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Repurposing Potential of 1st Generation H₁-specific Antihistamines as Anti-filovirus Therapeutics

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Abstract

Ebola and Marburg are filoviruses and biosafety level 4 pathogens responsible for causing severe hemorrhagic fevers in humans with mortality rates up to 90%. The most recent outbreak in West Africa resulted in approximately 11,310 deaths in 28,616 reported cases. Currently there are no FDA-approved vaccines or therapeutics to treat infections of these deadly viruses. Recently we screened an FDA-approved drug library and identified numerous G protein-coupled receptor (GPCR) antagonists including antihistamines possessing anti-filovirus properties. Antihistamines are attractive targets for drug repurposing because of their low cost and ease of access due to wide use. In this report we identify common over the counter antihistamines, such as diphenhydramine (Benadryl) and chlorcyclizine (Ahist) as potential candidates for repurposing as anti-filovirus agents. Furthermore, we demonstrate that this potential is wide-spread through the 1st generation of H₁-specific antihistamines but is not present in newer drugs or drugs targeting H₂, H₃ and H₄ receptors. We showed that the filovirus entry inhibition is not dependent on the classical antagonism of cell surface histamine or muscarinic acetylcholine receptors but occurs in the endosome, like the cathepsin inhibitor CA-074. Finally, using extensive docking studies we showed the potential for these drugs to bind directly to the EBOV-GP at the same site as toremifene. These findings suggest that the 1st generation antihistamines are excellent candidates

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for repurposing as anti-filovirus therapeutics and can be further optimized for removal of unwanted histamine or muscarinic receptor interactions without loss of anti-filovirus efficacy.

Keywords

Ebola; Marburg; Antiviral; Antihistamine; Repurposing

1. Introduction

Filoviruses are single-stranded, non-segmented RNA virus consisting of three members: Ebola virus (EBOV), Marburg virus (MARV), and Cuevavirus (Kuhn et al., 2010). EBOV and MARV are biosafety level 4 (BSL4) pathogens classified in the NIAID Biodefense Research Agenda as Category A Agents because of their stability in aerosolized form, high case-fatality rate and lack of FDA-approved vaccines or therapies. EBOV and MARV can cause severe hemorrhagic fevers in humans and are associated with up to 90% mortality rates in EBOV infection (Feldmann & Geisbert, 2011). Western Africa has recently suffered a lethal EBOV epidemic with approximately 11,000 deaths, which spread to other continents, including North America. Currently there is no approved vaccine or antiviral therapy against EBOV infections and any future outbreaks of this deadly virus or dissemination by bioterrorism could have global consequences. Therefore, the control of this pathogen and bioterrorist threat through vaccination or therapeutic means is of paramount importance.

One potential target for the development of potential therapeutics is filovirus entry, which is solely mediated by the viral surface glycoproteins (GPs). The GP exists as a homotrimer of heterodimers consisting of two subunits, GP1 and GP2 (Lee & Saphire, 2009; Manicassamy, Wang, Jiang, & Rong, 2005). GP1 is responsible for initial attachment to the target cell surface, most likely through interactions with heparin sulfate and other closely related glycosaminoglycans (O'Hearn et al., 2015; Salvador et al., 2013), or DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), L-SIGN (liver/lymph node-specific ICAM-3 grabbing non-integrin), mannose-binding lectin, LSECtin (liver and lymph node sinusoidal endothelial cell C-type 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). Following attachment, the virion is internalized via macropinocytosis and transported to the late endosome (Nanbo et al., 2010; Saeed, Kolokoltsov, Albrecht, & Davey, 2010). In the late endosome, the GP is proteolytically processed by the host endosomal proteases Cathepsin B and L (Brecher et al., 2012; Chandran, Sullivan, Felbor, Whelan, & Cunningham, 2005; Schornberg et al., 2006). Proteolytic processing removes the mucin-domain and glycan cap of GP1, which exposes the receptor binding domain for interaction with the endosomal EBOV receptor Niemann-Pick Type C1 (NPC1) triggering GP2 mediated viral membrane fusion with the endosomal membrane resulting in release of the RNA genome into the cytoplasm (Carette et al., 2011; Cote et al., 2011; Gong et al., 2016; H. Wang et al., 2016).

Recently, our group identified numerous G-protein-coupled-receptor (GPCR) antagonists with potent and specific anti-filovirus properties targeting filovirus entry. The majority of the GPCR antagonists with antifilovirus activity were classified as antihistamines/antimuscarinics, such as benztropine and chlorcyclizine. Further analysis, revealed that they inhibit a late entry step in the endosome(Cheng et al., 2015; Cheng et al., 2017). More recently, co-crystallization studies of several small molecules, such as, toremifene and benztropine revealed that they are capable of binding directly to the EBOV-GP causing protein destabilization(Ren, Zhao, Fry, & Stuart, 2018; Zhao et al., 2016). However, further work remains to rule out the possibility that classical GPCR antagonism plays a role in the observed anti-filovirus properties.

Histamine receptors are a class of GPCRs which primarily respond to histamine and are classified into four different receptors (H_1 , H_2 , H_3 , and H_4) based on mechanism of action and function. H_1 is expressed in the peripheral tissues as well as the central nervous system and is responsible for mediating most allergic responses(Thurmond, Gelfand, & Dunford, 2008). H_2 receptors are found extensively in the gastric tissues and are responsible for stimulating both gastric acid secretion and gastrointestinal motility(Shamburek & Schubert, 1992). H_3 is found primarily in the central nervous system and to a lesser extent the peripheral nervous system where it serves as an autoreceptor regulating the release of numerous neurotransmitters(Esbenshade et al., 2008). H_4 is widely expressed in bone marrow and white blood cells and regulates mast cell chemotaxis(Thurmond et al., 2008).

