Antiviral Research 135 (2016) 81–90

Contents lists available at ScienceDirect

Antiviral Research

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

Antiviral activities of niclosamide and nitazoxanide against chikungunya virus entry and transmission

Yu-Ming Wang [a, 1], Jeng-Wei Lu [b, 1], Chang-Chi Lin [a, c], Yuan-Fan Chin [a], Tzong-Yuan Wu [d, e], Liang-In Lin [b, f], Zheng-Zong Lai [a], Szu-Cheng Kuo [a, c, **], Yi-Jung Ho [a, g, *]

[a] Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan, ROC
[b] Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University, Taipei, Taiwan, ROC
[c] Department and Graduate Institute of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, ROC
[d] Department of Bioscience Technology, Chung Yuan Christian University, Chung-Li, Taiwan, ROC
[e] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan, ROC
[f] Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan, ROC
[g] School of Pharmacy, National Defense Medical Center, Taipei, Taiwan, ROC

ARTICLE INFO

Article history:
Received 4 June 2016
Received in revised form
24 August 2016
Accepted 9 October 2016
Available online 11 October 2016

Keywords:
Antiviral screening
Chikungunya virus
Insect cell fusion assay
Niclosamide
Nitazoxanide

ABSTRACT

Chikungunya disease results from an infection with the arbovirus, chikungunya virus (CHIKV). Symptoms of CHIKV include fever and persistent, severe arthritis. In recent years, several antiviral drugs have been evaluated in clinical trials; however, no registered antivirals have been approved for clinical therapy. In this study, we established a high-throughput screening (HTS) system based on CHIKV 26S mediated insect cell fusion inhibition assay. Our screening system was able to search potential anti-CHIKV drugs in vitro. Using this system, four compounds (niclosamide, nitazoxanide, niflumic acid, tolfenamic acid) were identified. These compounds were then further analyzed using a microneutralization assay. We determined that niclosamide and nitazoxanide exhibit ability to against CHIKV-induced CPE. The anti-CHIKV abilities of these compounds were further confirmed by RT-qPCR and IFA. Moreover, niclosamide and nitazoxanide were found to (1) limit virus entry, (2) inhibit both viral release and cell-to-cell transmission, and (3) possess broad anti-alphavius activities, including against two clinical CHIKV isolates and two alphaviruses: Sindbis virus (SINV) and Semliki forest virus (SFV). In conclusion, our findings suggested that niclosamide and nitazoxanide were able to inhibit CHIKV entry and transmission, which might provide a basis for the development of novel human drug therapies against CHIKV and other alphavirus infections.

1. Introduction

Chikungunya disease is caused by an infection with Chikungunya virus (CHIKV), and sporadic outbreaks have occurred in Africa and Asia for decades. In 2005, 2006, an outbreak of chikungunya disease on La Réunion Island in France resulted in one-third of residents becoming infected. During this outbreak, the virus was also transmitted to south India (Parola et al., 2006) and southern Europe, including Italy and France (Gould et al., 2010; Rezza et al., 2007). Furthermore, the Carribean has been affected by an outbreak of chikungunya disease since December 2013, and the virus has become a public health threat in central America (Weaver and Forrester, 2015). Together, the two aforementioned outbreaks has led to hundreds of deaths and caused more than one million people to fall ill (Mavalankar et al., 2007; Weaver and Forrester, 2015). Symptoms of chikungunya disease include high fever, arthritis, arthralgia, headache, nausea, vomiting, and conjunctivitis. Some patients also suffer from severe joint pain for weeks or months (Burt et al., 2012; Weaver and Lecuit, 2015).

CHIKV is an alphavirus belonging to the family Togaviridae and has been classified as a risk group-3 (RG-3) pathogen (Staples et al.,

* Corresponding author. Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan, ROC.
** Corresponding author.
E-mail addresses: szucheng1234@gmail.com (S.-C. Kuo), ejung330@gmail.com (Y.-J. Ho).
[1] YMW and JWL contributed equally to this work.

http://dx.doi.org/10.1016/j.antiviral.2016.10.003
0166-3542/

2009) that enters cells through receptor-mediated endocytosis (i.e. the binding phase). Upon entry, the endosome undergoes acidification, which can lead to conformational changes in CHIKV envelope glycoproteins E1 and E2 (Voss et al., 2010). Envelop glycoproteins then form trimers, which cause the viral membrane and the endosome membrane to fuse together, forming a pore (i.e. the fusion phase) (van Duijl-Richter et al., 2015). The viral genome is then released into the cytosol.

The CHIKV RNA includes 49S genomic RNA and 26S subgenomic RNA. The 49S genomic RNA encodes four non-structural proteins which are responsible for viral genome synthesis (i.e. genome synthesis). CHIKV envelope glycoproteins and capsid proteins are translated by 26S subgenomic RNA in the endoplasmic reticulum (ER) and then are delivered into Golgi apparatus for further processing and glycosylation. Those structural proteins form the spike complexes pE2-E1, which are transported to the plasma membrane. Finally, the spike complexes are assembled with the nucleocapsid cores (Schwartz and Albert, 2010; van Duijl-Richter et al., 2015); and the viruses bud out and release to become mature virions (i.e. the release phase). CHIKV can also be spread through cell-to-cell transmission, which allows virions to efficiently avoid attacks from the host immune system (Hahon and Zimmerman, 1970).

