Inhibition of Filoviral Cell Entry by Polyanionic Compounds

Thesis

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By

Ghada Mohammed Nour Eldin Ahmed Youssef, MSc.

Supervisors

Prof. Dr. Marie Abdel Aziz Marie

Prof. of Medical Microbiology and Immunology,

Faculty of Medicine, Cairo University

Prof. Dr. Azza Essam Eldin Badr

Prof. of Medical Microbiology and Immunology

Faculty of Medicine, Cairo University

Prof. Dr. Ashraf Elsayed Abdel Hamed Sorour

Prof. of Medical Microbiology and Immunology,

Faculty of Medicine, Cairo University

PD. Dr. Thomas Von Hahn

Institute of Molecular Biology

Hannover Medical School, Germany

Cairo University

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Abstract

Ebola and Marburg viruses are filoviruses characterized by being highly pathogenic and causative agents of viral haemorrhagic fever. Till date, no particular anti-viral therapy has demonstrated effectiveness in filoviral infection. Several polyanionic compounds proved to have a potent antiviral activity on many enveloped viruses.

In this work we investigated the effect of selected polyanionic compounds on filovirus glycoprotein (GP) - mediated cell entry.

Using lentiviral pseudoparticles bearing the GPs of Marburg virus (MARV) and Zaire Ebolavirus (ZEBOV), we found out that all the tested polyanionic compounds had an inhibitory effect on filovirus GP-mediated cell entry. The cytotoxic profile of our tested compounds was investigated and found to be satisfactory. DS 5000 was selected for further testing. Its half maximal inhibitory concentration (IC_{50}) on MARV and ZEBOV viral pseudoparticles cell entry was 4.46µg/ml and 0.8µg/ml, respectively. These concentrations are obviously much lower than the concentrations which can exert an anticoagulant activity. We also found a significant inhibitory effect of DS 5000 on other viral pseudoparticles which belong to filoviruses and also to other virus families. The inhibitory effect of DS 5000 on MARV and ZEBOV viral pseudoparticles cell entry was also confirmed in a variety of cell lines.

Other experiments were performed for more understanding of the mechanism by which DS 5000 inhibit the filoviral cell entry. These include: (1) testing the effect of DS 5000 on MARV and ZEBOV attachment to EAhy cells by measuring the concentration of p24 antigen using ELISA. This test revealed significant inhibition of MARV pseudoparticles cell attachment at DS 5000 concentrations of 5 and 50 µg/ml, whereas insignificant inhibition of cell attachment was observed with ZEBOV viral pseudoparticles at DS 5000 concentrations of 50 µg/ml, (2) virus binding assays using amicon tubes. This revealed significant inhibition of ZEBOV and MARV pseudoparticles cell entry, when the pseudoparticles were incubated with DS 5000 50µg/ml for 1 hour before centrifugation in amicon tubes and (3) testing the effect of DS 5000 pre-treatment of EAhy cells on the viral pseudoparticles cell entry. This test revealed no significant effect of the cell pre-treatment on the pseudoparticles cell entry. Our findings suggest that DS 5000 acts most probably on the viral glycoproteins preventing virus interaction with the cell surface receptors.

It can be hoped that in the near future more attention will be given to improving the *in vivo* effectiveness of these polyanionic compounds, which have shown to be potent and broad-spectrum antiviral agents *in vitro*.

Key words: Marburgvirus-Ebolavirus-polyanionic compounds

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List of abbreviations

Ad	-Adenoviruses
ATP	-Adenosine triphosphate
APTT	-Activated Partial Thromboplastin Time
BDBV	-Bundibugyo Ebolavirus
BUN	-Blood urea nitrogen
CADs	-Cationic amphiphilic drugs
CatB	-Cathepsin B
CatL	-Cathepsin L
CBC	-Complete blood count
CDC	-Centers for Disease Control and Prevention
CO_2	-Carbondioxide
DCS	-Dendritic cells
DC-SIGN	-Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dH_2O	-Distilled Water
DIC	**-**Disseminated intravascular coagulation
DMEM	-Dulbecco's modified eagle medium
DMSO	-Dimethyl sulfoxide
DRC	-Democratic Republic of the Congo
DS	-Dextran Sulphate
EBOV	-Ebola virus
EDTA	-Ethylenediaminetetraacetic acid
EGTA	-Ethylene glycol tetraacetic acid
ELISA	-Enzyme-linked immunosorbent assay
FCS	-Fetal calf serum
FRα	-Folate receptor alpha