H_1 receptors as the primary mediators of allergic reactions have been the most extensively studied in terms of drug development. H_1 -specific antihistamines have been developed and refined since the 1970's, resulting in the production of numerous FDA-approved drugs. These drugs are further classified into three separate generations based on their ability to interact with muscarinic acetylcholine receptors and their ability to cross the blood-brain barrier. The 1st generation drugs include diphenhydramine (Benadryl) and chlorcyclizine (Ahist) which readily interact with acetylcholine muscarinic receptors and cross the blood-brain barrier causing classic side effects such as dry mouth and drowsiness(Carson, Lee, & Thakurta, 2010). The 2nd generation drugs display little to no interaction with muscarinic receptors and limited ability to cross the blood-brain barrier resulting in few side effects, examples including: loratadine (Claritin) and cetirizine (Zyrtec)(Carson et al., 2010). The 3rd generation drugs are active metabolites or enantiomers of the 2nd generation drugs displaying increased efficacy and/or fewer side effects (ex fexofenadine)(Carson et al., 2010). Classically, these drugs have been utilized to treat a wide-variety of conditions including allergies (dermatitis, conjunctivitis, hypersensitivity reactions and urticaria), motion sickness, vertigo, and insomnia(Carson et al., 2010). As a result, they are widely utilized, and several of them are listed on the World Health Organization's (WHO's) list of essential medications. Thus, these drugs widely available and relatively cheap which makes them ideal candidates for drug repurposing.

Development of H_2 -specific antihistamines also began in the 1970's for the treatment of heartburn and peptic ulcers. However, these drugs have been replaced by more effective proton pump inhibitors(Lamers, 1996). Currently there are no FDA-approved H_3 - and H_4 -

specific antihistamines but some experimental compounds have been developed and are available for research purposes.

The hits from our initial GPCR antagonist screen were limited to antihistamines targeting the H₁ receptor. To truly understand the potential for antihistamines to be repurposed as anti-filovirus agents it is necessary to study a more diverse selection of compounds targeting the four different histamine receptors (H₁, H₂, H₃, and H₄). Additionally, the H₁-specific antihistamines can further be classified into 1st, 2nd, and 3rd generation drugs based on differences in their chemical properties and receptor specificity, which also needs to be explored for potential differences in anti-filovirus activity. In this study, we characterize the prevalence of the anti-filovirus properties of the antihistamines and begin to elucidate their mechanism of action. The findings reported here have important implications for future repurposing of these compounds for use as anti-filovirus therapeutics.

2. Materials and Methods

2.1 Cell culture

Human A549 lung epithelial cells (ATCC#CCL185) and 293T embryonic kidney cells (ATCC# CRL-1573) were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 100 units of penicillin and 100 µg/mL streptomycin (Invitrogen).

2.2 Pseudovirion production

Pseudoviruses for initial IC₅₀ drug screening were created using the following plasmids: hemagglutinin (HA) and neuraminidase (NA), isolated from highly pathogenic avian influenza virus, A/Goose/Qinghai/59/05 (H5N1) strain, Marburg virus glycoprotein, Ebola virus Zaire glycoprotein, and the HIV-1 pro-viral vector pNL4-3.Luc.R⁻E⁻ which were obtained through the NIH AIDS Research and Reference Reagent Program. All three types of pseudovirions HIV/EBOV, HIV/MARV, and HIVAIV were produced by transient co-transfection of 293T cells using a polyethyleneimine (PEI)-based transfection protocol. Five hours after transfection, cells were washed with phosphate-buffered saline (PBS), and 20 mL of fresh media was added to each 150 mm plate. Twenty-four hours post transfection, the supernatant was collected and filtered through a 0.45 µm pore size filter. Pseudovirion stocks were stored at 4 °C prior to use.

2.3 Chemical Reagents

Diphenhydramine, cetirizine, fexofenadine, tiotidine, impentamine, JNJ-7777120, and meclizine were purchased from Tocris. Chlorcyclizine, orphenadrine, cyclizine, and buclizine were purchased from Fisher Scientific Company LLC. Doxylamine and hydroxyzine were obtained from VWR. Carbinoxamine was obtained from Sigma-Aldrich, chlorphenoxamine from Medchem Express, and nor-diphenhydramine from Crescent Chemical Company. All compounds were dissolved in DMSO, except for diphenhydramine and chlorcyclizine which were dissolved in water, at a final concentration of 10 mM. All compounds were stored at -20 °C until use.

2.4 Measuring IC₅₀ and CC₅₀ against pseudovirus

Low passage A549 cells were seeded in 96-well plates at the density of 5,000 cell/well and let sit for 24 hours prior to infection. In the presence of drug concentrations ranging from 100 μ M to 0.4 μ M at 2-fold dilutions, A549 cells were infected with HIV/MARV, HIV/EBOV, or HIV/AIV pseudovirions containing a luciferase reporter gene. All drugs were dissolved in DMSO and final DMSO concentrations never exceeded 1%. Plates were incubated for 48 hours and viral infection was quantified by luminescence using the neolite reporter gene assay system (PerkinElmer). Virus with DMSO alone was used as a negative control and data was normalized to the negative control. Drug cytotoxicity was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in the A549 cells treated the same way as for antiviral screen. IC₅₀ and CC₅₀ values were determined by fitting dose-response curves with four-parameter logistic regression in Graphpad.