Antiviral drugs are developed to disrupt the life cycle of virus. As indicated, the four main steps of viral multiplication include binding, fusion, genome synthesis, and transmission (including release and cell-to-cell transmission) (Abdelnabi et al., 2015; Parashar and Cherian, 2014). Virus fusion is a critical step of CHIKV infection. The fusion process is dependent on low-pH and the presence of cholesterol (Kuo et al., 2011); (Kuo et al., 2012). Similar findings have been reported for the Sindbis virus (SINV) and the Semliki forest virus (SFV) (Boggs et al., 1989; Chanel-Vos and Kielian, 2004; Kempf et al., 1987; Lanzrein et al., 1993). Therefore, fusion inhibitors are potential candidates for anti-CHIKV drugs. In this report, we described the use of an CHIKV 26S mediated insect cell fusion inhibition assay that uses baculovirus-based expression as a high-throughput screening (HTS) system to search for novel anti-CHIKV drugs (Macarron et al., 2011). We also discussed the effects and mechanisms of two potential anti-CHIKV candidates, niclosamide and nitazoxanide. Our results suggested that the antiviral properties of niclosamide, and nitazoxanide made them promising compounds for further development of anti-CHIKV drugs.

2. Materials and methods

2.1. Cell lines and viruses

BHK-21 (ATCC CCL-10, baby hamster kidney cells) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, catalog # 10564–011) containing 5% fetal bovine serum (FBS, Invitrogen, catalog # 10082–147) at 37 °C and 5% CO_2. Sf21 cells were grown in Sf-900 II SFM (Invitrogen, catalog # 10902–096) containing 5% FBS at 27 °C. We used BHK-21 cells to propagate CHIKV strains S27 (ATCC-VR-64, African prototype), 0611aTw (Singapore/0611aTw/2006/FJ807896, provided by the centers for disease control, ROC [TCDC]), and 0810bTw (Malaysia/0810bTw/2008/FJ807899, with 226V mutant, provided by TCDC) as well as Sindbis virus (ATCC-VR-68, original strain) and Semliki forest viruses (ATCC-VR-67, original strain). S-WT (i.e. a baculovirus constructed with the CHIKV 26S genome) and vector baculovirus (which was used as a control) were propagated in Sf21 cells (Kuo et al., 2011).

2.2. Compounds

An FDA-approved 788 drug library (Selleckchem, catalog No.L1300, 10 mM in DMSO) was purchased from Selleckchem to facilitate the CHIKV 26S mediated insect cell fusion inhibition assay. Niclosamide (Sigma, N3510), nitazoxanide (Sigma, N0290) and suramin (Sigma, S2671) were purchased from Sigma-Aldrich. T1105 (3-hydroxy-2-pyrazinecarboxamide, Toronto Research Chemicals (TRC), D454150) were purchased from TRC.

2.3. CHIKV 26S mediated insect cell fusion inhibition assay

Sf21 cells were transduced with vAc-CHIKV 26S-Rhir-E (S-WT) (Kuo et al., 2011) at an MOI (multiplicity of infection) of 2 and held at 27 °C overnight. Vector baculoviruses (vAc-Rhir-E) were transduced at the same time and under the same conditions. To delay membrane fusion, on the second day, we replaced the old medium with fresh medium (at a pH of 6.8). On the third day, infected cells were pre-treated with 100 μM of the 788 FDA-approved drugs (pH 6.8) for 1 h. The medium was then replaced with fresh medium (pH 5.8) containing 0.1 mg/ml cholesterol and 100 μM of the 788 FDA-approved drugs, and cells were incubated for 2 h. Images of viral fusion were captured using an inverted fluorescence microscope (Olympus, IX71), and fusion area was analyzed by Image J software. The fusion index was calculated using the formula: 1 − [cell count/(total area of fused cells/average area of single cells)] (Ho et al., 2015). Related fusion indexs of test compounds were normalized with respect to the group of S-WT at pH5.8.

2.4. Microneutralization assay

BHK-21 cells were infected with CHIKV strain S27 at an MOI of 0.001 in the presence of compounds at indicated dosages in Fig. 2B and Fig. S1. After an incubation period of 3 days, infected cells were fixed and stained using 0.1% crystal violet solution.

2.5. Immunofluorescence assay (IFA)

Infected cells were fixed and incubated with rabbit anti-CHIKV E2 antibodies (1:200) (Kuo et al., 2011) or J2 anti-dsRNA IgG2a monoclonal antibodies (1:200; Scicons, catalog #J2-1406) for 1 h. These pre-stained cells were subsequently washed three times with PBS and incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:500; Invitrogen, A11037), Alexa Fluor 594-conjugated goat anti-mouse IgG (1:500; Invitrogen, A11032) or Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500; Invitrogen, A11029) for 1 h. After a final wash with PBS, images of cells were captured using an inverted fluorescence microscope (Olympus, IX71).

2.6. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Trizol reagent (Invitrogen, catalog # 15596–026). The QuantiTect SYBR Green RT-PCR kit (Qiagen, catalog # 52906) was used to quantify viral RNA and actin RNA. (Primer sequences are listed in Table S1(Fragkoudis et al., 2007; Ho et al., 2015; Sane et al., 2012). Specifically, RT-qPCR was conducted (using the Roche LightCycler 480 System) for 30 min at 50 °C, 15 min at 95 °C, and then for forty-five additional cycles (where 1 cycle consisted of 15 s at 95 °C, 25 s at 57 °C, and 10 s at 72 °C). Relative values were calculated using the ΔΔCt method, and each experiment was performed in triplicate.