GP	- Glycoprotein
HF	-Haemorrhagic fever
HIV	-Human immunodeficiency virus
hMGL	- Human macrophage c-type lectin specific for galactose
IC_{50}	- Half maximal inhibitory concentration
IFN	- Interferon
Ig	- Immunoglobulin
IL	-Interleukin
Kb	-kilobase
KDa	- kilodalton
LB	- Lysogeny Broth
LLOV	- Lloviu virus
LSECtin	-Liver/Lymph node sinusoidal endothelial cell c-type lectin
L-SIGN	-Liver/Lymph node-specific intercellular adhesion molecule-3-grabbing integrin
MARV	-Marburg virus
MCP	-Macrophage chemotactic protein
NaCl	-Sodium chloride
NaOH	- Sodium hydroxide
NC	- Nucleocapsid
NoEnv	- No envelope
nm	- Nanometer
NO	-Nitric oxide
NHP	-Nonhuman primate
NP	-Nucleoprotein
P-value	-Probability value
PAVAS	- Co-polymers of vinyl alcohol sulphate with acrylic acid

PBS	- Phosphate buffered saline
PEI	-Polyethylenimine
RAVV	- Ravn virus
PH	- Power of hydrogen
RBD	-Receptor binding domain
RLU	**-** Relative light units
rpm	- Rotation per minute
RNA	-Ribonucleic acid
RESTV	-Reston Ebolavirus
SERMs	-Selective estrogen receptor modulators
sGP	-Soluble Glycoprotein
siRNA	-Small interfering RNA
SUDV	- Sudan Ebolavirus
TAFV	- Taï Forest Ebolavirus
TAM	- tyro3/axl/mer
TIM	-T-cell immunoglobulin and mucin domain
TNF	-Tumour necrosis factor
VSV	- Vesicular stomatitis virus
ZEBOV	- Zaire Ebola virus

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Filoviruses constitute the family *Filoviridae* which belongs to the order *Mononegavirales* (Dolnik et al., 2008). Ebola and Marburg viruses are filoviruses characterized by being highly pathogenic and causative agents of viral haemorrhagic fever (Bixler and Goff, 2015). This filoviral haemorrhagic fever is characterized by a sudden epidemic occurrence as well as a high lethality with case-fatality rate ranges from 22 to 90% (Feldmann et al., 2001; Laminger and Prinz, 2010).

Although a reservoir host of the filoviruses has not been approved yet; many studies indicate bats as a potential reservoir of these viruses (Laminger and Prinz, 2010). Filoviral haemorrhagic fevers are typical zoonotic diseases transmitted accidentally by direct contact with live or dead animals (Leroy et al., 2011). Vectors involved in transmitting the virus from the bat reservoirs to humans may be either nonhuman primates or pigs; direct transmission via contact with bats was also reported (Ansari, 2014). Filoviruses transmission among humans occurs through direct human-to-human contact or contact with the infectious body fluids, particularly in the late stages of infection, when viral loads are highest (Carroll et al., 2013).

