Virus Research 217 (2016) 23–31

Contents lists available at ScienceDirect

Virus Research

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

A high-throughput screening system targeting the nuclear export pathway via the third nuclear export signal of influenza A virus nucleoprotein

Michinori Kakisaka, Takafumi Mano, Yoko Aida*

Viral Infectious Diseases Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

ARTICLE INFO

Article history:
Received 29 December 2015
Accepted 7 February 2016
Available online 4 March 2016

Keywords:
Influenza virus
NP
Nuclear export
NES
Compound
HTS

ABSTRACT

Two classes of antiviral drugs, M2 channel inhibitors and neuraminidase (NA) inhibitors, are currently approved for the treatment of influenza; however, the development of resistance against these agents limits their efficacy. Therefore, the identification of new targets and the development of new antiviral drugs against influenza are urgently needed. The third nuclear export signal (NES3) of nucleoprotein (NP) is the most important for viral replication among seven NESs encoded by four viral proteins, NP, M1, NS1, and NS2. NP-NES3 is critical for the nuclear export of NP, and targeting NP-NES3 is therefore a promising strategy that may lead to the development of antiviral drugs. However, a high-throughput screening (HTS) system to identify inhibitors of NP nuclear export has not been established. Here, we developed a novel HTS system to evaluate the inhibitory effects of compounds on the nuclear export pathway mediated by NP-NES3 using a MDCK cell line stably expressing NP-NES3 fused to a green fluorescent protein from aequorea coerulescens (AcGFP-NP-NES3) and a cell imaging analyzer. This HTS system was used to screen a 9600-compound library, leading to the identification of several hit compounds with inhibitory activity against the nuclear export of AcGFP-NP-NES3. The present HTS system provides a useful strategy for the identification of inhibitors targeting the nuclear export of NP via its NES3 sequence.

© 2016 Published by Elsevier B.V.

Contents

1. Introduction 24
2. Materials and methods 24
   2.1. Cells, transfection and antibodies 24
   2.2. Compounds 24
   2.3. Plasmid construction 24
   2.4. Indirect immunofluorescence 25
   2.5. Library screening using the HTS system targeting the nuclear export of NP-NES3 25
   2.6. Cell viability assay 25
   2.7. In vitro antiviral activity assay 25
   2.8. Statistical analysis 25
3. Results 25
   3.1. Construction of the plasmid expressing the AcGFP-NP-NES3 fusion protein 25
   3.2. Establishment of a stable cell line expressing AcGFP-NP-NES3 26

Abbreviations: IAV, influenza virus; NP, nucleoprotein; vRNA, viral RNA; vRNP, viral ribonucleoprotein; NLS, nuclear localization signal; NES, nuclear export signal; CRM1, chromosome region maintenance 1; LMB, leptomycin B; HTS, high-throughput screening; AcGFP, green fluorescent protein from aequorea coerulescens.

* Corresponding author.
E-mail address: aida@riken.jp (Y. Aida).

http://dx.doi.org/10.1016/j.virusres.2016.02.007
0168-1702/© 2016 Published by Elsevier B.V.

3.3. Establishment of a HTS system targeting the nuclear export of NP-NES3 28
3.4. Identification of novel nuclear export inhibitors using the HTS system targeting NP-NES3 nuclear export 28
4. Discussion 28
Acknowledgments 31
References 31

1. Introduction

Influenza A virus (IAV) remains a significant health threat because it can cause seasonal epidemics and because of the emergence of antigenically shifted or highly virulent strains that cause significant economic losses and are associated with high morbidity (Szucs, 2004; Taubenberger and Kash, 2010). IAV has shown resistance to available drugs, such as M2 channel inhibitors and neuraminidase inhibitors (Poland et al., 2009; Hu et al., 2013). The occurrence of drug resistant viruses underscores the need for the development of antiviral drugs based on a new mechanism of action. Nucleoprotein (NP) is a valid target for the development of antiviral drugs (Cianci et al., 2013). NP is the major component of viral ribonucleoprotein (vRNP), which consists of three polymerase subunits (PB2, PB1, and PA) and viral RNA (vRNA) (Arranz et al., 2012; Moeller et al., 2012). It contains many functional domains in its sequence, such as a nuclear localization signal (NLS), a nuclear export signal (NES), and an RNA binding site (Aida et al., 2012), and it regulates multiple processes during the viral lifecycle, including nuclear import and export of vRNP and viral genome transcription/replication (Aida et al., 2012). Small molecule compounds targeting NP inhibit the multiplication of IAV (Cianci et al., 2013). The mycalamide analog, a natural product derived from sponges of the mycale species, was identified by chemical array screening and shown to bind to NP via an unconventional NLS and inhibit IAV replication (Hagiwara et al., 2010). Nucleozin was identified as an NP inhibitor by a cell based influenza virus infection assay; it blocks the formation of higher-order NP oligomers by aggregation through the cross-linking of two NP molecules, resulting in a potent antiviral effect (Kao et al., 2010; Gerritz et al., 2011). These results indicate that the influenza virus NP is a valid target for the development of antiviral compounds. In addition, we previously identified NP-NES3 as a promising antiviral drug target out of three NESs (NP-NES1, E24–L49; NP-NES2, V183–I197; and NP-NES3, P248–S274) within a single viral NP (Yu et al., 2012). IAV possesses seven NESs in different viral proteins, including NP, M1, NS1, and NS2 (O'Neill et al., 1998; Huang et al., 2013; Paterson and Fodor, 2012; Li et al., 1998; Cao et al., 2012). Mutation of these NES sequences attenuated viral replication kinetics compared with those of wild-type virus; in particular, mutation of the NP-NES3 sequence blocked the ability to produce replicable viruses (Chutiwitoonchai et al., 2014). Moreover, nuclear export of NP is attenuated in mutant NP-NES3 carriers, whereas mutations of NP-NES1 and NP-NES2 have no significant effect on cytoplasmic localization (Chutiwitoonchai et al., 2014). These results indicate that NP-NES3 is essential for viral replication and the nuclear export of NP, and is therefore a potential novel target for antiviral drugs.

