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Antiviral activity of lanatoside C against dengue virus infection

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ABSTRACT

Dengue infection poses a serious threat globally due to its recent rapid spread and rise in incidence. Currently, there is no approved vaccine or effective antiviral drug for dengue virus infection. In response to the urgent need for the development of an effective antiviral for dengue virus, the US Drug Collection library was screened in this study to identify compounds with anti-dengue activities. Lanatoside C, an FDA approved cardiac glycoside was identified as a candidate anti-dengue compound. Our data revealed that lanatoside C has an IC_{50} of 0.19 μM for dengue virus infection in HuH-7 cells. Dose-dependent reduction in dengue viral RNA and viral proteins synthesis were also observed upon treatment with increasing concentrations of lanatoside C. Time of addition study indicated that lanatoside C inhibits the early processes of the dengue virus replication cycle. Furthermore, lanatoside C can effectively inhibit all four serotypes of dengue virus, flavivirus Kunjin, alphavirus Chikungunya and Sindbis virus as well as the human enterovirus 71. These findings suggest that lanatoside C possesses broad spectrum antiviral activity against several groups of positive-sense RNA viruses.

1. Introduction

Dengue virus (DENV), the most significant virus in the *Flaviviridae* family with the highest morbidity and mortality rates, poses a serious global threat in the past few decades. DENV is serologically subdivided into four serotypes, DENV-1, -2, -3 and -4. The enveloped virus contains a single-stranded, positive-sense RNA genome that is directly translated into a single polyprotein which is eventually cleaved into the ten individual viral proteins. The three structural proteins, envelope (E), capsid (C) and premembrane (prM) proteins form the virion particles (Mukhopadhyay et al., 2005). The remaining seven non-structural proteins are primarily involved in viral RNA replication within the infected cells.

Dengue infection can manifest itself either as a mild, self-limiting clinical infection or the more severe dengue hemorrhagic fever (DHF) and even the life-threatening dengue shock syndrome (DSS). Currently, there are numerous candidate compounds (Lim et al., 2013), including the well-known mycophenolic acid and ribavirin (Diamond et al., 2002; Takhampunya et al., 2006) that are reported to possess anti-DENV activities. Nevertheless, none of them are approved yet for the treatment of the disease. Therefore, the development of safe and effective antiviral treatments is now a pressing issue to combat this medically important mosquito-borne viral pathogen.

Lanatoside C, a US Food and Drug Administration (FDA) approved cardiac glycoside that acts by inhibiting the Na^+-K^+-ATPase pump was recently found to inhibit several negative-strand RNA viruses including the influenza virus, vesicular stomatitis virus and Newcastle disease virus (Hoffmann et al., 2008). Another well studied cardiac glycoside, ouabain that acts similarly by inhibiting the Na^+-K^+-ATPase pump also inhibited herpes simplex virus (Dodson et al., 2007), Sendai virus (Nagai et al., 1972; Tomita and Kuwata, 1978) and murine leukemia virus (Tomita and Kuwata, 1978). Increased intracellular Na^+ and reduced intracellular K^+ concentrations have both been demonstrated to affect replication of several negative-strand viruses, DNA viruses and retroviruses (Nagai et al., 1972; Hartley et al., 1993; Chen et al., 2004; Hoffmann et al., 2008; Bertol et al., 2011).

In our high-throughput screen of the US Drug Collection library for anti-DENV compounds, lanatoside C was identified as a potent inhibitor of dengue virus infection. The inhibitory effect of lanatoside C on DENV-2 was examined by measuring the production of infectious virus particles and viral RNA as well as viral protein expression. The study was also extended to the different DENV

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serotypes and other positive-strand RNA viruses (Kunjin virus, Chikungunya virus, Sindbis virus and Human enterovirus 71). Our data showed dose-dependent inhibition of DENV-2 virus infection, viral RNA production and viral protein synthesis following treatment with increasing concentrations of lanatoside C. Other viruses within the *Flaviviridae*, *Togaviridae* and *Picornaviridae* families were also found to be inhibited by lanatoside C.

2. Materials and methods

2.1. Cell lines and viruses

Human umbilical vein endothelial (HUVEC) cells were purchased from Clonetics, USA. Baby hamster kidney fibroblast (BHK21) cells, human muscle rhabdomyosarcoma (RD) cells and mosquito cell line C6/36 from *Aedes albopictus* were obtained from American Type Culture Collection (ATCC). The human leukemic monocyte lymphoma (U937) cell line was kindly provided by Professor Ng Mah Lee, Department of Microbiology, National University of Singapore (NUS). The human hepatoma (HuH-7) cell line was a kind gift from Dr. Priscilla Yang, Department of Microbiology and Immunology, Harvard Medical School. HUVEC, BHK21 and U937 cells were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS). HuH-7 and RD cells were maintained in DMEM (Sigma–Aldrich) supplemented with 10% FCS. C6/36 cells were maintained in L-15 medium (Sigma–Aldrich) supplemented with 10% heat inactivated FCS. BHK21, U937, RD and HuH-7 cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. C6/36 cells were maintained at 28 °C, in the absence of CO_2.