2.5 Infectious virus assays

Experiments using live authentic Ebola (Kikwit) were performed in biosafety level 4 (BSL-4) facilities at the United States Army Medical Research Institute for Infectious Disease (USAMRIID). Infectious assays were performed in two different cells lines (human foreskin fibroblasts (HFF-1) and HeLa cells) and different plaque forming units (pfu) to achieve similar detectable infection rates after 48 hours of incubation.

2.5.1 EBOV infection assay of Human Foreskin Fibroblasts and HeLa cells—

Two hours after administration of compounds, 384-well plates containing HFF-1 or HeLa cells were transferred to the BSL-4 suite and infected with Ebola/Kikwit at a multiplicity of infection of 2.0 pfu per cell for HeLa cells (calculated for 6,000 cells/well, assuming one complete round of replication at 15–20 h after cell seeding) and 24 pfu per cell for HFF-1 cells (calculating for the cell density at the time of seeding) in 10 μ L of media. The assay plates were incubated in a tissue culture incubator for 48 h before infection was terminated by fixing the samples in formalin solution prior to immunostaining.

2.5.2 Immunostaining Assay—Immunostaining was used to visualize infection in cells treated with unmodified authentic EBOV and MARV isolates. After formalin fixation steps all wells on the assay plates were treated with viral antigen specific monoclonal antibody (mm 6D8 anti-GP for EBOV) at 1 to 1000 dilution for 1 h and followed by staining with Dylight488 conjugated anti-mouse IgG (Thermo) (1–1000 dilution) in blocking buffer containing 3% BSA in PBS. All wells also were stained with Draq5(Biostatus) for nuclei and cytoplasm detection. Five images from each well on the assay plate were acquired on the Opera confocal imaging plate reader (Perkin Elmer) using 10x Air objective. Signal from anti-virus staining was detected at 488 nm emission wavelength and signal for nuclei and cytoplasm at 640 nm and used for cell count and masking correct location of the viral staining. Image analysis is performed using PE Acapella algorithms. The number of infected cells were calculated for five images in each well and converted into presence of infection. The plate-based normalization was done using the average of the low signal non-infected control wells (16 wells) and high signal infected control wells (16 wells) for each plate using Gene data analytical software. Analysis of the dose response curve to determine EC₅₀ was performed using GeneData software by applying the Levenberg-Marquardt algorithm

(LMA) for curve fitting. Most of the curve-fitting were results of a 4- or 3-parameter non-linear regression analysis $Y = \text{bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogCC}_{50} - X) * \text{HillSlope}))}$ where the Top and Bottom were fixed at 100 and 0, respectively. Assay plates were not used for analysis if Z' for control wells (n=16) were <0.4.

2.6 Competition Assay

A549 cells were seeded in 96-well plates at a density of 5,000 cells/well. Twenty-four hours later cells were treated with 400 μM histamine or 2 mM acetylcholine and let incubate for 1 hour. After 1 hour, the media containing histamine or acetylcholine was removed and new media containing drug, pseudovirus, and 400 μM histamine or 2 mM acetylcholine was added. Drug concentrations ranged from 100 μM to 0.4 μM in 2-fold dilutions. Forty-eight hours post infection, virus infection levels were measured using the neolite reporter gene assay system (PerkinElmer). Virus with drug at the same concentrations was used as a comparison. Virus with DMSO was used as a negative control. Virus with histamine or acetylcholine alone was used to measure any effects the agonists might have on virus entry. Cells with 400 μM histamine or 2 mM acetylcholine were used to measure cytotoxicity using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

2.7 CRISPR-Cas9 generation of H₁ and H₄ knockout A549 cell lines

Target sequences (two different sequences for each receptor gene) were selected to minimize off-target effects, based on the rating of the CRISPR Design Tool (<http://crispr.mit.edu>). Based on target sequences, single guide RNA (sgRNA) oligos were designed and synthesized by Integrated DNA Technologies (25nmole DNA oligo, no modification needed) (sgRNA oligos and sequences of target sites are summarized in Table S1 and S2, respectively). The top and bottom strands were synthesized separately and phosphorylated, as well as, annealed before use. To prepare sgRNA oligos inserts, the oligos of either strand were dissolved and diluted to final concentration of 100 μM . A mixture of 1 μL sgRNA oligos of both strands respectively, 1 μL 10X T4 ligation buffer (New England Biolabs, MA), 1 μL T4 PNK (New England Biolabs, MA), and 6 μL ddH₂O was then added in a PCR tube and put in a thermocycler (Bio-Rad, CA) set to the following program: 37 °C for thirty minutes; 95 °C for five minutes; ramp down to 25 °C at 5 °C per minute. The phosphorylated and annealed oligos inserts were then 1:200 diluted by ddH₂O and cloned into a CRISPR vector, pSpCas9(BB)-2A-Puro, purchased from Addgene (Addgene plasmid ID: 62988).

