Antiviral Research 145 (2017) 24–32

Contents lists available at ScienceDirect

Antiviral Research

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

Identification of a coumarin-based antihistamine-like small molecule as an anti-filoviral entry inhibitor

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ARTICLE INFO

Article history:
Received 10 May 2017
Received in revised form
12 June 2017
Accepted 18 June 2017
Available online 20 June 2017

Keywords:
Ebola
Marburg
Antiviral
Antihistamine
Histamine receptor antagonist

ABSTRACT

Filoviruses, consisting of Ebola virus, Marburg virus and Cuevavirus, cause severe hemorrhagic fevers in humans with high mortality rates up to 90%. Currently, there is no approved vaccine or therapy available for the prevention and treatment of filovirus infection in humans. The recent 2013–2015 West African Ebola epidemic underscores the urgency to develop antiviral therapeutics against these infectious diseases. Our previous study showed that GPCR antagonists, particularly histamine receptor antagonists (antihistamines) inhibit Ebola and Marburg virus entry. In this study, we screened a library of 1220 small molecules with predicted antihistamine activity, identified multiple compounds with potent inhibitory activity against entry of both Ebola and Marburg viruses in human cancer cell lines, and confirmed their anti-Ebola activity in human primary cells. These small molecules target a late-stage of Ebola virus entry. Further structure-activity relationship studies around one compound (cp19) reveal the importance of the coumarin fused ring structure, especially the hydrophobic substituents at positions 3 and/or 4, for its antiviral activity, and this identified scaffold represents a favorable starting point for the rapid development of anti-filovirus therapeutic agents.

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1. Introduction

Filoviruses are single-stranded, negative-sense RNA viruses with filamentous morphology, which cause severe hemorrhagic fevers in humans and nonhuman primates. Filoviruses consist of three members: Marburgvirus (MARV), Ebolavirus (EBOV), and Ceuvavirus (Kuhn et al., 2010). The first filovirus was recognized and named Marburg virus for its outbreak origin in Marburg, Germany in 1967. That outbreak led to 7 deaths among the 31 reported cases (Siegert et al., 1967). Since then, fatal filovirus outbreaks, particularly Ebolavirus, occurred sporadically, with mortality rates up to 90% (Feldmann and Geisbert, 2011). The latest 2013–2015 West Africa Ebola epidemic was the most widespread outbreak of EBOV in history and persisted for about two years. It led to 28,652 infections (including suspected, probable and confirmed cases) and 11,325 reported deaths from the 15,261 laboratory-confirmed cases with a mortality rate of 74% (as of April 13th, 2016 updated by the World Health Organization). Since there is no FDA-approved vaccine or therapy available against these viruses for humans, development of antiviral therapies is urgently needed to prevent and control future outbreaks and potential bioterrorism attacks.

Viral entry, the first step of the viral replication cycle, represents a potential target for antiviral drug development. Filovirus entry is mediated by a single viral glycoprotein (GP), which is composed of two subunits, GP_1 and GP_2 (Lee and Saphire, 2009; Manicassamy et al., 2005). GP_1 mediates the initial attachment of virion to the host cell surface, likely by binding with heparan sulfate and other similar glycosaminoglycans (O'Hearn et al., 2015; Salvador et al., 2013), or C-type lectin family members like LSECtin (liver and lymph node sinusoidal endothelial cell C-type lectin), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), L-SIGN (liver/lymph node-specific ICAM-3 grabbing

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http://dx.doi.org/10.1016/j.antiviral.2017.06.015
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nonintegrin), mannose-binding lectin, and hMGL (human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin) (Alvarez et al., 2002; Gramberg et al., 2005; Takada et al., 2004). The virus is then internalized via macropinocytosis and trafficked to late endosomes/lysosomes (Nanbo et al., 2010; Saeed et al., 2010), where the GP_1 is trimmed by the cysteine proteases cathepsin B and L in the low pH environment (Brecher et al., 2012; Chandran et al., 2005; Schornberg et al., 2006). The mucin-like domain and the glycan cap of GP_1 are removed and the receptor binding domain is exposed for the interaction with the internal EBOV receptor, Niemann-Pick C1 (NPC1), which triggers GP_2 conformational change and leads to viral-endosomal membrane fusion (Carette et al., 2011; Cote et al., 2011; Gong et al., 2016; Wang et al., 2016).