DENV serotype 1–4 (Singapore isolates) were grown in C6/36 mosquito cell line derived from *A. albopictus*. Sindbis virus (SINV) and Kunjin virus (KUNV) were kind gifts from Professor Ng Mah Lee, Department of Microbiology, National University of Singapore. Human Enterovirus 71 (5865/SIN/000009) (HEV71) was kindly provided by A/P Vincent Chow, Department of Microbiology, National University of Singapore and Chikungunya virus (D1225Y08) (CHIKV) was kindly provided Dr. Ng Lee Ching, Environmental Health Institute, National Environment Agency.

2.2. US drug collection screen

The US Drug Collection (Microsource, Discovery Systems Inc., Gaylordsville, CT, USA) library consisting of a total of 1040 compounds was screened for compounds that effectively inhibit DENV infection. Briefly, HuH-7 cells were infected with DENV-2 at multiplicity of infection (MOI) of 1 and incubated at 37 °C for 1 h to allow virus adsorption. The cells were then incubated with the different compounds and subsequently processed for immunofluorescence assay as described previously (Low et al., 2011). Inhibition of DENV-2 by the various compounds, relative to the DMSO-treated control was expressed as the percentage of DENV antigen positive cells. The top 20 positive hits displaying greater than 50% inhibition against DENV-2 were selected for further validation of their anti-DENV properties. HuH-7 cells were infected and treated with the selected compounds for validation. The supernatant was collected for quantification of infectious viral titre via plaque assay.

2.3. Cell viability assay

AlamarBlue® cell viability assay (Invitrogen, Carlsbad, CA, USA) was used to measure the cell viability of the various cell lines following treatment with different concentrations of lanatoside C (Sigma–Aldrich) following manufacturer's instructions. Sodium azide and 0.1% DMSO were used as experimental controls. The fluorescence intensity was measured by the Infinite M200 microplate reader (Tecan, Männerdorf, Switzerland) using excitation and emission wavelengths of 570 nm and 585 nm, respectively. The data was then processed by the i-control 1.6 software (Tecan).

2.4. Dose-dependent studies

2.4.1. Infection of cells

For DENV-1, DENV-2, DENV-3, DENV-4 and Kunjin virus (KUNV), adherent HuH-7 and HUVEC cells were seeded on 24-well plates at 5×10^4 and 1×10^5 cells/well one day prior to infection. Cells were then infected with the different viruses at MOI of 1. For the suspension cell line, U937, cells were seeded at 8×10^5 cells/well on the day of infection before being infected by DENV-2 (MOI of 1). The cells were then incubated at 37 °C for 1 h to allow virus adsorption. For CHIKV and SINV virus, BHK21 cells were seeded on 24-well plates at 7.5×10^4 cells/well one day prior to infection. The cells were infected at MOI of 1 as described above for 1 h (SINV) and 1.5 h (CHIKV). For HEV71, RD cells were seeded on 24-well plates at 2×10^5 cells/well one day prior to infection. The cells were infected at MOI of 1 as described above for 1 h. Following infection, the cells were rinsed with PBS before treatment with lanatoside C.

2.4.2. Lanatoside C treatment

Appropriate volumes of medium (supplemented with 2% FCS) containing various concentrations of drug were added to the infected cells. 0.1% DMSO was used as the vehicle control for comparison with the drug-treated infected cells. The cells were then incubated at 37 °C for either 12 h (SINV and HEV71 infected BHK21 and RD cells), 24 h (CHIKV infected BHK21 cells), 48 h (DENV-1, DENV-2, DENV-3, DENV-4 and KUNV infected HuH-7, HUVEC and U937 cells) before they were harvested for plaque assay.

2.5. Time-of-addition study

For the pretreatment assay, HuH-7 cells were treated with lanatoside C for 2 h before infection by DENV-2. For the time-of-addition study, HuH-7 cells were first infected with DENV-2 at MOI of 1 and lanatoside C was then added at 0, 4, 6, 8, 10, 12, 24 and 48 hpi. The infected cells were processed for immunofluorescence detection at 72 hpi and the percentage of DENV antigen positive cells were quantified.

2.6. Quantitative RT-PCR

At 24 and 48 hpi, DENV-2 viral RNA was extracted from the samples, using the RNeasy Mini Kit (QIAGEN), following the manufacturer's protocols. Briefly, the nucleic acid was first precipitated using 100% ethanol. The nucleic acid was subsequently allowed to bind to the RNeasy spin column before excess salt was removed by three washing steps using the washing buffers provided. The DENV-2 viral RNA was then eluted with 30 μL of RNase-free water. Finally, DNA was removed using one unit of RQ1 RNase-Free DNase (Promega). The purified RNA was stored at −20 °C. Reverse transcription was first performed in which the cDNA of both the positive and negative viral RNA strands were transcribed with primers (DEN12F: 5′-cttaaatacattcaccaacatggaag-3′ and DEN12R: 5′-acctgtcatctatgggtttcac-3′) specifically targeting the DENV-2 viral genome using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Forward primers were used to transcribe cDNA from the negative-strand RNA, whereas reverse primers were used to transcribe cDNA from the positive-strand RNA. Primers and viral RNA were incubated at 70 °C for 5 min and then placed on ice for 2 min. cDNA was then synthesized at 42 °C for 60 min, and then

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