To knock out the targeted genes using pSpCas9 plasmids, A549 cells or HeLa cells were plated into one well of a six-well plate and were about 80% – 90% confluent before transfection. Each well was transfected with 600 ng of plasmid and 6 μL of Lipofectamine 2000 (Invitrogen) in 500 μL of Opti-MEM. Plasmids and Lipofectamine 2000 were separately pre-incubated in 250 μL of Opti-MEM in room temperature for 5 minutes before mixing thoroughly and added into cell culture (changed to 2 mL/well Opti-MEM before transfection). After six hours, cell culture was replaced by fresh medium. For each batch of transfection experiment, two control wells were added. One is transfected with an empty GFP plasmid, the other was not transfected at all. Forty-eight hours post transfection, GFP fluorescence was checked under a microscope to roughly calculate transfection efficiency,

and fresh medium containing puromycin (Invitrogen, 1.5 µg/mL for A549 cells or 1.0 µg/mL for HeLa cells) was added. After three days, when all cells in the control wells died, medium containing puromycin was removed and replaced with fresh medium without puromycin. Surviving cells became confluent about one and a half weeks later. Confluent cells were treated with trypsin (Gibco) and monoclonal cell lines were isolated by limited dilution. Briefly, cells were diluted to 2.5 cells/mL, and 200 µL of cells were added to one well of a 96-well plate. About one week after plating, the colonies were inspected under a light microscope and the wells that might have been seeded with multiple cells were marked off. Single cell colonies were selected for further expansion and genotyping.

For each candidate monoclonal cell line, their genomic DNA was extracted until cell number reached 5×10^6 , using a DNAeasy Blood and Tissue Kit (Qiagen). DNA fragments of each gene covering the region targeted by sgRNA were amplified by PCR using gene-specific primers (Table S1). PCR products were cleaned up using a QIAquick PCR purification kit (Qiagen). The purified PCR products were sent for Sanger sequencing using the same forward gene-specific primers (HRH1-Fwd or HRH4-Fwd) that have been used during PCR reaction. Cells with frame-shift mutations (deletion or insertion) in all targeted alleles were selected for future functional tests, based on sequencing results and statistical analysis performed by a quantitative method named Tracking of Indels by Decomposition (TIDE).

2.8 Time of addition experiment

A549 cells were seeded in 96-well plates at a density of 5,000 cells/well. Twenty-four hours later, cells were infected with HIV/EBOV at 4 °C for 1 hour to allow viral attachment to the cell surface. Virus was then removed, and the cells were washed twice with cold PBS before fresh media was added. The temperature was then shifted to 37 °C to initiate viral internalization. Diphenhydramine (25 µM), chlorcyclizine (25 µM), and cathepsin inhibitor CA-074 (100 µM) were added at various time points ranging from 0 h (time of internalization) to 5 hours post internalization at 1-hour intervals. Triplicate wells were used for each time point and virus with DMSO alone was used as a negative control. Virus infection was measured at 48 hours post infection as previously described.

2.9 GLIDE Docking

We used the commercial docking software GLIDE (Schrödinger 10.0, Portland, OR) within Schrödinger Suite 2014 that applies a two-stage scoring process to rank the best conformations and orientations of the ligand based on its interactions with the Ebola GP (PDB code, 5JQ7)(Friesner et al., 2006). Three-dimensional coordinates of the ligands and their isomeric, ionization, and tautomeric states were calculated using the LigPrep (including Ionizer) module within the Schrödinger Suite 2014 programs. The protein was prepared using the “protein preparation tool”, and the structures were minimized with MacroModel software within Schrödinger Suite 2014. A grid file (20 Å by 20 Å) encircling toremifene in the cavity of GP was generated. Conformational flexibility of the ligands was handled via an exhaustive conformational search. Initially, we used Schrödinger’s proprietary GlideScore scoring function in standard precision (SP) mode. We selected top-scored compounds to dock again in extra precision (XP) mode to score the optimized position, and used UCSF CHIMERA to view the binding positions and generate pictures(Pettersen et al., 2004).

3. Results

3.1 Only H₁ specific antihistamines possess potent anti-filovirus properties

To identify broad-spectrum filovirus inhibitors in a biosafety level 2 (BSL-2) facility, our lab developed a human immunodeficiency virus (HIV-1) based surrogate assay (J. Wang et al., 2014). The assay utilizes a replication incompetent HIV-1 core and the surface protein of highly pathogenic enveloped viruses such as Ebola virus, Marburg virus, and avian influenza virus H5N1 (AIV). Using this strategy, we initially tested antihistamines with inhibition specificity for a single histamine receptor referred to as H₁, H₂, H₃, and H₄ receptors against HIV/EBOV, HIV/MARV, and our specificity control HIV/AIV. From this initial screen only the H₁ specific inhibitors (diphenhydramine and chlorcyclizine) showed strong inhibitory effects on the entry of HIV/EBOV and HIV/MARV at 12.5 μ M (75% inhibition) with no effect on HIV/AIV infection (Figure 1A). Diphenhydramine and chlorcyclizine produced typical S-shaped dose-response curves with clear separation of antiviral activity and cell toxicity with selectivity indices (SI) $\geq$ 10, except for diphenhydramine against HIV/MARV (SI=7.7) (Figure 1B and 1C, Table 1). Diphenhydramine and chlorcyclizine had IC₅₀'s of 2.6 μ M and 2.7 μ M, respectively, against HIV/EBOV and IC₅₀'s of 7.6 and 4.6 μ M, respectively, against HIV/MARV (Table 1). H₂ and H₃ specific antihistamines (tiotidine and impentamine, respectively) displayed no anti-filovirus activity up to a concentration of 100 μ M (Table 1). The experimental H₄-specific antihistamine, JNJ-7777120, displayed modest anti-filovirus activity against both HIV/EBOV and HIV/MARV with IC₅₀'s of 16.6 μ M and 20.6 μ M, respectively (Table 1), with SI values of 5 and 6.9, respectively (Table 1).