Because of the lack of specific treatment or vaccines, the filoviruses potential use as bioweapons, drives continued research in the industrial countries (Manuscript, 2012). The structural similarity of Ebola virus glycoprotein (GP) to those of the retroviral envelopes has allowed the generation of pseudotyped recombinant retroviral particles that have been used to explore important aspects of the Ebola virus biology (Alvarez et al., 2002). Until now the mechanism of filoviral cell entry is poorly understood, though several cellular proteins have been reported as filoviral receptors or co-receptors including several lectins and integrin ß1 (Aleksandrowicz et al., 2011). Several studies revealed that some polyanionic compounds such as dextran sulfates, pentosan polysulfates, fucoidan and carrageenans are proved to be potent inhibitors of cell entry of many enveloped viruses including herpes simplex virus, human cytomegalovirus and human immunodeficiency virus (Baba et al., 1988b).

In this work we aimed at:

- Testing nine different polyanionic compounds including three co-polymers of vinyl alcohol sulfate with acrylic acid (PAVAS 6, PAVAS 14 and PAVAS 15) and six dextran sulfate Na salts in different molecular weights (DS 1000, DS 3400, DS 5000, DS 10000, DS 40000 and DS 70000) for their inhibitory effect on two filoviruses, namely Marburg virus (MARV) and Zaire Ebola virus (ZEBOV), cell entry.
- Assessment of the cytotoxicity of all the tested polyanionic compounds.
- Depending on our findings, one polyanionic compound with significant inhibitory effect on the filoviral cell entry and acceptable cytotoxic profile will be selected for further investigations. These include:
  - Constructing concentration-response curves for the inhibitory effect of this compound on MARV and ZEBOV cell entry to determine its half maximal inhibitory concentration (IC_{50}).
  - Testing the effect of this compound on MARV and ZEBOV cell entry using different types of cell lines.
  - Testing the effect of this compound on other viruses which belong to the family *Filoviridae* as well as to other viral families.
  - Investigating the mechanism of action of this compound on filoviral cell entry.

Filoviruses

1. Taxonomy and Classification

Filoviruses constitute the family *Filoviridae,* which belongs to the order *Mononegavirales* (Dolnik et al., 2008). The order *Mononegavirales*, derived from the Greek "*monos"* —alone or single, referring to the single-stranded RNA genome of order members; the Latin "negare" —to negate, referring to the negative polarity of the single-stranded RNA genomes of order members; the suffix –virales—ending denotes a virus order. These viruses, the mononegaviruses, are enveloped and contain a linear, nonsegmented, single-stranded RNA genome. The members of this order include the families Bornaviridae, Filoviridae, Paramyxoviridae, and Rhabdoviridae (Kuhn et al., 2011). Filoviruses differ from other mononegaviruses in that they have longer genomes (≈19 kb) than most other members of the order, their genomes encode two unusual proteins: VP30 and VP24 and additionally they are the only mammal-infecting members of the order *Mononegavirales*. Consequently, filoviruses have been assigned to their own family, *Filoviridae*, derived from the Latin "*filo*", meaning "threadlike" (Kiley et al., 1982).

The taxonomy of the family *Filoviridae* has changed several times since the discovery of its members, resulting in a plethora of species, virus names and abbreviations. The family *Filoviridae* is currently divided into three accepted genera, namely *Ebolavirus* (EBOV), *Marburgvirus* (MARV) and *Cuevavirus* (Kuhn et al., 2014).

The genus Marburgvirus was discovered in 1967 when an outbreak of viral haemorrhagic fever was reported among laboratory workers in Europe, who had been exposed to tissues and blood from imported African green monkeys (Dolnik et al. 2008). Its name is derived from Marburg, the city in Germany, where this virus was first isolated. Within this genus, there is a single species (Marburg virus) which consists of two distinct viruses, Marburg virus (MARV) and Ravn virus (RAVV), derived from Ravn—last name of the Danish patient from whom this virus was first isolated. They are approximately 20% divergent from one another (Kuhn et al., 2014).

