphoinositide 3-kinase; IFN, interferon; PKR, protein kinase R; OAS, 2′5′-oligoadenylate synthetase; dsRNA, double-stranded RNA; mAbs, monoclonal antibodies.

* Corresponding author at: Department of Microbiology, Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore, MD4, 5 Science Drive 2, Singapore 117597, Singapore. Tel.: +65 65163692; fax: +65 67766872.

E-mail address: Yee_Joo_TAN@NUHS.edu.sg (Y.-J. Tan).

http://dx.doi.org/10.1016/j.antiviral.2015.01.015
0166-3542/

polyadenylation and specificity factor 30 (CPSF30), inhibition of cellular apoptosis by interaction with the p85β regulatory subunit of phosphoinositide 3-kinase (PI3K), limitation of interferon (IFN) production and the IFN-induced proteins, such as protein kinase R (PKR) and 2′5′-oligoadenylate synthetase (OAS)/RNase L by binding to double-stranded RNA (dsRNA) (Hale et al., 2008; Min and Krug, 2006), and inhibition of mRNA splicing by binding to U6 snRNA (Qiu et al., 1995; Wang and Krug, 1998). NS1 has two functionally distinct domains: the N-terminal RNA binding domain (RBD), consisting of three α-helices, and the C-terminal effector domain, consisting of seven β-strands and three α-helices. The RBD domain binds with low affinity to several RNA species in a sequence independent manner (Chien et al., 2004; Hatada and Fukuda, 1992; Qian et al., 1995), and effector domain predominantly interacts with host-cell proteins and also functionally stabilizes RBD domain (Wang et al., 2002).

NS1, a well conserved protein, is expressed at very high levels in infected cells (Krug and Etkind, 1973; Palese and Shaw, 2007). Therefore, NS1 protein is a good target for therapeutics development and several small molecules have been found to inhibit NS1 function resulting in reduced viral replication (Engel, 2013; Nayak et al., 2014; Woo et al., 2013). In our previous study, we generated a panel of new monoclonal antibodies (mAbs) against the RNA binding domain of NS1 (NS1(RBD)) (Tan et al., 2010). Here we report the biophysical characterization of one of these mAbs, named as 2H6, and NS1(RBD) protein interaction, and inhibition of viral replication by targeting NS1 protein in infected cells. 2H6 has been shown to bind to NS1 of different IAV subtypes, namely H5N1, H3N2 and H1N1 (Tan et al., 2010).

2. Materials and methods

2.1. Cell lines and virus

A549, 293T and MDCK cells were purchased from American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. A/Puerto Rico/8/1934(H1N1) (PR8) virus was obtained from the American Type Culture Collection and grown in embryonated chicken eggs as previously described (Narasaraju et al., 2011).

2.2. Generation of anti-M1 monoclonal antibody

The cDNA encoding for the M1 gene from a H5N1 isolate (A/chicken/Hatay/2004(H5N1)), GenBank accession number AM040045) was cloned into the pGEX-6P1 vector (GE Healthcare, Uppsala, Sweden). Glutathione S-transferase (GST)-fused M1 protein was then expressed in *Escherichia coli* BL21(DE3) (Novagen, EMD Chemicals, Inc., Madison, WI, USA) and purified as previously described (Tan et al., 2010). The GST-fusion protein was then used to immunize mice and generate hybridomas as previously described (Oh et al., 2010). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Biological Resource Centre, A*STAR, Singapore (Protocol Number: 110693). All the procedures were carried out in strict accordance with the recommendations of the National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines in Singapore. All efforts were made to minimize suffering and euthanasia was performed using carbon dioxide.

2.3. Ascites production

Ascites was produced by injecting hybridoma cells into the peritoneal cavities of pristine-primed BALB/c mice. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Biological Resource Centre, A*STAR, Singapore (Protocol Number: 110694). All the procedures were carried out in strict accordance with the recommendations of the NACLAR guidelines in Singapore. All efforts were made to minimize suffering and euthanasia was performed using carbon dioxide.

2.4. Expression and purification of NS1 in bacteria

The gene encoding N-terminus of NS1 (1–73 aa) of A/chicken/Hatay/2004(H5N1) was cloned into modified pET-28a expression vector (Novagen) with N-terminal His-SUMO tag. The wild-type NS1(RBD) and its mutants (R38AK41A, S42A and T49A) were expressed in *E. coli* BL21(DE3)-RILP overnight at 20 °C, and protein expression was induced using 0.4 mM isopropyl β-D-thiogalactoside (IPTG). Cells were harvested by centrifugation and the cell pellet was resuspended in lysis buffer which was followed by passing it through a cell disruptor (www.avestin.com) for five times. After ultracentrifugation at 40,000 rpm for 1 h, the supernatants were loaded onto Ni–NTA affinity column for purification. Pooled eluted fractions were dialysed overnight against dialysis buffer (20 mM Tris (pH 7.4), 100 mM NaCl) supplemented with Ulp1 protease for His-SUMO cleavage. The fractions were reloaded onto Ni–NTA affinity column to remove His-SUMO tag. The flow through was then loaded onto HiLoad 26/60 Superdex 75 column (GE Healthcare), equilibrated in 25 mM Tris (pH 7.4), 500 mM NaCl and 10 mM DTT for further purification. The purified proteins were dialysed against dialysis buffer again and concentrated to 3 mg/mL in a Centriprep-10 (Amicon). Purified NS1 fragments were then subjected to Tricine–SDS–PAGE on a 15% gel which was stained using coomassie blue to visualize the purity of proteins.

2.5. Purification of 2H6 whole antibody and preparation of 2H6-antigen-binding fragment (Fab)

Antibody was purified from ascites by HiTrap Protein A column (GE Healthcare) according to manufacturer's instructions. Briefly, ascites was diluted in 1× phosphate buffer saline (PBS) and injected in pre-equilibrated Protein A column. The whole antibody was eluted from the column using 25 mM glycine (pH 2.2) elution buffer and then subjected to papain cleavage according to manufacturer's instructions. In brief, whole antibody was incubated with immobilized papain (Thermo Scientific) in digestion buffer (20 mM sodium phosphate (pH 7), 10 mM EDTA, 20 mM cysteine) overnight at 37 °C which was followed by Fab purification by HiTrap Protein L column (GE Healthcare) according to manufacturer's instructions.

2.6. Circular dichroism (CD) spectroscopy

Far-UV CD spectra (260–190 nm) were recorded using a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan). The cuvette chamber and instrument optics were continuously purged with 30 L of nitrogen/min before and during the measurements. The spectra were recorded using a scanning speed of 50 nm/min, a resolution of 0.1 nm and a bandwidth of 1 nm. NS1(RBD) (10 μM) and 2H6-Fab (5 μM) were dissolved in 1 mM phosphate buffer (pH 7.4) and placed in a cuvette with 0.1 cm path length. An average of three scans was taken to increase the signal to noise ratio and the baseline was subtracted.

2.7. Gel filtration chromatography

The NS1(RBD) and 2H6-Fab were mixed in 1:1 M ratio and incubated overnight at 4 °C. The NS1(RBD) and 2H6-Fab complex formation was examined by gel filtration chromatography on a