3.2 Validation of H₁-specific antihistamine antifilovirus properties using infectious Ebola Virus

To validate the efficacy of the H₁-specific antihistamines, we further evaluated their ability to block replication of infectious Ebola virus strain Ebola/Kikwit in tissue-cultured HFF-1 and HeLa cells under BSL-4 containment conditions. Both diphenhydramine and chlorcyclizine showed typical S-shaped dose-response curves in both HFF-1 and HeLa cells (Figure 2). The IC₅₀'s against infectious Ebola virus for diphenhydramine and chlorcyclizine were similar in both HeLa (5.7 μ M and 5.7 μ M, respectively) and HFF-1 cells (2.2 μ M and 3.2 μ M, respectively) (Figure 2). The cell toxicities remained relatively similar across both cell lines, with marginally better separation of antiviral activity from cell toxicity in the HFF-1 cells (Figure 2).

3.3 Only the 1st generation H₁-specific antihistamines possess anti-filovirus properties

There are three generations of H₁-specific antihistamines possessing different levels of specificity for the histamine receptors and ability to cross cell membranes (Table 2). The H₁ antihistamines which showed antiviral activity, as described above, all belong to the 1st generation of drugs. Thus, we decided to test the 2nd and 3rd generation drugs (Cetirizine and Fexofenadine, respectively) to determine if the anti-filovirus properties were maintained. Neither compound had detectable anti-filovirus activity up to 100 μ M suggesting the anti-filovirus properties are not preserved across the three generations of H₁-specific antihistamines (Table 1). To determine the prevalence of anti-filovirus activity within the 1st generation H₁-specific antihistamines, we tested nine commercially-available analogs of

diphenhydramine and chlorcyclizine. From this screen, we identified 2 diphenhydramine analogs (orphenadrine and chlorphenoxamine) and 2 chlorcyclizine analogs (hydroxyzine and cyclizine) displaying similar degrees of anti-filovirus activity with clear separation of antiviral activity from cell toxicity (SI 10) (Table 3). Chlorphenoxamine and hydroxyzine displayed nearly identical IC_{50} 's as diphenhydramine and chlorcyclizine against both HIV/EBOV and HIV/MARV (1.1/3.4 μ M and 6.2/6.1 μ M, respectively), while orphenadrine and cyclizine showed similar values against HIV/EBOV but markedly worse IC_{50} 's against HIV/MARV (2.1/5.0 μ M and 17.5/18.9 μ M, respectively) (Table 3). The results of this screen and our previously published work strongly suggest that the anti-filovirus properties described in this report are extremely prevalent within the 1st generation of H₁-specific antihistamines but are not carried through to the 2nd and 3rd generations (Cheng et al., 2015; Cheng et al., 2017).

3.4 Structure-Activity-Relationship analysis of diphenhydramine and chlorcyclizine analogs

Using the data in Table 3 we can ascertain some details about the structural-activity-relationship of the different 1st generation antihistamines analogous to diphenhydramine and chlorcyclizine. Analysis of diphenhydramine and its analogs reveals that loss of a methyl substituent from the terminal amine group (Nor-diphenhydramine), as well as, substitution of an aromatic carbon with a nitrogen group (Chlorphenoxamine and Doxylamine) significantly reduces drug efficacy (Table 3). Analysis of chlorcyclizine and its analogs revealed that loss of the chlorine group (Cyclizine) led to a significant reduction in MARV inhibition but not EBOV inhibition (Table 3). Additionally, bulky substitutions at the terminal amine group (Buclizine and Meclizine) generally led to a decrease in potency against both EBOV and MARV infections (Table 3). No modifications found in either the diphenhydramine or the chlorcyclizine analogs appeared to lead to a significant increase in potency.

3.4 Competition assays and CRISPR-Cas9 suggest a mechanism of filovirus inhibition independent of classical GPCR antagonism

Our initial assay revealed that only the 1st generation H₁-specific antihistamines possess strong anti-filovirus properties ($IC_{50} < 5 \mu$ M) (Table 1). These antihistamines are known to classically interact with both the H₁ receptor and to lesser extent the muscarinic acetylcholine receptors (Liu, Zheng, & Farley, 2006). To determine if antagonism of these receptors was responsible for the observed anti-filovirus properties we performed a competition assay. Cells were treated with either 400 μ M histamine or 2 mM acetylcholine for one hour prior to infection, to saturate the cell-surface histamine or acetylcholine receptors, respectively. Then the media was removed, and new media was applied containing either 400 μ M histamine with drug and virus or 2 mM acetylcholine with drug and virus. Diphenhydramine and chlorcyclizine showed 100% inhibition of virus infection at 25 μ M drug concentration with or without the presence of histamine or acetylcholine (Figure 3A and 3B). The inhibition of virus by the H₄-specific antihistamine, JNJ-7777120, was more variable (due to being closer to the IC_{50} concentration) but did not show significant differences in inhibition (60–70%) with or without the addition of agonist (Figure 3A and 3B). The results of this assay show that treatment with histamine or acetylcholine has no

effect on the antiviral activities of these antihistamines, suggesting that antagonism of these two receptors is not necessary for the observed anti-filovirus properties. To confirm our results, we utilized CRISPR-Cas9 technology to create A549 cell lines lacking the H₁ or H₄ receptors (Table S1 and S2). At 25 μ M drug concentration we observed 100% virus inhibition by diphenhydramine and chlorcyclizine in the H₁ knockout cells, H₄ knockout cells, and wild type A549 cells, as expected (Figure 3C). JNJ-777120 displayed increased variability in the inhibition rates (40–70%) among the 3 cell lines but this variation was not statistically significant (Figure 3C). The results of these two experiments strongly suggest that the mechanism of action for the anti-filovirus properties of antihistamines is not linked to their classical antagonism of histamine or acetylcholine receptors.