The genus *Ebolavirus* derived its name from Ebola—name of the headstream of the Mongala River in Zaire (today the Democratic Republic of the Congo), where this virus was thought to be first encountered. The first ebolaviruses were discovered in 1976, when simultaneous viral haemorrhagic fever outbreaks occurred in Zaire and Sudan (Johnson et al., 1977). In 1983, convincing data were published demonstrating that the viruses causing the two outbreaks were antigenically related, but not identical (Richman, Cleveland, McCormick, & Johnson, 1983). In the following years, two additional ebolaviruses were discovered that, while antigenically cross-reactive with the Zaire and Sudan viruses, were unique: the first in 1989 in Reston (the town in Virginia, USA) and the second in 1994 in the Republic of Côte d'Ivoire (Le Guenno et al., 1995). Today, full-length genomic sequences are available for isolates of all of these viruses. Their comparison reveals that the genomes of the four viruses differ from each other by 36.7–42.3% (Kuhn et al., 2011).

Due to the genomic sequence diversity and the fact that the four viruses are endemic in different geographic areas and possibly have different reservoir hosts, the creation of several different *Ebolavirus* species was accepted. The names of these species were derived from the places, where its members were first encountered: Zaire Ebolavirus (ZEBOV), Sudan Ebolavirus (SUDV), Reston Ebolavirus (RESTV) and Taï Forest Ebolavirus (TAFV), Taï Forest: derived from Parc National de Taï [Taï National Park]—a place in the Republic of Côte d'Ivoire (Kuhn et al., 2011).

A fifth Ebolavirus species was described, whose genomic sequence was different from previously recognized viruses by 31.7–42.4%. It is called Bundibugyo Ebolavirus (BDBV), which derives its name from Bundibugyo—name of the town of Bundibugyo in the Republic of Uganda, where members of this species were first encountered (Towner et al., 2008). An Ebola-like filovirus, Lloviu virus (LLOV), derived from Lloviu, referring to the name of the cave in Spain, where members of this species were first encountered, is the only species in the third distinct genus in *Filovirdea* , *Cuevavirus*. The name of this genus is derived from spanish word la cueva —which means “cave” (Negredo et al., 2011).

2. Epidemiology

Filoviruses are among the most lethal human pathogens in the world. Of the eight known species of filoviruses, six are known to cause disease in humans. The disease caused by BDBV, ZEBOV, SUDV and TAFV is called Ebola virus disease and that caused by MARV and RAVV is called Marburg virus disease. There have been no demonstrable illnesses with RESTV infection in humans, which is only pathogenic for nonhuman primates (NHPs), whereas live LLOV has never been recovered; the virus was discovered by gene sequencing of tissues from bat populations and the pathogenicity in humans and NHPs is unknown (Anthony and Bradfute, 2015).

Ebola- and Marburg viruses are highly pathogenic viruses and causative agents of viral haemorrhagic fever (Bixler and Goff, 2015). Filoviral haemorrhagic fever is characterized by a sudden epidemic occurrence as well as a high lethality with case-fatality rates ranging from 22 to 90% (Feldmann et al., 2001; Laminger and Prinz, 2010). Ebola Zaire virus is considered the most lethal with a 90% case fatality rate (Adalja, 2014).

Because of the lack of specific treatment or vaccines, the filoviruses' potential use as a bioweapon drives continued research in the industrial countries (Manuscript, 2012).

The high pathogenicity of these viruses is reflected in their designation as category A agents and the requirement that laboratory work with them must be conducted with the highest level of safety (Adalja, 2014).

Filoviruses are primarily african in origin (fig.1), with the exception of Reston virus, which has been found in the Philippines and China. However, all filoviruses can spread by travel of infected individuals (Anthony and Bradfute, 2015). Since the first cases of filoviral infections were documented in 1967, there have been 11 documented outbreaks of Marburg virus and 24 Ebola virus outbreaks. The recent outbreak of Ebolavirus infection in several adjoining countries in Africa in 2014 with a high mortality rate (51%) has provided serious concern to public health officials worldwide and has prompted a sense of urgency to develop effective chemotherapeutic agents and vaccines as tools to halt this and future outbreaks of filoviral infections (Ansari, 2014).