NP-NES3 is recognized by a specific cellular transport receptor, chromosome region maintenance 1 (CRM1), whereas the transport of NP-NES1 and NP-NES2 is CRM1-independent (Chutiwitoonchai et al., 2014). CRM1 binds to the NP-NES3 consensus sequence, and the CRM1 inhibitor leptomycin B (LMB) inhibits the nuclear export of NP by blocking the interaction between CRM1 and NP, thereby exerting an antiviral effect (Chutiwitoonchai et al., 2014; Elton et al., 2001). However, LMB exhibits substantial cytotoxicity in vivo due to the irreversible nature of the modification via the covalent binding to C528 of CRM1 (Sun et al., 2013; Mutka et al., 2009), suggesting that it is not suitable as a therapeutic agent. Therefore, novel nuclear export inhibitors capable of blocking the interaction between CRM1 and NP-NES3 could be effective as anti-influenza agents.

In the present study, we developed a high-throughput screening (HTS) system for the identification of nuclear export inhibitors targeting NP-NES3. Our system uses a MDCK cell line stably expressing NP-NES3 fused to a green fluorescent protein from aequorea coerulescens (AcGFP-NP-NES3) and a cell imaging analyzer, CELAVIEW RS100, which offers the following advantages: (i) the established cell line allows monitoring of the intracellular distribution of NP-NES3 by observing the AcGFP fluorescence signal; (ii) CELAVIEW RS100 can quantitatively measure the nuclear fluorescence signal and simultaneously acquire the intracellular fluorescent image in a large number of cells, enabling the HTS assay; and (iii) validation analyses determined a setting of CELAVIEW RS100 that can measure the inhibition of nuclear export by specific compounds and can be used for HTS. Several hit compounds with inhibitory activity against AcGFP-NP-NES3 nuclear export were successfully identified using this newly developed HTS system. These results suggest that our newly constructed HTS system is a useful tool for the identification of novel nuclear export inhibitors targeting NP-NES3.

2. Materials and methods

2.1. Cells, transfection and antibodies

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing penicillin, streptomycin, and glutamine (PSG, Gibco, Los Angeles, CA, USA), and 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). MDCK cells stably expressing AcGFP (AcGFP-MDCK cells) or AcGFP-NP-NES3 (NES3-MDCK cells) were established by transfection with pAcGFP1-Hyg-N1 containing a hygromycin resistance gene and encoding the original AcGFP sequence (control vector) or pAcGFP1-Hyg-C/AcGFP-NP-NES3 containing a hygromycin resistance gene and encoding (AcGFP-NP-NES3). Stable transfectants were selected in DMEM containing 0.8 mg/mL hygromycin B (Sigma-Aldrich), followed by limited dilution. Transfections were performed using Lipofectamine 2000 (Invitrogen). Anti-NP monoclonal antibody (MAb) (Santa Cruz, Dallas, TX, USA) and goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) were purchased from the indicated suppliers.

2.2. Compounds

The chemical library for HTS screening was provided by Open Innovation Center for Drug Discovery, the University of Tokyo (http://www.ocdd.u-tokyo.ac.jp/library_e.html). Oseltamivir carboxylate and T-705 were purchased from Kemprotec and PharmaBlock R&D, respectively. LMB was purchased from Sigma. All compounds were dissolved in dimethyl sulfoxide (DMSO) and kept at −20 °C.

2.3. Plasmid construction

AcGFP coded in the pAcGFP1-Hyg-N1 vector (Takara Bio, Otsu, Japan) was amplified by polymerase chain reaction (PCR) using Prime STAR Max DNA Polymerase

Download English Version:

https://daneshyari.com/en/article/6142144

Download Persian Version:

https://daneshyari.com/article/6142144

Daneshyari.com