3.5 Time-of-addition reveals that antihistamines block filovirus entry in the endosome in a similar fashion to the human endosomal cathepsin inhibitor CA-074

Since the competition assay and CRISPR-Cas9 experiments above revealed that the anti-filovirus properties of antihistamines are not linked to their classical GPCR antagonism, a time-of-addition experiment was performed to elucidate which step in viral entry is inhibited. To determine if the compounds blocked cell-surface attachment, we incubated the A549 cells with pseudotyped HIV/EBOV and either 25 μ M antihistamine or 100 μ M CA-074 for 1 hour at 4°C. Unattached virions were removed by washing the cells and viral entry was triggered by shifting the temperature from 4°C to 37°C. Similar to what we observe with the endosomal cathepsin inhibitor CA-074, neither diphenhydramine nor chlorcyclizine blocked virus internalization (Figure 4A). Next, compounds were added at various time points ranging from 0 (time of infection) to 5 hours post infection. (Figure 4B). Once again, the results were similar to that of the cathepsin inhibitor CA-074 which is known to inhibit viral entry in the endosome (Misasi et al., 2012; Montaser, Lalmanach, & Mach, 2002). Overall, these results suggest that the antihistamines diphenhydramine and chlorcyclizine do not inhibit viral attachment to the cell surface but rather block a stage of viral entry in the endosome prior to fusion.

3.6 Analysis of binding potential for 1st generation H₁-specific antihistamines to the same binding cavity of toremifene on the EBOV-GP using docking studies

Recently, several small molecule inhibitors of EBOV entry were co-crystallized with EBOV-GP; including, the selective estrogen receptor modulator (SERM), toremifene, and antimuscarinic, benztropine (Ren et al., 2018; Zhao et al., 2016). Initial assays revealed that benztropine and toremifene blocked an entry step in the endosome similar to what we have reported for the 1st generation H₁-specific antihistamines described here (Cheng et al., 2015; Johansen et al., 2013). These structures revealed that residues Y517, L184, L558, and E100 are critical for toremifene binding to the GP cavity (Figure 5A). Therefore, we assessed the potential for these antihistamines to bind to the same cavity and interact with these residues using docking studies. The chlorcyclizine tested in our experiments exists as a racemic mixture of the (S)-chlorcyclizine and (R)-chlorcyclizine enantiomers. Docking revealed significant differences in binding orientation within the cavity between the (S)- and (R)-enantiomers. The (R)-enantiomer (Figure 5C) maintains strong interaction with Y517 (distance < 4 Å) but not L184 and L558, whereas the (S)-enantiomer (Figure 5B) of chlorcyclizine is oriented such that interactions with the critical hydrophobic Y517, L184

and L558 are only loosely maintained because the size of the 4-chlorophenyl ring shifts the compound away from these residues by 2 Å. Both enantiomers maintain strong electrostatic interactions with residue E100 (<5Å). Diphenhydramine binding orientation suggests that the critical interactions with Y517, L184, L558, and E100 are all conserved (Figure 5D).

4. Discussion

Previously, we reported a diverse array of GPCR antagonists possessing broad-spectrum anti-filovirus properties, the majority of these GPCR antagonists were classified as antihistamines (Cheng et al., 2015). There are numerous FDA-approved antihistamines classified by histamine receptor specificity, the ability to cross the blood-brain barrier, and the ability to interact with muscarinic acetylcholine receptors. In this report, we characterized the anti-filovirus properties of each class and generation of antihistamines. The findings reported here provide important insights into the potential repurposing of antihistamines as anti-filovirus agents.

Antihistamines are first categorized by which of the histamine receptors (H₁, H₂, H₃, and H₄) they preferentially antagonize. So, we screened antihistamines specific for each of the receptors against both pseudotyped EBOV and MARV. From this initial screen we determined that only H₁-specific antihistamines possessed anti-filovirus properties with clear separation from cell toxicity (SI>10) (see Table 1, Figure 1). The anti-filovirus properties of the H₁-specific antihistamines were confirmed using infectious EBOV/Kikwit strain (see Figure 2). Since JNJ-7777120 only displayed modest inhibition of filovirus and since, there are no FDA-approved H₄-specific antihistamines and very few experimental compounds it is difficult to further evaluate the antifilovirus potential for this class of antihistamines.