We previously screened an FDA-approved drug library, identified several groups of GPCR antagonists as potent entry inhibitors, and revealed antihistamines as the most enriched GPCR antagonists (Cheng et al., 2015). In this report, we screened a library of 1220 antihistamine-like compounds (designed with ligand-based approach) and identified multiple compounds as potent inhibitors of filovirus entry. These small molecules exhibit potent inhibitory activity against both pseudotyped EBOV and MARV in adenocarcinomic human alveolar basal epithelial cells (A549). More importantly, the anti-Ebola activities of cp15 and cp19 were further validated with human primary cells including peripheral blood mononuclear cells (PBMC) derived macrophages and human foreskin fibroblasts (HFF). The compound cp19, which has an IC_{50} value of 3.4 μM in PBMC derived macrophages and an IC_{50} value of 1.2 μM in HFF against infectious EBOV infection, was further evaluated with structure-activity relationship (SAR) studies. Our results suggest that the coumarin fused ring structure of cp19 is important for its anti-filovirus activity and that the hydrophobic substituents at positions 3 and/or 4 confer an important interaction with its target.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cells (239T, ATCC# CRL-1573), human lung epithelial cells (A549, ATCC#CCL185) and Hela cells (ATCC#CCl-2) were cultured in DMEM (Cellgro) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 μg/mL of streptomycin and 100 units of penicillin (Invitrogen). The HFF-1 (SCRC-1041) cells was cultured in MEM supplemented with 10% FBS and 0.5 mM sodium pyruvate for limited amount of passages not exceeding 16. Human peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats obtained from healthy volunteers (New York blood center, Leukocytes E3752V00) and maintained in RPMI-1640 with GlutaMAX™ (Gibco) supplemented with 10% FBS, 10 mM Hepes, 100 units/mL penicillin and streptomycin. Cells were purified using ficoll columns from 50 mL of blood and cryopreserved at 2×10^6 cells/vial.

2.2. Generation of pseudovirions

The pseudovirions were created by the following plasmids: hemagglutinin (HA) and neuraminidase (NA), isolated from A/Goose/Qinghai/59/05 (H5N1) strain, Marburgvirus glycoprotein, Ebolavirus Zaire envelope glycoprotein, Lassa virus envelope glycoprotein and the HIV-1 proviral vector pNL4-3.Luc.R^-E^- (obtained through the NIH AIDS Research and Reference Reagent program). All four types of pseudovirions, HIV/MARV, HIV/AIV, HIV/EBOV, and HIV/LASV were produced by transient cotransfection of human 293T cells using a polyethylenimine (PEI)-based transfection protocol. Plasmids encoding MARV GP, hemagglutinin (HA)/neuraminidase (NA), EBOV GP, LASV GP and replication-defective HIV vector (pNL4-3.Luc.R^-E^-) were used for transient cotransfection into 293T producing cells. Six hours after transfection, cells were washed with phosphate-buffered saline (PBS) and 20 mL of fresh media was added to each plate (150 mm). Twenty-four hours post-transfection, the supernatants were collected and filtered through 0.45 μm pore size filter (Nalgene). The pseudovirion stocks were stored at 4 °C prior to use.

2.3. Compound library and chemical reagents

The histamine receptor antagonist library of 1220 compounds was obtained from OTAVA chemicals (Vaughan, Canada). On each 384-well plate, 320 unique compounds were arrayed at 5 mg/mL in DMSO from column 3 to 22, leaving columns 1, 2, 23 and 24 with DMSO. The positive control, an HIV reverse transcriptase inhibitor Azidothymidine (AZT, Sigma), was solubilized at 10 mM in DMSO and used at a final concentration of 5 μM for the screen. Benztropine mesylate (Santa Cruz Biotech) was dissolved in DMSO at 10 mM. All chemicals were stored at −80 °C until use.

2.4. High-throughput screen

The antihistamine library was screened in 384-well plates with a final concentration of 6.25 μg/mL in 0.125% DMSO (v/v) to identify Marburg virus (MARV) entry inhibitors. Low-passage A549 cells were seeded at the density of 1000/well in 384-well plates 24 h before infection. In the presence of compounds, A549 cells were infected by HIV/MARV or HIV/LASV pseudotyped virus, which contained luciferase reporter gene. Plates were incubated for an additional 48 h and infection was quantified by the luciferase activity of infected A549 cells using the neolite Reporter Gene Assay System (PerkinElmer). Virus alone with DMSO was used as the negative control; and virus with 5 μM AZT, an HIV reverse transcriptase inhibitor, was used as the positive control. Data was normalized by plate median and the criterion of average > 80% inhibition in duplicate plates was applied for picking hits.

The selected active compounds were then reformatted into new 384-well plates and tested against HIV/MARV, HIV/EBOV, HIV/LASV or HIV/AIV at 6.25 μg/mL in 0.125% DMSO (v/v) to confirm the primary results and to identify filovirus-specific hits. The cytotoxicity of hit compounds was examined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in the A549 cells treated the same way as for antiviral screen. The signals in the 16 negative control wells (DMSO) were averaged and used to data normalization.

The hit compounds were serial diluted for IC_{50} and CC_{50} evaluation. IC_{50} and CC_{50} values were determined by fitting the dose-response curves with four-parameter logistic regression in GraphPad.

2.5. Time-of-addition experiment

A549 cells were incubated with HIV/EBOV at 4 °C for 1 h to allow virus attachment to the cells. Virus was removed and cells were washed with cold PBS twice before fresh media was added. The temperature was shifted to 37 °C to trigger virus entry. At different time points of virus entry, cp15 (10 μg/mL), cp19 (10 μg/mL), benztropine (25 μM), or AZT (1 μM) was introduced to assess their impact on virus entry. Triplicate wells were used for each treatment at each time point. Control infected cell cultures were treated with drug vehicle (DMSO) only. Virus infection was measured 48 h post-infection as described above.

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