H₁-specific antihistamines are further categorized into three generations of drugs separated by their ability to interact with muscarinic acetylcholine receptors and their ability to cross the blood-brain barrier. Testing the 2nd and 3rd generation H₁-specific antihistamines cetirizine and fexofenadine, respectively, against HIV/EBOV and HIV/MARV revealed that neither of these compounds displayed anti-filovirus properties up to 100 µM drug concentration. The loss of anti-filovirus activity could be attributed to either the loss of antimuscarinic activity or the decreased ability to cross cell membranes, both of which are the result of the 2nd and 3rd generation antihistamines being zwitterions (highly polar) at physiological pH (Liu et al., 2006). Based on the results of our competition assays and CRISPR-Cas9 experiments we conclude that the anti-filovirus properties of antihistamines are not linked to their antagonism of histamine or acetylcholine receptors; therefore, the loss of anti-filovirus activity in the 2nd and 3rd generation antihistamines is most likely due to decreased cell permeability (see Figure 3). Correlation of decreased drug cell permeability and loss of anti-filovirus activity suggests that the drug target is intracellular. The results of the time-of-addition assay support this hypothesis and strongly suggest that antihistamines target an endosomal step in filovirus entry similar to the cathepsin inhibitor CA-074 which is known to block proteolytic processing of the EBOV-GP in the endosome (see Figure 4) (Misasi et al., 2012; Montaser et al., 2002).

Recent publications provide two possible mechanisms of action for these compounds within the endosome. The first is the result of these compounds being cationic amphiphilic drugs (CADs) which are characterized by a hydrophobic ring structure with a hydrophilic side chain containing a terminal amine group (Miller, Adhikary, Kolokoltsov, & Davey, 2012; Salata, Calistri, Parolin, Baritussio, & Palu, 2017). The terminal amine group becomes positively-charged in the low pH environment of the endosome which helps the drug accumulate. As the drug accumulates in the endosome it disrupts endosomal processes crucial to viral entry. In the case of filovirus inhibition, CADs may induce acid sphingomyelinase to detach from the endosomal membrane preventing the conversion of sphingomyelin to sphingosine (Fan et al., 2017). As a result, sphingosine levels decrease and prevent the normal outflow of calcium through the two-pore calcium channels (Fan et al., 2017). Calcium accumulation is hypothesized to prevent EBOV-GP and MARV-GP conformational changes necessary for viral fusion and escape from the endosome (Fan et al., 2017).

A second possible and more likely mechanism of action for the reported antihistamines, is direct binding to the filovirus GP. Recently, several small molecule inhibitors of filovirus entry, including the antihistamine/antimuscarinic benztropine, were shown to bind to the EBOV-GP at the GP1/GP2 interface (Ren et al., 2018; Zhao et al., 2016). Binding to this location caused protein destabilization which is hypothesized to prevent the virus from fusing and escaping from the endosome by promoting a premature fusion ready state (Zhao et al., 2016). Docking studies of (R)/(S)-chlorcyclizine and diphenhydramine with the EBOV-GP strongly suggest that at least some of the antihistamines described here can bind the same pocket as toremifene but in different orientations (Figure 5). However, these two mechanisms are not mutually exclusive.

Using pharmacokinetic and pharmacodynamic (PK/PD) data from past clinical trials, we evaluated the potential for the 1st generation antihistamines to be repurposed without modification. The typical adult dosing regimens for diphenhydramine, chlorcyclizine and related compounds is one 25/50/100 mg tablet 2–4 times per day (Blyden, Greenblatt, Scavone, & Shader, 1986; Fouda, Hobbs, & Stambaugh, 1979; Griffin & Baselt, 1984; Labout, Thijssen, Keijser, & Hespe, 1982). The maximum plasma concentration (C_{max}) for the maximum recommended doses of these compounds in adults is on average 100 ng/L which is significantly lower than our reported IC₅₀'s between >1 μ M (Blyden et al., 1986; Fouda et al., 1979; Griffin & Baselt, 1984; Labout et al., 1982). Therefore, it is unlikely these compounds would provide the desired antiviral effect at the FDA-approved doses without modifications. However, the data presented here provides a strong platform for future work focused on modifying these antihistamines to increase their potency into the nM range.

In conclusion, the results of this study strongly suggest that the 1st generation H₁-specific antihistamines represent a vast reservoir of potential candidates for drug repurposing for use as anti-filovirus therapeutics with modifications to improve their potency. Since their anti-filovirus properties do not rely upon their classical GPCR antagonism these compounds would be excellent candidates for further optimization focused on removing these unwanted interactions and potentially reducing their side-effects. If co-crystallization work supports

the results of our docking studies, then future efforts can be made to optimize the binding affinity of these compounds for the cavity at the GP1 and GP2 subunit interface to improve the potency and drug like properties of these antihistamines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Antihistamines spanning multiple classes and generations were screened for anti-filovirus activity.
- Only 1st generation H₁-specific antihistamines displayed anti-filovirus activity in both human immortalized cell lines and human primary cells.
- Competition Assays and CRISPR Cas9 experiments suggest anti-filovirus activity is independent of classical GPCR antagonism.
- Time-of-Addition experiments suggest antihistamines block an endosomal entry step.

Figure 1. 1st generation H₁-specific antihistamines display potent anti-filovirus properties against pseudotyped EBOV and MARV

(C.) Drug specificity for diphenhydramine and chlorcyclizine was determined by comparing percent virus infection of HIV/EBOV, HIV/MARV, and HIV/AIV at drug concentration 12.5 μ M. Dose-response curves for diphenhydramine (B.) and chlorcyclizine (C.) against pseudotyped HIV/EBOV and HIV/MARV in A549 cells. Blue curves represent cell-cytotoxicity at the doses tested. Error bars represent the average S.D. of three independent experiments

Figure 2. 1st generation H₁-specific antihistamines inhibit infectious Kikwit Ebolavirus strain
Dose- response curves for diphenhydramine (A.) and chlorcyclizine (B.) against infectious Kikwit EBOV strain (red) in HeLa cells. Dose-response curves for diphenhydramine (C.) and chlorcyclizine (D.) against infectious Kikwit EBOV strain (red) in human foreskin fibroblast (HFF-1) cells. Blue curves represent cell-cytotoxicity at the doses tested. Error bars represent the average S.D. of three independent experiments

Figure 3. The anti-filovirus properties of antihistamines do not rely on antagonism of the histamine receptors

Inhibitory effects of antihistamines on pseudotyped HIV/EBOV entry in A549 cells given two doses of 400 μ M histamine (A.) or 2 mM acetylcholine (B.). The first dose was given 1 hour prior to infection and the second dose at the time of virus and antihistamine administration. (C.) H1 or H4 receptor was knocked out in A549 cells using CRISPR-Cas9 and the inhibitory effects of antihistamines were measured against HIV/EBOV at 25 μ M concentrations. Error bars represent the average S.D. of three independent experiments

Figure 4. Antihistamines block Ebolavirus entry in the endosome

(A.) A549 cells were treated with pseudotyped HIV/EBOV and 25 μ M antihistamines or 100 μ M CA074 (cathepsin inhibitor) for 1 hour at 4°C to prevent virus internalization. Cells were then washed with ice-cold PBS to remove unbound virus and then the temperature was shifted to 37°C to promote viral entry. (B.) A549 cells were treated with 25 μ M antihistamines or 100 μ M CA074 at various time points during infection with pseudotyped HIV/EBOV. Error bars represent S.E.M. from three independent experiments.

Figure 5. Predicted binding position of chlorcyclizine and diphenhydramine to Ebola GP and superimposition with toremifene

(A) Crystal structure of Ebola glycoprotein trimer in complex with toremifene (grey; 5JQ7); (B) Binding pose of S-chlorcyclizine (green) to toremifene binding site, wherein hydrophobic interactions with Y517, L184 and L558 were loosely maintained, while electrostatic interaction with E100 remains within 5 Å; (C) Binding pose of R-chlorcyclizine (magenta) to toremifene binding site, showing that the phenyl ring of both molecules are closely overlapped and forms strong interactions with Y517; (D) Binding position of diphenhydramine (pink) to toremifene binding site, wherein hydrophobic interactions with Y517, L184 and L558 were conserved, as well as electrostatic interaction with E100.

Table 1

Characterization of antihistamines as anti-filovirus agents

Compound	Target Receptor	Drug Generation	HIV/EBOV		HIV/MARV	
			IC ₅₀ (μ M)	SI	IC ₅₀ (μ M)	SI
Diphenhydramine	H ₁	1	2.6 $\pm$ 0.6	20	7.6 $\pm$ 1.6	7.7
Chlorcyclizine	H ₁	1	2.7 $\pm$ 1.2	37	4.6 $\pm$ 0.1	10
Cetirizine	H ₁	2	>100	1	>100	1
Fexofenadine	H ₁	3	>100	1	>100	1
Tiotidine	H ₂	NA	>100	1	>100	1
Impentamine	H ₃	NA	>100	1	>100	1
JNJ777120	H ₄	NA	16.6 $\pm$ 3.9	5	20.6 $\pm$ 7.9	6.9

Table 2

Characteristics of different generations of H1-specific antihistamines.

Generation	Drug Name	Structure	Interact with central H ₁ receptors	Interact with Muscarinic Receptors	Acetylcholine	Zwitterion
1	Chlorcyclizine		Yes	Yes		No
	Diphenhydramine
2	Cetirizine		No	No	No	Yes
3	Fexofenadine		No	No	No	Yes

Table 3
Anti-filovirus Properties of Diphenhydramine and Chlorcyclizine Analogs (Pseudotyped Virus)

Diphenhydramine Analogs						
Compound	IC ₅₀ (μM) EBOV	IC ₅₀ (μM) MARV	CC ₅₀ (μM)	SI EBOV	SI MARV	
Diphenhydramine	2.6 ± 0.6	7.6 ± 1.6	52.6 ± 9.4	20	7	
Nordiphenhydramine	12.2 ± 0.5	42.5 ± 14.9	>100	>8	>2	
Orphenadrine	2.1 ± 1.0	17.5 ± 0.8	>100	>47	>6	
Chlorphenoxamine	1.1 ± 0.5	6.2 ± 0.8	55.3 ± 3.4	50	9	
Carbinoxamine	29.5 ± 8.1	38.8 ± 12.1	>100	>3	>3	
Doxylamine	14.7 ± 2.1	>100	>100	>7	>6	
Chlorcyclizine Analogs						
Compound	IC ₅₀ (μM) EBOV	IC ₅₀ (μM) MARV	CC ₅₀ (μM)	SI EBOV	SI MARV	
Chlorcyclizine	2.7 ± 1.2	4.6 ± 0.1	43.3 ± 6.7	16	10	
Hydroxyzine	3.4 ± 2.2	6.1 ± 2.8	74.1 ± 27.9	21	12	
Cyclizine	5.0 ± 1.4	18.9 ± 1.5	>100	>20	>5	
Bucizine	12.3 ± 5	12.7 ± 11.5	62.0 ± 17.5	4	4	
Meclizine	6.7 ± 1.8	10.2 ± 3.7	61.7 ± 14.3	9	6	

IC₅₀, half maximal inhibitory concentration; CC₅₀, 50% cytotoxic concentration; SI, selectivity index; NA, not applicable.

Standard deviation calculation from three independent experiments.